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The Role of GAPDH in maintaining the functional state of the DNA repair enzyme APE1

par

Emily Ayoub

Biochimie

Faculté de Médecine

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The Role of GAPDH in maintaining the functional state of the DNA repair enzyme APE1

Présenté par :

Emily Ayoub

a été évalué par un jury composé des personnes suivantes :

Président-raporteur : Martine Raymond

Directeur de recherche : Pascal Chartrand

Co-directeur de recherche : Dindial Ramotar

Membre du jury : El Bachir Affar

Résumé

Les sites apuriniques/apyrimidiniques (AP) sont des sites de l'ADN hautement mutagène. Les dommages au niveau de ces sites peuvent survenir spontanément ou être induits par une variété d'agents. Chez l'humain, les sites AP sont réparés principalement par APE1, une enzyme de réparation de l'ADN qui fait partie de la voie de réparation par excision de base (BER). APE1 est une enzyme multifonctionnelle; c'est une AP endonucléase, 3'-diestérase et un facteur redox impliqué dans l'activation des facteurs de transcription. Récemment, il a été démontré qu'APE1 interagit avec l'enzyme glycolytique GAPDH. Cette interaction induit l'activation d'APE1 par réduction. En outre, la délétion du gène GAPDH sensibilise les cellules aux agents endommageant l'ADN, induit une augmentation de formation spontanée des sites AP et réduit la prolifération cellulaire. A partir de toutes ces données, il était donc intéressant d'étudier l'effet de la délétion de GAPDH sur la progression du cycle cellulaire, sur la distribution cellulaire d'APE1 et d'identifier la cystéine(s) d'APE1 cible(s) de la réduction par GAPDH. Nos travaux de recherche ont montré que la déficience en GAPDH cause un arrêt du cycle cellulaire en phase G1. Cet arrêt est probablement dû à l'accumulation des dommages engendrant un retard au cours duquel la cellule pourra réparer son ADN. De plus, nous avons observé des foci nucléaires dans les cellules déficientes en GAPDH qui peuvent représenter des agrégats d'APE1 sous sa forme oxydée ou bien des focis de la protéine inactive au niveau des lésions d'ADN. Nous avons utilisé la mutagénèse dirigée pour créer des mutants (Cys en Ala) des sept cystéines d'APE1 qui ont été cloné dans un vecteur d'expression dans les cellules de mammifères. Nous émettons l'hypothèse qu'au moins un mutant ou plus va être résistant à l'inactivation par oxydation puisque l'alanine ne peut pas s'engager dans la formation des ponts disulfures. Par conséquent, on anticipe que l'expression de ce mutant dans les cellules déficientes en GAPDH pourrait restaurer une distribution cellulaire normale de APE1, libérerait les cellules de l'arrêt en phase G1 et diminuerait la sensibilité aux agents endommageant l'ADN. En conclusion, il semble que GAPDH, en préservant l'activité d'APE1, joue un nouveau rôle pour maintenir l'intégrité génomique des cellules aussi bien dans les conditions normales qu'en réponse au stress oxydatif.

Mots clés : APE1, GAPDH, délétion de GAPDH, dommage à l'ADN, cysteine, site AP.

Abstract

Apurinic/apyrimidinic (AP) sites are highly mutagenic DNA lesions occurring either spontaneously or by the action of DNA damaging agents. In human cells, AP sites are processed by the major DNA repair enzyme APE1 through the base excision repair (BER) pathway. APE1 is a multifunctional protein that has AP endonuclease/3'-diesterase activities in addition to its role as a redox factor in activating many transcription factor. Recently, it has been shown that APE1 interacts with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an interaction that results in the activation of APE1 by reduction. Interestingly, depletion of GAPDH sensitized the cells to DNA damaging agents and induced an increase in spontaneous AP sites frequency. Moreover, cells knocked-down for GAPDH showed defects in proliferation. Here we set up to investigate the effects of GAPDH knockdown on cell cycle progression, APE1 subcellular localization and to identify the cysteine residue(s) of APE1, target(s) of GAPDH reduction. Our studies showed that GAPDH deficient cells arrested in G1 phase of the cell cycle. The defect in cell cycle progression is most probably due to accumulation of DNA damage which activates checkpoints leading to a delay in the cell cycle to allow DNA repair. Furthermore, in GAPDH deficient cells, APE1 formed nuclear foci-like structures that could represent aggregates of the oxidized form of APE1 or inactive APE1 foci on DNA lesions. Using site-directed mutagenesis, we created seven APE1 cysteine to alanine mutants which were cloned into a mammalian expression vector. We expect that at least one of these mutants is likely to resist the inactivation by oxidation as it cannot engage in disulfide bridge formation. Therefore, the expression of this mutant(s) in GAPDH knockdown cells is expected to restore a normal APE1 cellular distribution, rescue the cell cycle defects, and render the cells less sensitive to DNA damaging agents. In conclusion, our results show a new role of GAPDH in maintaining genomic stability under oxidative stress by maintaining APE1 in its functional state.

Key words: APE1, GAPDH, GAPDH knockdown, DNA damage, cysteine, AP sites.

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3MeA	3-methyladenine
4NQO	4-Nitroquinoline 1-oxide
7MeG	7-methylguanine
8oxoG	8-oxo-7,8-dihydrodeoxyguanosine
A	Adenine
AAG	Alkyladenine-DNA glycosylase
AD	Alzheimer's disease
AID	Activation-Induced cytidine Deaminase
AP site	Apurinic-apyrimidinic site
AP-1	activator protein-1
APE1	AP endonuclease 1
APOBEC1	Apolipopro tein B mRNA Editing Catalytic subunit1
Asp	aspartic acid
AT	ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
BER	Base excision repair
C.elegans	*Caenorhabditis elegans*
CPDs	cyclobutane pyrimidine dimmers
CS	Cockayne syndrome
Cys	Cysteine
DDR	DNA damage response
DHT	Dihydrothymidine
DNA	deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dRP	Deoxyribosephosphate
DSB	double strand breaks
dsDNA	double-stranded DNA
DTT	Dithiothreitol
E.Coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Endo IV	Endonuclease IV
ER	endoplasmic reticulum
EXO III	Exonuclease III
EYFP	enhanced yellow fluorescent protein
G	Guanine

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione *S*-transferase
H_2O_2	Hydrogen Peroxide
HD	Huntington's disease
HDACs	histone deacetylases
HIF-1α	hypoxia inducible factor-1α
His	Histidine
HR	Homologous recombination
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	ionizing radiation
kb	Kilo bases
kDa	Kilo Dalton
LB	Luria Broth
LP	Long patch
MDM2	mouse double minute 2
MMR	Mismatch repair
MMS	methyl methanesulfonate
MTS	mitochondrial targeting sequence
MW	molecular weight
NAC	N-acetyl cysteine
NCA	Lucanthone and 7-Nitroindole-2-carboxylic acid
nCARE	negative calcium regulatory element
NER	Nucleotide Excision Repair
NF-κB	nuclear factor-κB
NHEJ	Non-homologous end joining
NIR	Nucleotide Incision Repair
NLS	Nuclear localization signal
OGG1	8-oxoguanine glycosylase
PAGE	Polyacrylamide gel electrophoresis
PARP1	poly(ADP-ribose) polymerase 1
PCNA	proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic Acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	Revolutions Per Minute
SAM	*S*-adenosylmethionine
SDS	Sodium Dodecyl Sulfate
siRNA	small interference RNA

SP	Short patch
SSB	Single strand breaks
ssDNA	single-stranded DNA
TRX	Thioredoxin
UDG	Uracil-DNA glycosylase
UV	UltraViolet
V(D)J	Variable Diversity Joining
XP	Xeroderma Pigmentosum

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To Mom, Dad, Tina and Perla...

For the good times and the time yet to come ...

Chapter 1: Introduction

1. General Overview

140 years after its discovery, deoxyribonucleic acid (DNA), the genetic material and the blueprint of the cell, is known to be responsible for holding the information needed to constitute all the components of a living cell, hence of a whole organism. Today, we are still studying its structure, its transmission from one generation to the other, its interaction with its environment and its regulation by different known and unknown factors in a cell's micro- and macro-environment.

The cell faces many challanges throughout its life, leaving it with different kinds of damaging agents that cause damage to proteins (enzymes, structural proteins, etc...), to DNA and to other important components. The cellular response to damage depends on the cell type and the nature of damage. It either resolves the problem or bypasses it, leaving in the latter case a potential permanent damage [1]. As one would expect, damage to DNA is the most harmful damage that a cell might undergo due to many facts. First, DNA replication will allow this damage, or "mutation" in other terms, to be transmitted to daughter cells; second, DNA transcription will give a modified protein product that might be non-functional. In fact, damage to DNA is considered to be the early step to carcinogenesis, mutagenesis, and aging [2, 3]. Consequently, the cell has evolved DNA repair pathways to overcome these challenges, as one of its surviving tools.

2. DNA Damage

Genome integrity is continuously challenged by endogenous and exogenous agents, which might generate different unwanted insults. Identifying the sources and nature of these insults is of crucial importance for the cell to determine the damage-specific strategy in order to remove a given lesion.

2.1 Sources of DNA damage

2.1.1 Endogenous origin

While the cell struggles to protect its genome from different damaging agents, it produces, ironically, its own "homemade" DNA damaging products, mainly reactive oxygen species (ROS) and reactive nitrogen species (RNS).

ROS are oxygen-derived free radicals that have one or more unpaired electrons. Superoxide (O_2^-), Hydrogen peroxide (H_2O_2) (non radical), hydroxyl radical ($OH\cdot$) among others belong to ROS family. On the other hand, RNS are nitrogenous products of nitric oxide synthases, ranging from Nitric Oxide to Nitrates [4]. ROS and RNS are produced by cellular metabolisms as by-products of the mitochondrial electron transport reactions and some metal-catalyzed reactions. They are also produced by immune system cells like neutrophils and macrophages during inflammation. Moreover, their formation can be induced by external factors like ultra-violet (UV) light, gamma radiation and X-rays. ROS can exist as pollutants in the atmosphere as well [5]. Once in the cell, these species may damage proteins, lipids and nucleic acids [6]. The harmful effects of ROS

can be balanced by an antioxidant system which includes enzymatic (e.g. superoxide dismutase SOD) and non-enzymatic antioxidants (e.g. vitamin E). Oxidative stress occurs when the balance between antioxidant and ROS production favours the latter. In DNA, ROS primarily induces oxidized bases and single-strand breaks (See section 2.2 for details). Paradoxically, at low concentrations, ROS and RNS can benefit the cell by increasing defence against infectious agents [6].

In addition to ROS, some naturally occurring phenomena, such as meiosis, V(D)J and class switch recombination in immunoglobulin rearrangements may also cause damage to DNA [7].

2.1.2 Exogenous Sources

Besides endogenous sources of DNA damage, the cell is subject to many exogenous factors that are either naturally occurring in a given cellular environment, like viral infection, UV-light from the sun and ionizing radiations (e.g. gamma radiation, X-rays...), or that are human-made like many mutagenic/carcinogenic substances (e.g. tobacco, DNA intercalating agents like ethidium bromide, etc...). These factors might act directly on DNA or indirectly by inducing an increase in ROS levels which, subsequently will damage the DNA. Chemotherapeutic drugs targeting the DNA are another example of man-made damaging agents [6]. They are divided into many categories: alkylating agents, antimetabolites, anthracyclines, plant alkaloids and topoisomerase inhibitors. All of these drugs lead to accumulated damage on the DNA which might interfere with cancer cell division and survival. Many secondary effects were associated with this type

of cancer treatment and the main reason is the untargeted mode of action of many of these drugs and their impact on healthy cells [8].

2.2 Types of DNA damage

DNA damage refers to any structural alteration of the DNA that will interfere with its normal conformation and composition leading to errors in transcription and replication. The types of DNA lesions vary depending on the origin of the damage and the targeted part of the DNA. DNA damaging agents can affect the nitrogenous bases as well as the sugar backbone. Base damage includes: oxidation, alkylation and deamination. Bases can also be spontaneously lost by hydrolysis. The DNA backbone can be broken on one or both strands leading to single strand breaks (SSBs) and double strand breaks (DSBs) [9].

2.2.1 Spontaneous base loss and AP sites

The N-glycosidic bonds that hold the nitrogenous bases to the sugar-phosphate backbone are weak points in the structure of DNA. Hydrolysis of this bond by a water molecule leads to the loss of the base and creating an apurinic-apyrimidinic (AP) site, also known as abasic site **(figure1)** [10]. The double ring structure of purines, adenine (A) and guanine (G), makes them more susceptible for hydrolysis than pyrimidines [10, 11]. AP sites are highly mutagenic lesions that interfere with DNA transcription and replication by blocking DNA and RNA polymerases. Some DNA polymerases bypass AP sites, incorporating an untemplated nucleotide into the newly synthesized strand, introducing

mutations [12, 13]. AP sites can also be generated as intermediates in the repair of base lesions by the base excision repair (BER) pathway, the main DNA repair pathways that repairs AP sites. (See section 4.1.1).

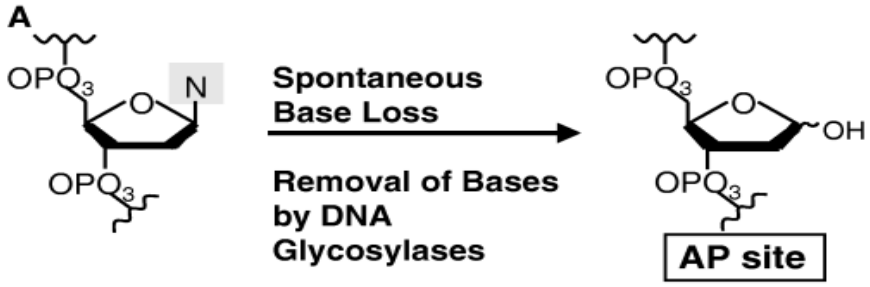

Figure 1. Abasic site. Adapted from [14].

2.2.2 Base modification

2.2.2.1 Base oxidation

DNA bases are subject to oxidation by different radicals, the most prominent of which is hydroxyl radical (OH·). Oxidation usually occurs on carbons 5 and 6 of pyrimidines and carbons 4 and 8 of purines [15]. Bases can be oxidized either within the DNA strand or in the dNTP pool before incorporation [16]. Some common oxidized bases include 8-oxoguanine (8-oxo-G), thymine glycol, uracil glycol, FapyG, and 5-formyluracil (**figure 2**). High levels of 8-oxo-G and thymine glycol have been reported under normal conditions as well as under oxidative stress in both nuclear and mitochondrial DNA [17]. They are highly mutagenic, if left unrepaired, they will lead to mutations that are lethal in some cases. The DNA polymerase might bypass this type of lesions, inserting the wrong nucleotide, thus creating transversion mutations as a consequence. Moreover, they can

lead to replication fork arrest after blocking the DNA polymerase, which might induce checkpoint responses and apoptosis. Modified bases can be removed by DNA glycosylases which cleave the N-glycosylic bond between the sugar and the base, leaving behind an AP site [16]. It is noteworthy that the sugar moiety of the DNA might undergo oxidation as well. In this latter case, oxidized AP sites and SSBs with 3' blocking groups are the possible outcomes [18] (see section 2.2.4).

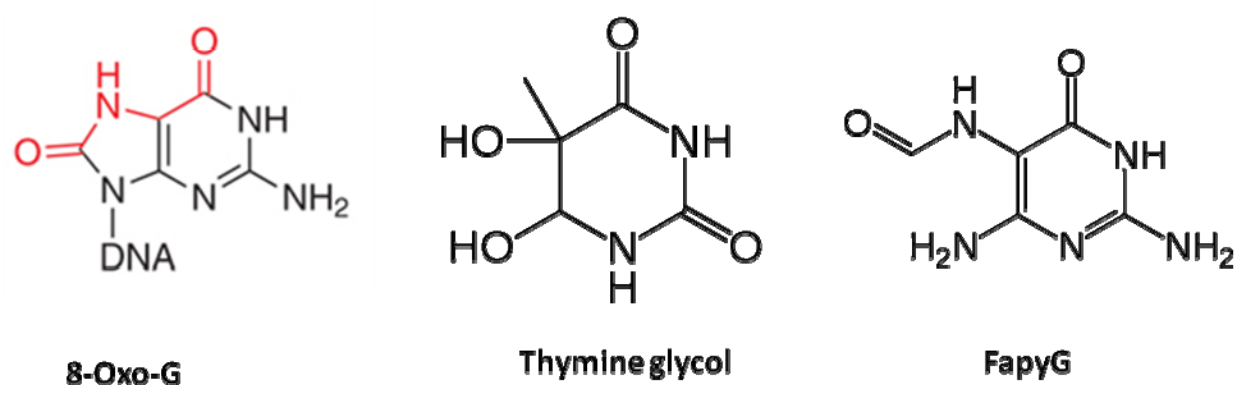

Figure 2. Examples of Oxidized bases.

2.2.2.2 Base alkylation

The transfer of an alkyl group to a nitrogenous base is another common base modification [19]. *S*-adenosylmethionine (SAM) is a well-characterized endogenous source of methylated bases, as it has a reactive methyl group that can be transferred to a DNA base, giving rise to 7-methylguanine (7-MeG) or 3-methyladenine (3-MeA) (**figure 3**) [20]. The drug methyl methanesulfonate (MMS) also induces these lesions [21]. 3-MeA blocks RNA and DNA polymerases and is highly toxic intermediates, whereas 7-MeG seems harmless. Alkyladenine-DNA glycosylase (AAG) is the only human enzyme that excises

3MeA from DNA, and creates AP sites as a consequence [22].

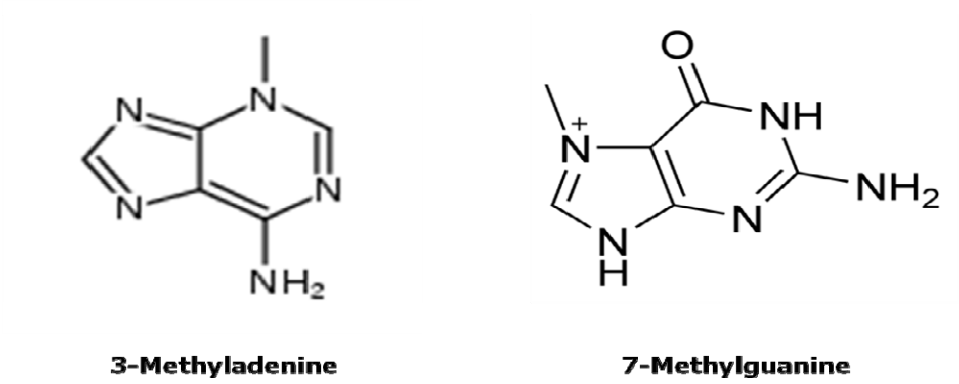

Figure 3. **Examples of alkylated bases**

2.2.2.3 Base deamination

Base deamination is the removal of the amine group, usually by hydrolysis, from DNA nucleobases. Hydrolytic deamination of cytosine generates uracil, which leads to GC to AT transversion mutations after DNA replication. Uracil-DNA glycosylase (UDG) hydrolyses the glycosylic bond between the uracil and the sugar, creating an AP site. 5-Methylcytosine can undergo a spontaneous deamination, generating thymine. Consequently, C to T transversions are the most common single point mutations. Such mutations are particularly dangerous because the thymine is not recognized as a damaged base [23]. Mismatch repair pathway allows the recognition and repair of mismatched bases as well as some insertions and deletions that occur during DNA replication or recombination [24]. Another way to deaminate DNA bases is by the action of reactive nitrogen species (RNS) in addition to several proteins such as activation-induced cytidine deaminase (AID) and Apolipoprotein B mRNA editing catalytic subunit 1 (APOBEC1)[25, 26].

2.2.3 Bulky lesions and cross links

Bulky lesions and inter- or intra-strand cross links are caused by the UV-light. The main lesions created by UVB are cis-syn cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) – pyrimidone photoproducts (**figure 4**), which connect adjacent pyrimidine molecules present in the same or different strands of the DNA to create a distortion in the DNA structure . 4-Nitroquinoline 1-oxide (4NQO) is a UV-mimetic drug that causes the above mentioned DNA lesions that are repaired by the nucleotide excision repair (NER) pathway. Deficiency in repairing such lesions has been associated with important human DNA repair syndromes such as xeroderma pigmentosum (XP) and Cockayne syndrome (CS) [27].

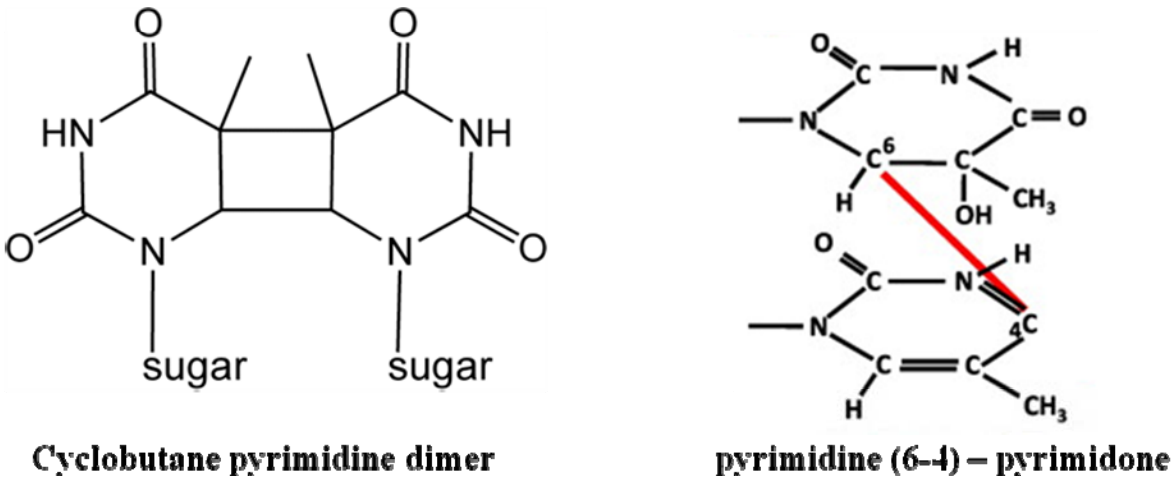

Figure 4. Main DNA lesions induced by UV-light.

2.2.4 Sugar-phosphate backbone breakage

2.2.4.1 Single strand breaks

SSBs are discontinuities in one strand of the DNA duplex. They occur as a result of spontaneous hydrolysis of the sugar-phosphate backbone, as a consequence of ionizing radiation (IR), or elevated ROS levels. SSBs can form directly by the removal of the oxidized sugar or indirectly during the base-excision repair (BER) of damaged bases. In the latter case, a DNA glycosylase cleaves off the damaged base to create an AP sites which is then cleaved by an AP endonuclease or lyase to create a SSB [28-30]. In addition to the sources mentioned above, some chemotherapeutic drugs directly induce SSBs. For example, bleomycin creates SSBs with 5'-phosphate and 3'-phosphoglycolate or 3'-phosphate termini [31].

If not repaired rapidly or appropriately, SSBs create a serious threat to chromosomal stability and hence cell survival. Consequently, cells have evolved rapid and efficient mechanisms for their repair during later stages of the BER pathway for instance [32]. (See section 4.1.1 for more details).

2.2.4.2 Double strand breaks

DSBs are the most dangerous DNA lesions because they can lead to large-scale genomic rearrangement and chromosomal fusions (translocations, deletions). A lot of naturally occurring phenomena prompt the formation of DSBs like: meiosis, mating type switching (in yeast), V(D)J and immunoglobulin class switch recombination. External factors like IR, drugs like bleomycin and chemicals also contribute to the formation of DNA DSBs. They are repaired mainly by two pathways: Homologous recombination (HR) and Non-homologous end joining (NHEJ) [7, 33-35].

3. DNA Damage response

Cells have developed elaborate mechanisms to sense and respond to DNA damage. These mechanisms are collectively called DNA damage response (DDR). DDR is today known to be a signal transduction pathway as it appeared to be a kinase cascade activated by DNA damage and replication stress. The main kinases of this pathways are ataxia telangiectasia and Rad3-related (ATR) and Ataxia telangiectasia mutated (ATM), a defect in which was associated with ataxia telangiectasia (AT) disease[36]. Although it was thought for years that the purpose of this cascade is to regulate the cell cycle transition, in the last decade it was shown that the DDR is versatile and that cell cycle control, at checkpoints level, is only one of many outcomes controlled by this sensory network whose central goal is the repair of DNA [37].

In human cells, DNA damage activates p53 which then induces the transcription of p21, a major inhibitor of the Cyclin-dependent kinases (CdKs). The inhibition of CdKs induces cell cycle arrest [38]. Moreover, Chk1 and Chk2, the two serine/threonine kinases that act downstream of ATR/ATM, are activated by phosphorylation in response to DNA damage. The activation of Chk1 and Chk2 leads to the inhibition of Cdc25, a dual specificity phosphatase that ensures cell cycle progression by promoting phase transition [39, 40]. Chk1 is the primary effector of the intra-S and G2/M phase checkpoints, whereas Chk2 plays a minor role on the intra-S and G1/S checkpoints [41]. **Figure 5** represents a simplified scheme of the cell cycle checkpoint pathways and the main key proteins involved and their effect on the cell cycle progression.

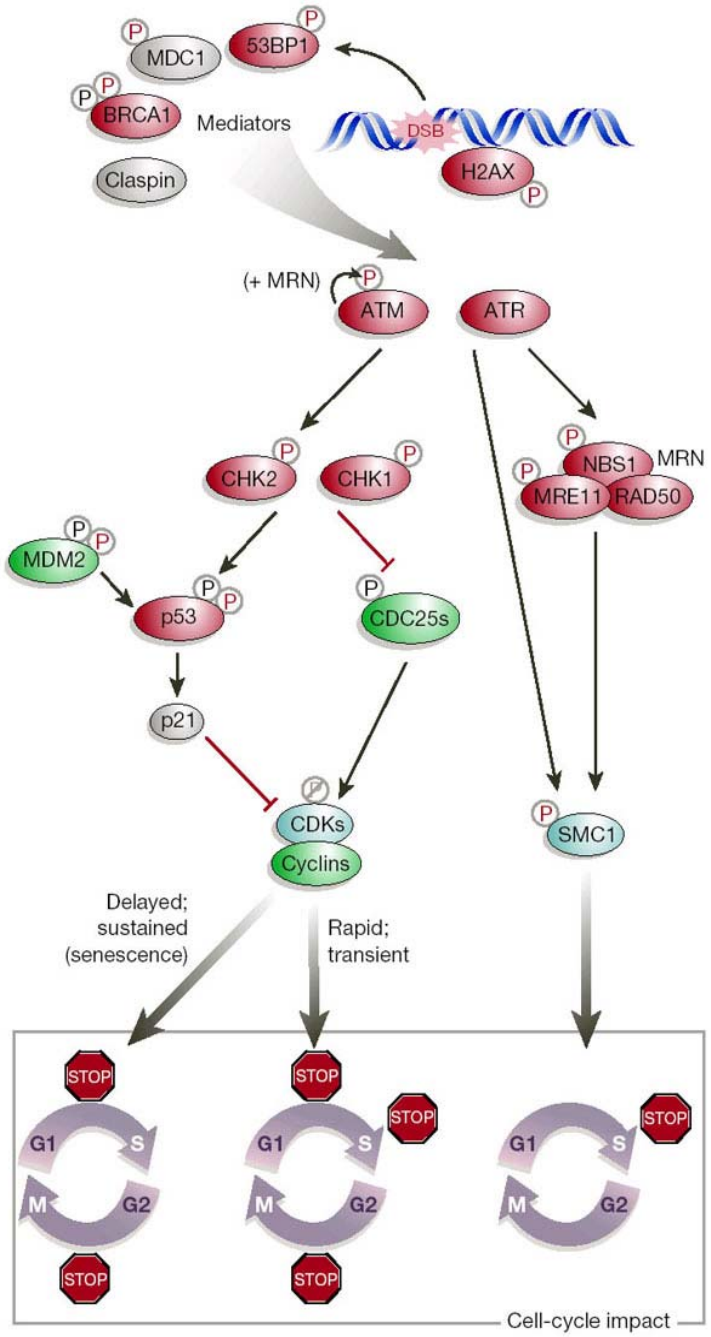

Figure 5. Cell cycle checkpoint pathways induced in response to DNA damage. In red: Tumor suppressors ; in green: proto-oncogenes. Adapted from [42].

4. DNA repair: a crucial line of defence

4.1 DNA repair pathways

The diversity and specificity of DNA repair pathways come from the need to repair different types of lesions that arise in the DNA. DNA repair pathways are damage-specific as shown in **figure 6** that summarizes different types of damages, types of lesions created and the major DNA repair pathway involved.

4.1.1 Base excision repair

This section will summarize BER in more details, focusing mainly on the pathway as it occurs in humans.

Base excision repair (BER) is a vital DNA repair pathway for living cells since it is responsible for correcting most common types of damages occurring in the genome: damaged bases (e.g. deaminated, oxidized and alkylated bases), AP sites, as well as single strand breaks with 3'-blocked termini [43, 44]. Given this role, BER pathway is considered one of the major DNA repair pathways that deals with most DNA damage [45, 46]. Moreover, this pathway is well conserved from bacteria to human in terms of the core components of its DNA repair machinery [47, 48].

The BER pathway was discovered nearly 35 years ago by Thomas Lindahl who searched for an enzymatic activity that catalyzed the removal of uracil from DNA. In *E.coli*, he identified uracil DNA glycosylase (UDG) [49, 50], and other groups subsequently demonstrated that this enzyme is conserved in most organisms [51, 52].

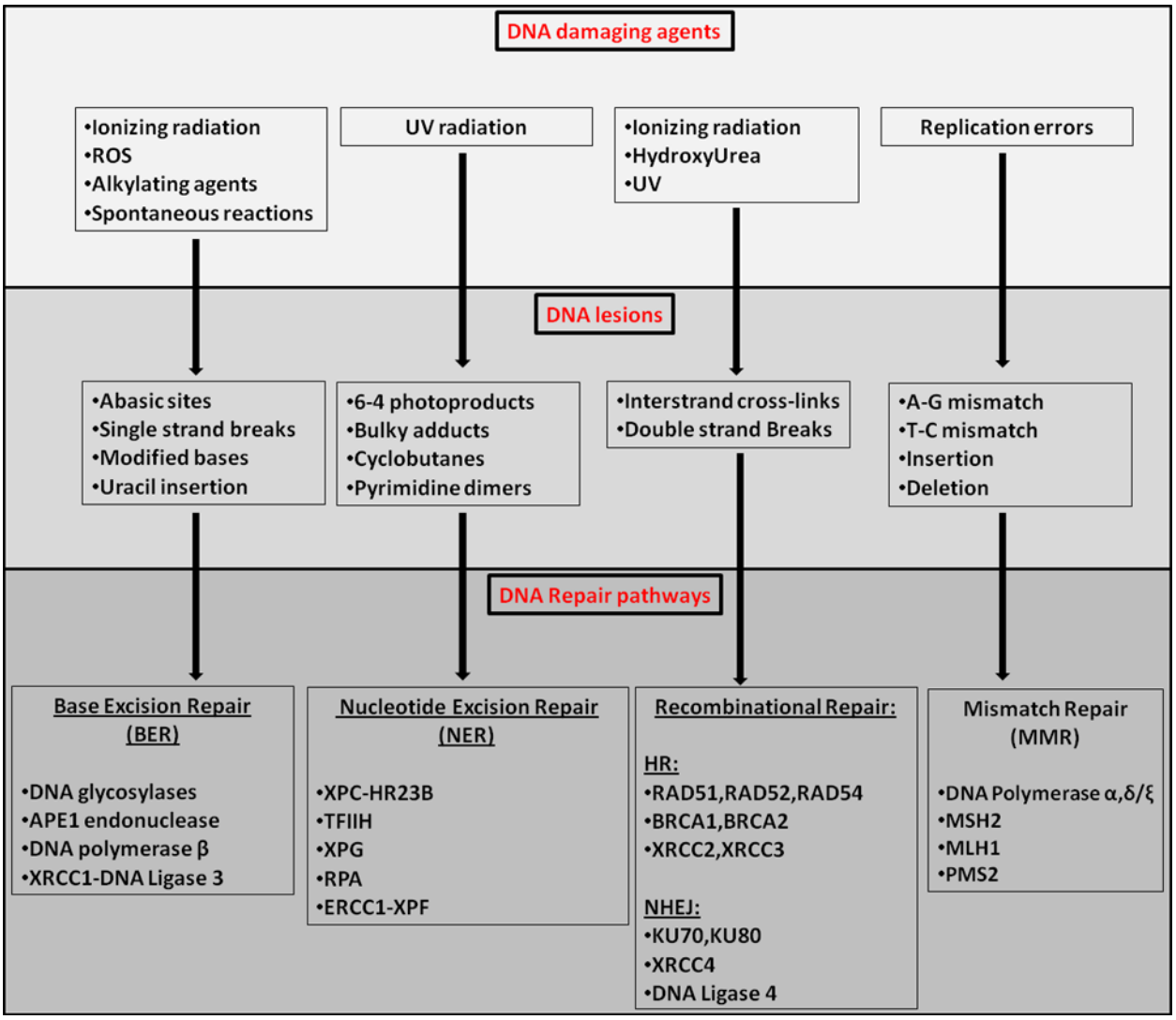

Figure 6. The main damage-specific DNA repair pathways in human cells. DNA-damaging agents (top), examples of DNA lesions (middle), and the Corresponding DNA repair pathways (bottom). The essential genes involved in each DNA repair pathway are shown below the corresponding titles. HR, homologous recombination; NHEJ non-homologous end-joining. Adapted from [53], [54] and [55].

Subsequent work over the next two decades identified more glycosylases specific for other damaged bases as well as AP endonucleases [56, 57].

BER occurs in five major steps: (1) Recognition and removal of the damaged base, (2) enzymatic incision of the AP site, (3) processing of the ends of the SSB by AP site incision, (4) repair DNA synthesis, and finally (5) ligation of nick to restore the continuity of the DNA strand [58]. These will be discussed in detail below, with an emphasis on step 2. **Figure 7** represents an overview of the pathway.

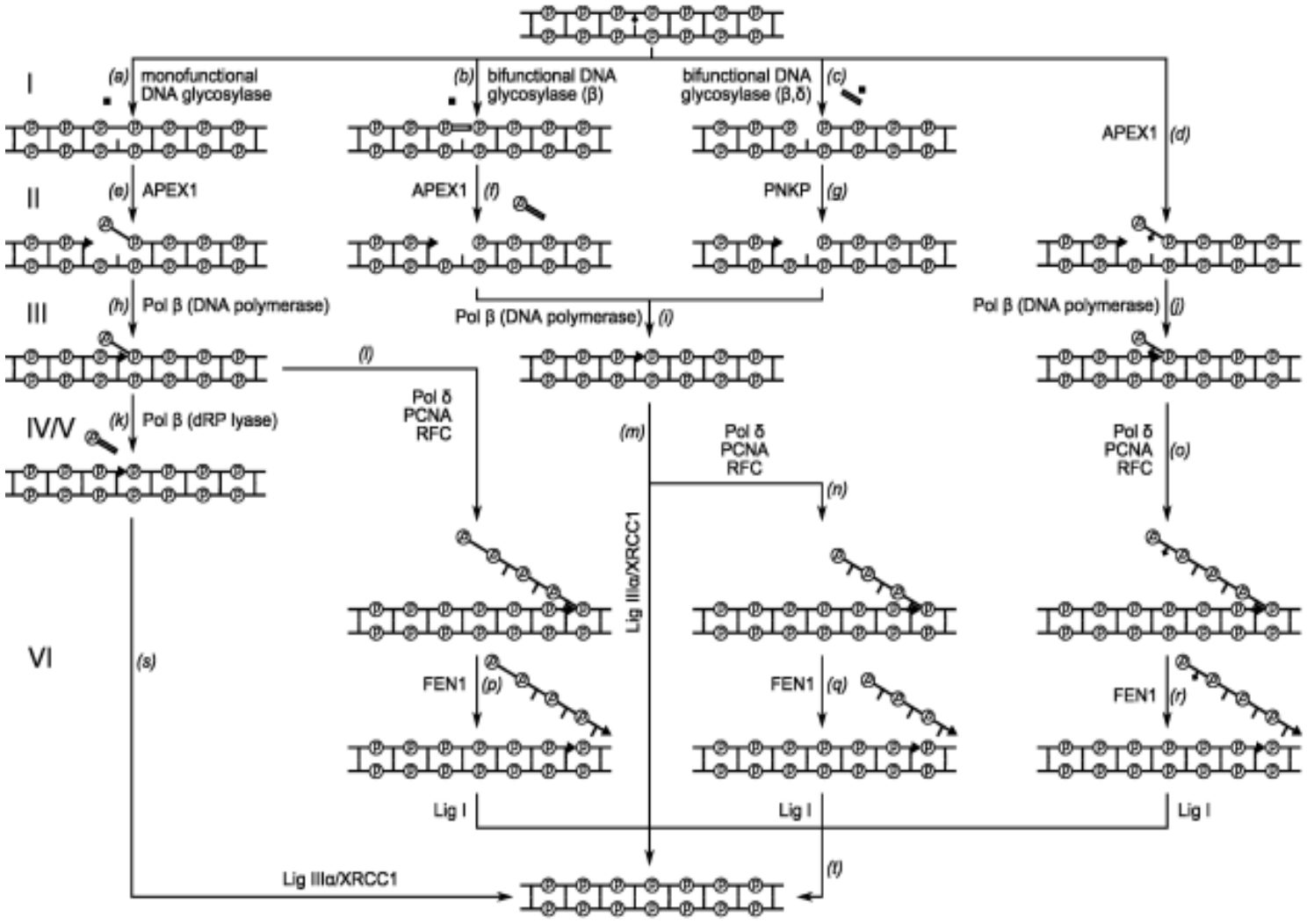

Figure 7. Overview of the BER pathway and its subpathways. Adapted from [47]

4.1.1.1 Damage Recognition

The first step in BER is the recognition of the modified base by a DNA glycosylase. Glycosylases cleave the N-glycosylic bond linking the damaged base to its corresponding deoxyribose sugar. DNA glycosylases are classified as either mono or bi-functional. While both classes of glycosylases hydrolyze the N-glycosylic bond, bifunctional glycosylases, such as 8-oxoguanine glycosylase (OGG1) and NEIL1/2/3, are endowed with an additional AP lyase activity. These enzymes can further process the AP site through β or β-δ-elimination to generate SSB with 3'-polyunsaturated aldehyde or 3'-phosphate termini, respectively [59-61]. Although DNA glycosylases are substrate specific, the recognition of different modified bases by the same glycosylase, or the same damaged base by multiple glycosylases still occur, thus providing redundancy [62].

4.1.1.2 AP sites and blocking groups processing

The removal of the modified base by the DNA glycosylase generates the highly mutagenic AP site intermediate. These lesions are substrates of the catalytic activity of AP endonuclease family, AP lyases and delta lyases. While delta lyases create an incision at AP sites and process 3'-aldehyde to create 3'-phosphate termini, AP endonuclease enzymes incise the phosphodiester backbone at the 5'side of AP sites to generate 3'-hydroxyl and 5'-deoxyribosephosphate (dRP) termini [61]. These unconventional termini have to be restored to 3'-OH and 5'-P through deoxyribose-phosphatase diesterase

(dRPase) activity of Pol β (5'-dRP), 3' diesterase activity of the Apurinic/apyrimidinic endonuclease 1 APE1 (3'-PUA) and the phosphatase activity of PNKP in order to allow further repair [14, 58].

However, between the AP endonucleases and the AP lyases cleavage of AP sites, the AP endonuclease incision is the predominant pathway [61]. The importance of AP endonucleases functions over the AP lyases has also been supported by findings showing that endonuclease deficient yeast strains are more sensitive to MMS, but not AP lyase deficient yeast strains [63, 64]. AP endonuclease families will be discussed in further details in sections 5.

4.1.1.3 BER sub-pathways: the short and long patch

4.1.1.3.1 Short Patch BER

In SP-BER, DNA polymerase β (POL β) performs a one-nucleotide gap-filling reaction and removes the 5'dRP via its lyase activity. This is then followed by sealing of the remaining nick by the XRCC1–ligase3 complex. XRCC1 is a scaffold protein that interacts with several of the BER core components and may therefore be important in protein exchange [65].

4.1.1.3.2 Long Patch BER

In long-patch BER (LP-BER), APE1 catalyzes the formation of a nick 5' to the AP site. This action recruits POL β or DNA polymerase δ (POL δ), PCNA, flap structure-specific endonuclease 1 (FEN1), and DNA ligase 1 (LIG1). In a PCNA-dependent manner POL β performs displacing synthesis and polymerizes a tract of DNA from ~2-10 bases, which produces a flapped substrate that is refractory to ligation. FEN1 cleaves the flap at the ssDNA-dsDNA junction, creating a ligatable nick, which is then sealed by DNA ligase I [66].

It was concluded that the short-patch BER (SP-BER) repair is probably the dominant subpathway of BER. [53]. However, it was shown that many factors such as cell state (cell cycle phase, differentiation stage), BER protein–protein interactions and the types of lesions are all involved in the selection between SP- and LP-BER [67].

4.1.1.4 BER and cancer

Cancer is, without any doubt, the disease of the past century. Since AP sites are considered the most occurring type of lesions arising in the DNA, they are major contributors to DNA mutations. Accumulated mutations often lead to subsequent activation of proto-oncogenes and inactivation of tumour-suppressor genes, the hallmarks of cancer. Therefore, BER implication as a cancer-prevention pathway is essential. Indeed, numerous links have been identified between oncogenesis and acquired or inherited alterations in the genome "guardians", highlighting the key role of DNA repair

systems in tumour prevention. In the last decade, evidence of association of BER deficiency with cancer has started to emerge. For instance, studies have demonstrated a link between defects in the adenine–DNA glycosylase MYH and adenomatous colorectal polyposis and colorectal cancer risk [68]. Reduced activity of OGG1 was associated with elevated lung cancer [69].

Moreover, BER enzymes are attractive targets for inhibitor-based cancer therapy. Many chemotherapeutic drugs act by damaging the DNA, leading to cell death. Therefore, selective inhibition of repair in cancer cells could sensitize them to the drug [70].

5. AP Endonuclease/3’ -Diesterase

AP sites are very common because, besides being formed spontaneously, they are intermediates in repair of most types of base damage. AP sites are repaired primarily by AP lyases and AP endonucleases. AP endonuclease family members cleave the sugar-phosphate bond 5′ of an AP site to produce a 3′ hydroxyl group and a 5′-deoxyribose phosphate [71, 72]. The first evidence of the presence of an enzyme that is capable of processing AP sites emerged from studies in *E.coli*. These studies showed that an enzyme termed exonuclease III, first described as a 3’ to 5’ exonuclease with a 3’-phosphatase activity, also possesses an AP endonuclease activity [73, 74]. To date, two families of AP endonucleases / 3’-diesterases, named Endonuclease IV (Endo IV) and Exonuclease III (Exo III) after the ancestral bacterial enzymes, have been well characterized [61]. In addition to AP endonucleases, AP lyases are also capable of processing AP sites. In fact, the *E. coli* endonuclease III is an AP lyase that cleaves on the 3′ side of the AP site

through a β-elimination reaction to produce a SSB with 3' α,β-unsaturated aldehyde, processed by AP endonucleases [75, 76].

5.1 Two Distinct families of AP endonucleases/3' Diesterase

Despite a certain degree of redundancy between the Exo III and Endo IV AP endonucleases families, some differences in expression and substrate specificity do exist. However, the key feature used to differentiate between the two families is their magnesium dependency. While members of the Endo IV family are Mg^{2+} independent, Exo III family members require magnesium for their DNA repair activity [61]. Interestingly, these two proteins use completely different structural folds to accomplish basically the same set of activities [77].

In *E.coli*, Exo III is encoded by the *xth* gene and is the major AP endonuclease accounting for around 90% of the total AP endonuclease activity in extracts. Exo III possesses four different catalytic activities. First, Exo III is an apurinic/apyrimidinic endonuclease activity. Second, it has a 3' to 5' exonuclease with an activity specific for dsDNA allowing it to degrade blunt ends and 5' overhangs. Third, it can also act as a 3' -phosphodiesterase that removes groups including, phosphates and phosphoglycolate in addition to the aldehydes left by AP lyases [78-80] to allow the DNA polymerase to prime DNA synthesis from a 3' -OH [71]. Fourth, it has an RNase H activity to degrade RNA in DNA-RNA hybrids [57, 81, 82].

While Exo III family members are present in all kingdoms of life, Endo IV homolog exist in prokaryotes and lower eukaryotes, but not in plants, mammals and many other vertebrates. Like Exo III family members, the Endo IV enzymes possess 3'-diesterase, 3' -exonuclease and RNase H activities [61].

In *E.coli* Endo IV is encoded by the *nfo* gene. It accounts for only 10% of the basal AP endonuclease activity but is induced by oxidative stress [57, 82]. In contrast, Apn1, the *S. cerevisiae* Endo IV homolog, is the major AP endonuclease in yeast and shares 41% sequence identity with the bacterial enzyme [83]. Yeast *apn1Δ* cells show less than 1% of AP endonuclease/3' diesterase activities of wild-type, a 60 fold increase in AT→GC transversion mutations, and hypersensitivity to alkylating agents and chemical oxidants[84, 85]. Apn2, the *E.coli* Exo III homolog in yeast was discovered as the second and the minor AP endonuclease in yeast after Apn1 [86]. As in *E.coli,* yeast Apn2 was shown to possess AP endonuclease, 3' diesterase and 3' to 5' exonuclease activities that require magnesium [63]. The transcription of Apn2 gene was found to be induced up to six-fold by treatment with MMS [87] and its 3'-diesterase and 3'→5' exonuclease activities stimulated by proliferating cell nuclear antigen (PCNA) [88].

In human cells, AP endonuclease 1 (APE1) and AP endonuclease 2 (APE2) are the human homologues of the Exo III family. APE1 is the major human AP endonuclease/3' diesterase and since the research project concentrates on the DNA repair capacity of APE1, this enzyme will be discussed in more details in section 6 below. This enzyme has previously been known as APEX in mice [89], "HAP1" for Human AP endonuclease 1 [90], and redox factor-1 (Ref-1) for its role as a redox regulator [91], but will be referred to as APE1 here.

Based on genomic databases from various organisms, APE2 was identified as a nuclear and mitochondrial AP endonuclease in human cells[92]. APE2 was found to possess a very weak AP endonuclease activity and an even weaker (7-fold lower) 3' diesterase activity [93]. Moreover, APE2, unlike APE1, could not complement yeast AP endonuclease mutants [94]. While APE1 has been reported to have no functional mitochondrial targeting sequence (MTS), it was shown that APE2 possesses a MTS and has a potential role in the repair of mitochondrial DNA lesions [92]. Moreover, it was reported that APE2 has a long C-terminal domain that contains a PCNA binding motif, similar to the one present in *S. cerevisiae* Apn2, suggesting a possible role in LB-BER [92]. However, the presence of APE2 did not rescue the lethal phenotypes of the knockout and the knockdown of APE1, which rule out possible significant roles for APE2 in DNA repair. The *in vivo* role of APE2 remains unclear; it might be a pseudogene or an evolutionary relic.

Many attempts failed to identify the Endo IV homolog in human. The highest organism where both AP endonucleases were identified and characterized is *Caenorhabditis elegans* (*C.elegans*) [95, 96]. In addition, Exo III and Endo IV were both identified but not yet characterized in the frog *Xenopus tropicalis* and the fish *Danio rerio* [97].

6. APE1

APE1 was first characterized and purified from HeLa cells in 1981 by the group of Stuart Linn [98]. Later, cDNAs encoding several mammalian AP endonucleases (human,

murine and bovine) were published [99-102]. The APE1 protein is closely related to other mammalian AP endonucleases (91-93% identity) and more distantly to *E.coli* Exo III (28% identity). Like Exo III, APE1 has a Mg^{2+}- dependent AP endonuclease activity *in vitro* [98, 103].

6.1 APE1: a DNA repair enzyme

As described earlier, APE1 is considered of major importance in protecting the cell from the effects of cytotoxic and mutagenic unrepaired AP sites.

The expression of the APE1 protein in human cells is activated by DNA damaging agents and ROS, in order to process the resulting AP sites during the BER [104]. The second major role of APE1 in the BER is its 3' diesterase activity. Although the diesterase activity of APE1 is about 200-fold lower than its AP endonuclease activity, this function allows it to remove a variety of 3' blocking groups including 3'phosphate and 3' phosphoglycolate occurring at single-strand breaks created by oxidative agents, ionizing radiation or by a chemotherapeutic agent like Bleomycin which creates 3' phosphoglycolate [105, 106].

APE1 is one of the major enzymes orchestrating the mammalian BER. In addition to its AP endonuclease/3' –diesterase activity, APE1 may have also an implication in the selection between the SP- and LP-BER subpathways. It was proposed that APE1 is active in both LP- and SP-BER and that it interacts with several proteins in these two subpathways including OGG1, XRCC1, PCNA, FEN1, and DNA polymerase β. Hence, APE1, owing to its interactions with other BER proteins and to its structural mechanism

of recognition of the AP site, it serves as an assembly and coordination factor for subsequent proteins after its initial cleavage of the DNA [107, 108].

In the first step of BER, after the DNA glycosylase removes the damaged base, it remains bound and presents the AP site to APE1. Due to the extensive surface of APE1-DNA interaction that covers both DNA strands and the DNA kinking, APE1 displaces the glycosylase allowing it to release the damaged base. After cleaving the AP site, the nicked DNA is exposed to DNA polymerase and/or XRCC1. This product-substrate exchange might be partially mediated by APE1 N-terminal and the DNA polymerase dRP-diesterase domain. Following repair, the DNA can no longer accommodate the bending and the component enzymes likely dissociate [109]. It appears that APE1 interactions with other BER proteins show a high level of DNA repair organization for maximal efficiency in a relatively large mammalian genome.

Although they belong to different AP endonucleases families and to two different species, it is noteworthy that the human APE1 shares many common features with the yeast Apn1. First, each one of them is the major AP endonuclease/3' diesterase enzyme in its corresponding cellular context. Second, they are both capable of incising the phosphodiester bond at the 5' of an AP site and of removing similar 3' blocking groups, *e.g.* 3' phosphate, 3'phosphoglycolate that are associated with Ionizing radiation (IR) and ROS [71, 110, 111]. However, in contrast to what might be predicted based of the biochemical repair activities of APE1 and Apn1, APE1 knockout mouse blastocysts and antisense-expressing human cells are hypersensitive to γ rays and oxidizing agents, whereas Apn1 yeast mutants are at best mildly sensitive to γ rays and oxidizing agents like (e.g. H_2O_2) [105]. These results suggest that there might be other species-specific

factors implicated in alternative repair mechanisms that need to be taken into consideration when assessing the right model (yeast or mammalian cells) to study DNA damage response to certain genotoxic agents.

Besides its activity in the BER pathway, APE1 was shown to be the damage-specific endonuclease in the nucleotide incision repair (NIR) pathway, a known feature in Endo-IV family of endonucleases [112]. NIR pathway was proposed as an alternative pathway to classic BER. In this pathway, APE1 nicks oxidatively damaged DNA (e.g. 5,6-dihydro-2'-deoxyuridine, 5,6-dihydrothymidine (DHT), 5-hydroxy-2'-deoxyuridine, alpha-2'-deoxyadenosine and alpha thymidine adducts) in a DNA glycosylase-independent manner, generating 3'-OH and 5' phosphate. This mechanistic feature is distinct from DNA glycosylase-mediated BER and has the advantage of avoiding the genotoxic intermediates (i.e. AP sites) generated in the BER pathway [113].
A recent study showed that APE1 appears to suppress the activation of poly(ADP-ribose) polymerase 1 (PARP1) during oxidative damage repair which promotes cell survival [114].

Furthermore, some groups showed that APE1 also possesses 3' to 5' exonuclease activity which plays a role in the excision of deoxyribonucleoside analogs from the DNA [115, 116]. If this is the case, the inhibition of this activity could have implications in treating some cancers where nucleoside analogs such as gemcitabine are used. Conflicting data are present in the literature regarding this latter activity of APE1. Some groups showed that this activity is structure specific and presented a preference for 3'-mismatched nucleotides [115, 117, 118], while others showed that it presented no exonuclease activity against blunt ended dsDNA [93, 119]. Interestingly, the yeast Apn1 is endowed with a

well-characterized 3' to 5' exonuclease activity that is capable of removing incorporated 8-oxo-G from the DNA, a function that possibly APE1 might have [120].

Given the many DNA repair functions of APE1, it is clear that this enzyme is fundamental to the maintenance of the genome and survival of cells. Hence the observation that even in the absence of exogenous DNA damage, siRNA directed against APE1 results in a decrease in proliferation, an increase in AP sites and increased levels of apoptosis [121].

6.2 APE1: a Redox enzyme

In addition to its role in DNA repair, APE1 has also been implicated in transcription regulation by reducing and thus activating many transcription factors. APE1 was first shown to reduce the conserved cysteine residues in Fos and Jun. Fos and Jun form a heterodimeric complex that regulates gene transcription by binding to the activator protein-1 (AP-1) DNA sequence motif. APE1 activation of Fos and Jun stimulates AP-1 DNA-binding activity [91].

Later reports have revealed a similar role of APE1 in activating p53, Fos, Jun, nuclear factor-κB (NF-κB), PAX (paired box-containing family of genes), hypoxia inducible factor-1α (HIF-1α), HIF-1-like factor and others [105]. The biological implication of these transcription factors activation is no fully clear yet, but what is known so far that APE1 redox function facilitates the DNA binding of some of these transcription factors or change the transcriptional activity of others [105]. Interestingly, it was demonstrated

that p53 binding to DNA and the subsequent transcriptional activation of p21, BAX or cyclin G is dependent on APE1. Moreover, APE1 protein was reported to stimulate p53 activity by both redox-dependent and -independent means [122].

APE1 has also been implicated in a number of other redox activations, one of which is the activation of bioreductive drugs requiring reduction for their activity [123]. Hence APE1 acts as a redox factor that senses the redox state of the cell.

APE1 redox activity requires the N-terminal region of the protein as the truncation of the 62 N-terminal residues renders the enzyme redox deficient (**Figure 15 and 16**) [124, 125]. It is noteworthy that human APE1 has 61 N-terminal additional amino acids that are not present in the *E.coli* Exo III. It was proposed that APE1 redox activity involves a cysteine residue(s) [124] that according to LJ Walker *et al.* was Cys 65 that forms a disulfide bridges with Cys 93 [125]. However, several reported crystal structures of APE1 showed three different observations contradicting the latter finding. First, no disulfide bridges were found in the crystal structures. Second, Cys 65 is buried inside the protein and is not accessible by the target transcription factors reduced by APE1 [77, 126, 127]. Third, all the reported APE1 crystallographic lattices are similar, suggesting the absence of conformational changes that could reposition Cys 65 [77]. Moreover, mice in which Cys 64 (equivalent to human Cys 65) is mutated to Ala show no developmental defects [128]. Thus, the mechanism by which Cys 65 acts in APE1 reduction of transcription factor needs to be further investigated.

6.3 Importance of APE1 cellular functions

Given the mild phenotype seen in other AP endonuclease-deficient organisms, lethality of APE1 -/- mice and knockout cells was a big surprise.

Unlike bacteria and yeast, which tolerate the complete loss of AP endonuclease activity with little consequence unless challenged with DNA damaging agents, APE1 knockout in mice is lethal at early embryonic stages, E3.5–E9.5 [129, 130].

However, heterozygous mutant mice developed into adulthood without any apparent abnormalities [129]. Although it was not clear at the time which function(s) of APE1 is contributing to the lethality phenotype seen in mice, a recent study using a specific inhibitor of APE1 redox function demonstrated that APE1 may play a role in normal embryonic hematopoiesis and that its redox function, but not the DNA repair endonuclease activity, is critical in normal embryonic hematopoietic development [131]. On the contrary, evidence for importance of DNA repair function was presented by another group who showed that epiblast cells have fragmented nuclei and undergo apoptosis and that explanted homozygous APE1-null blastocysts displayed increased sensitivity to γ-irradiation [130].

Further research showed that loss of APE1 is lethal at the level of the cell. Human cells in which APE1 is deleted by a microinjection of a Cre expression plasmid, undergo apoptosis within 48 hours [132].

Since APE1 deletion is lethal, many groups manipulated APE1 expression levels by either downregulation or overexpression. RNA interference (RNAi) knockdown resulted

in decreased cell proliferation and apoptosis which was correlated with an accumulation of AP sites [121]. Moreover, lower levels of APE1 render mammalian cells hypersensitive to MMS and H_2O_2 [133]. Importantly, the defects seen in knockdown cells were rescued by the expression of the yeast Apn1. Thus, the AP endonuclease activity of APE1 was shown to be essential for cell viability.

To date, many types of inhibitors of APE1 activities have been discovered. Lucanthone and 7-Nitroindole-2-carboxylic acid (NCA) were shown to be direct inhibitors of APE1 DNA repair activity [70, 134]. Recently, a novel quinone derivative that inhibits APE1 redox activity was identified, namely E3330 ([(2E)-3-[5-(2,3dimethoxy-6-methyl-1,4-benzoquinolyl)]-2-nonyl-2-propenoic acid]) [135].

In this context, a beneficial effect of the reduction of APE1 levels is to sensitize cells to chemotherapeutic agents such as bleomycin, carmustine, gemcitabine [136-138].

On the other hand, APE1 overexpression in human cells enhances tumour resistance to DNA damaging drugs and radiotherapy, as expected, a phenotype seen in many cancer cells [139].

6.4 Regulation of APE1 Activity

APE1 is regulated at two different levels: transcriptional and post-translational. Regulation of the activity of an essential enzyme like APE1 is of great importance for the cell to respond properly to DNA damage and to other diverse stimuli where it is involved.

6.4.1 Gene expression regulation

APE1 transcription is known to be induced by ROS. This regulation was proposed to happen in two steps: first APE1 translocates from the cytoplasm to the nucleus. Second, *de novo* protein synthesis takes place via transcriptional activation at the promoter level [104, 140-142]. Interestingly, APE1 was shown to autoregulate its own transcription by binding to its own promoter followed by inhibition of transcription at the level of its negative calcium regulatory element (nCARE) [143].

6.4.2 Post-translational modifications

APE1 is an abundant protein (~10^4–10^5 copies/cell) within eukaryotic cells and with a relatively long half-life of about 8 hours [144]. Therefore, the needed regulation of this multifunctional enzyme must lie in the many post-translational modifications that this enzyme undergoes.

6.4.2.1 APE1 structure

The N-terminal region contains the nuclear localization signal (NLS) and also appears to regulate the DNA binding activity of many transcription factors in vitro. Particularly, the residues 43-62 are necessary for APE1 redox activity [91, 122, 124, 125].

The active site residues of APE1 have been determined by site-directed mutagenesis and by analysis of sequence conservation between APE1 and Exo III [72, 145, 146]. There is

an absolute requirement for divalent metal ions for APE1 catalytic activity, with a distinct preference for magnesium [145]. **Figure 8** highlights APE1 active site, illustrating the main residues implicated in its catalytic activity along with the ones critical for binding to AP sites in DNA.

Comparison of the crystal structures of APE1 and Exo III reveals three loop regions that act in AP site recognition and cleavage. While many structural similarities between APE1, Exo III and the bovine endonuclease Deoxyribonuclease I (DNase I) have been reported, DNase I does not have an AP endonuclease activity since it has different loops and α-helices from APE1 and Exo III. These loops contribute to the positively charged grooves that form the DNA-binding faces of APE1 and Exo III thus dictating their specificity as AP endonucleases [77, 81, 109].

Although they are structurally unrelated enzymes, analysis of the crystal structures of APE1 and the bacterial Endo IV binding to an AP site shows that both enzymes orient the AP site via positively charged surfaces and insert loops into the DNA base stack, bending and kinking the DNA to promote flipping of the AP site into a sequestered enzyme pocket that contains only the damaged nucleotide [109].

His309 is considered the catalytic residue of APE1. It acts as the general base to abstract a proton from a water molecule, while Asp283 orients the imidazole ring of His309 and stabilizes its transiently- positive charged state along with Mg^{2+}. The resulting hydroxide ion then attack the 5' phosphate of an AP site via an inversion of configuration. The mutations of His309 to Asn and of Asp283 to Ala result in the elimination of enzymatic activity, showing the importance of these two residues for APE1 activity [77]. The

mechanism of the phosphodiester bond cleavage at the 5' of an AP site is summarized in **Figure 9.**

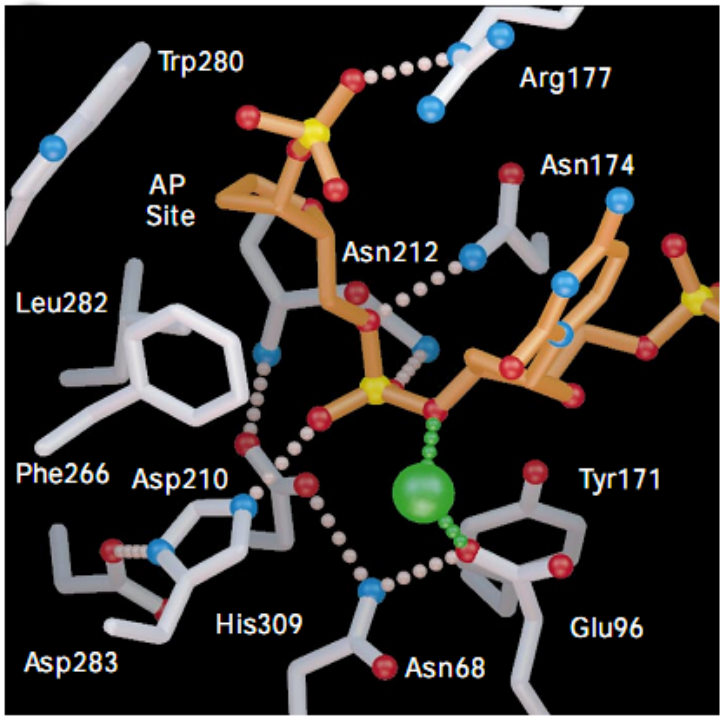

Figure 8. APE1 active site interactions with the AP site. Adapted from [77].

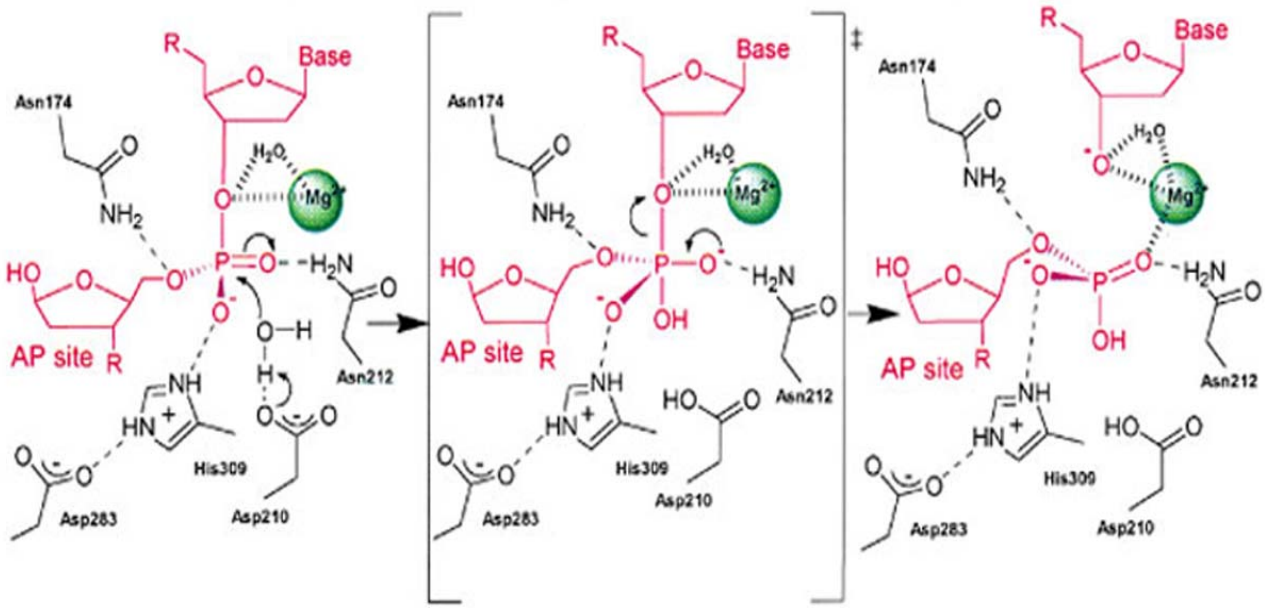

Figure 9. AP site cleavage reaction mechanisms by APE1catalytic site residues.

Adapted from [77].

6.4.2.2 Phosphorylation

It was demonstrated that APE1 is a substrate for phosphorylation by different kinases such as serine/threonine casein Kinases (CK) I and II and protein kinase C. However, phosphorylation uniquely by CK II was shown to inhibit the AP endonuclease activity of APE1 on AP sites [147]. Conversely, two years after this discovery another group showed that this phosphorylation by CK II does not have any impact on the DNA repair capacity of APE1, instead it enhance the redox function of APE1 shown by the increase of AP-1 binding to DNA [148].

6.4.2.3 Acetylation

p300 is a transcriptional co-activator that was shown to acetylate APE1 at Lys6 and Lys7 *in vitro* and *in vivo* in a Ca^{2+}-dependent manner. This modification enhances APE1 binding to nCARE which was shown to downregulate the parathyroid hormone (PTH) expression [149]. This acetylation is reversed by class I histone deacetylases (HDACs). Moreover, it was demonstrated that APE1 acetylation is needed for the activation of the Phosphatase and tensin homolog (PTEN) gene [150].

6.4.2.4 Ubiquitination

A recent discovery identified APE1 as a novel target of ubiquitination. This modification occurs on Lys residues of the N-terminus of the protein and involves the E3 ubiquitin ligase mouse double minute 2 (MDM2), in a p53-dependent manner. The ubiquitinated APE1 was shown to be predominantly present in the cytoplasm compared to nuclear localization of the unmodified protein. Although it was proposed that cytoplasmic localization of the ubiquitinated APE1 might be an indication of either its degradation or an alteration of its function, the effect of ubiquitination on APE1 is still not clear [151].

6.4.2.5 Nitrosylation

Oxidative stress was shown to have an effect on APE1 activity. Interestingly, nitrosative stress was also shown to modify this enzyme. The nitrosation of APE1 on Cys93 and Cys310 induces the translocation of APE1 to the cytoplasm, a phenomenon reversed by treatment with reducing agents [152]. The biological implication of this modification needs more investigation.

6.4.2.6 Redox regulation

In addition to the post-translational modifications listed above, APE1 diverse activities were found to be regulated by the reduction and oxidation. Thioredoxin (TRX) is a pleiotropic cellular factor that has thiol-mediated redox activity implicated in many cellular processes, including the reduction and activation of APE1 redox function [153].

The reduction of oxidized APE1 by TRX leads to intensified APE1-mediated p53 activation and enhanced AP-1 DNA binding [154]. Although the target residues of the reduction on APE1 are not known, they may include Cys65 and Cys93 since these sites are thought to be redox sensitive [125]. Moreover, it was shown that the redox state of APE1 affects its AP endonuclease activity *in vitro* and that this modification involves a specific cysteine residue of this enzyme [155].

Recently in our laboratory, we showed that APE1 interacts directly with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which converts the oxidized form of APE1 to the reduced active form, thus maintaining its AP endonuclease activity to repair AP sites [156]. Investigating the implications and aspects of this novel finding will be the object of my research project.

7. GAPDH: a multifunctional protein

GAPDH is a tetrameric enzyme (**Figure 10**) that uses the oxidized form of nicotinamide adenine dinucleotide (NAD^+) to converts glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, releasing NADH [157]. GAPDH is regarded as a housekeeping gene whose transcript level remains constant under most experimental conditions, and it has been frequently used as an internal control in studying the regulation of gene expression. Mounting evidence, however, has started to support the multi-functionality of this enzyme, identifying roles in a variety of cellular processes, independent from its glycolytic role.

GAPDH was shown to have phosphotransferase/kinase activity that serves to phosphorylate other proteins [158-162]. Interestingly, GAPDH was shown to have autophosphorylation activity as well [163]. Other new functions includes regulation of the cytoskeleton [164], membrane fusion and transport [165, 166], transport of nuclear RNA [167], glutamate accumulation into presynaptic vesicles [168], activation of transcription in neuronal cells [169], and protection of telomeres against chemotherapy-induced degradation [170]. Moreover, GAPDH exhibited a DNA repair activity by acting as a Uracil DNA glycocylase (UDG) which removes mis-incorporated uracil from the DNA [171, 172].

Notably, GAPDH was demonstrated to play a crucial role in neuronal apoptotic cell death that has pathophysiological implications in many neurodegenerative diseases such as Alzheimer's disease (AD) [173], Huntington's disease (HD) [174] and Parkinson's disease (PD) [175].

Under oxidative stress, GAPDH undergoes oxidation and manifests a reduction in its enzymatic activity [176]. This oxidation also enhances the binding of GAPDH to RNA and DNA [177]. Treatment with H_2O_2 induces the translocation of GAPDH from the cytoplasm to the nucleus in mammalian cells [178]. Moreover, a recent study showed that nitric oxide (NO) induces GAPDH nuclear localization followed by its acetylation by p300/CBP that will result in the transcriptional activation of many genes such as p53 [179]. Considering all of these alterations of function upon oxidative conditions, GAPDH seems to be playing an important role in cellular response to oxidative stress.

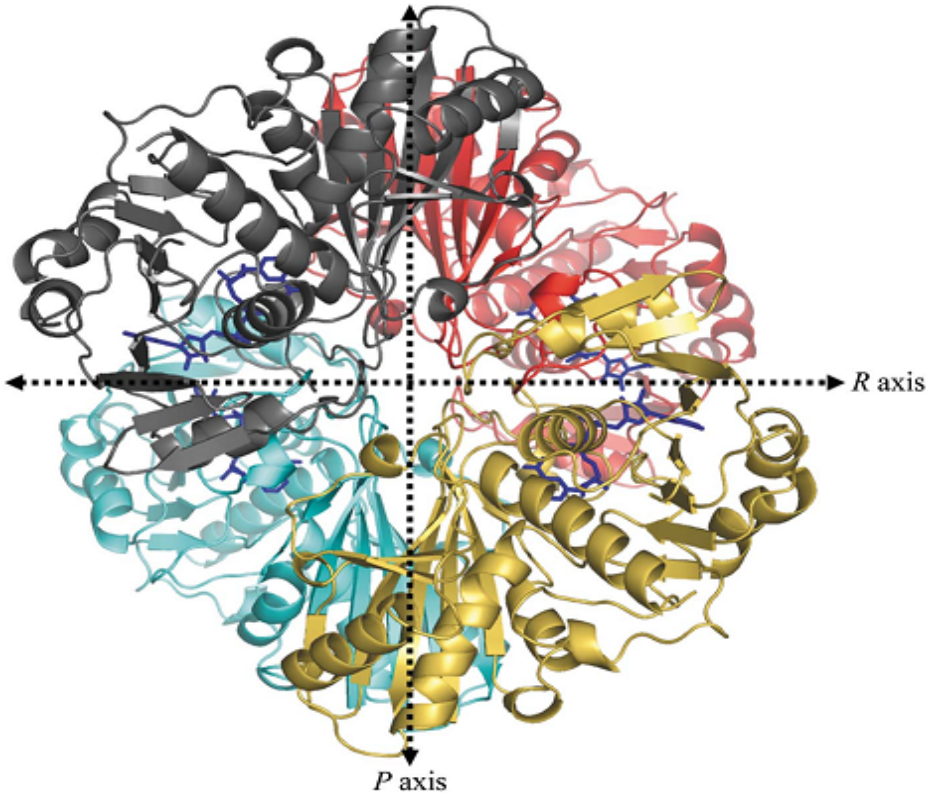

Figure 10. Overall structure of GAPDH homotetramer. Adapted from[180].

8. Research project

8.1 Background

As described earlier, APE1, a member of the Exo III family, was found to be the major AP endonuclease present in mammalian cells, unlike the Endo IV family members that are only present in lower organisms. Many previous attempts to identify an Endo IV enzymatic activity in mammalian cells that acts independently of Mg^{2+} failed to detect it.

It was recently documented in our laboratory [156] a trial to identify such activity using total cell extracts derived from human lung fibroblast that resulted in the detection of a weak Mg^{2+}- independent that was reflected by the cleavage of a 42-mer [γ-^{32}P] labeled

synthetic oligonucleotide substrate containing a single AP site. Subsequent purification steps followed by mass spectrometry analysis of the protein fraction having this activity revealed the presence of GAPDH protein. Purified recombinant GAPDH lacks an AP endonuclease activity, the only explanation to the observed activity is that GAPDH was co-purified with another protein possessing this activity, namely APE1. Since APE1 has an Mg^{2+}-dependent activity, a possible explanation is the EDTA concentration in the buffer used was not enough to chelate all the Mg^{2+} from the extract in the reaction, leaving some APE1 enzymes active, contributing to the detected AP endonuclease activity.

Further experiments showed that GAPDH interacts with APE1 *in vivo* and *in vitro*. This interaction causes the conversion of the oxidized and AP endonuclease deficient APE1 to a reduced and active protein capable of cleaving AP sites. This reducing potential of GAPDH was shown to be dependent on Cys 152 residue of its active site. Other reports showed that APE1 exists as heterogeneous molecules that are either reduced or oxidized [153]. Moreover, it was reported that APE1 can be oxidized by H_2O_2 to the inactive form, a phenomenon partially reversed by the treatment with dithiothreitol (DTT) [155].

Interestingly, siRNA knockdown of GAPDH in HCT116 cells caused sensitivity to MMS and bleomycin, two drugs known to create DNA lesions that are repairable by APE1, but not to UV irradiations which produces bulky lesions. A similar phenotype was observed when using siRNA directed against APE1 [138]. Furthermore, total extracts of these cells showed a reduced AP endonuclease activity. They also exhibited an increase in spontaneous AP sites frequency in their genomic DNA.

8.2 Hypothesis

Since GAPDH interaction with APE1 activates the AP endonuclease activity of this enzyme *in vitro*, and since the deficiency in GAPDH sensitized cells to DNA damaging drugs, induced an increase in AP sites formation and reduced the AP endonuclease capacity, we hypothesize that deficiency in GAPDH affects cell cycle progression and APE1 cellular distribution.

Moreover, since GAPDH activates APE1 by reduction, we postulate that this reduction occurs at the level of disulfide bridge(s) formed between APE1 cysteine residues.

8.3 Objectives

<u>Objective 1:</u> To determine whether activation of APE1 by GAPDH is required for efficient cell cycle progression

It is critical for dividing cells to ensure their DNA integrity in order to avoid any inherited mutations in daughter cells. DNA lesions activate checkpoint pathways that regulate specific DNA repair mechanisms during different phases of the cell cycle. Checkpoint-arrested cells resume their cell cycle progression once the damage has been repaired, whereas cells with unrepaired DNA lesions undergo permanent cell cycle arrest or apoptosis.

Since GAPDH Knockdown cells showed a defect in proliferation and since this knockdown affected the DNA repair capacity of the cells, i.e. AP endonuclease activity,

we studied the cell cycle progression of GAPDH knocked down cells and we found that GAPDH knockdown cells had a delay in progressing through the S phase as compared to control cells**.**

Objective 2: To determine whether depletion of GAPDH affects the subcellular localization of APE1

Although APE1 is mainly a nuclear protein, it has been reported that its cellular distribution varies from normal cells to cancer cells, as it was shown that APE1 localizes to the cytoplasm in cancer cells as in the case of lung [181], ovarian [182], thyroid[183], and breast cancer[184]. This localization has been linked to higher aggressiveness of the tumor. It was also shown to be present in the endoplasmic reticulum (ER) and the mitochondria [185, 186].

Moreover, upon oxidative stress some DNA repair enzymes like OGG1 and APE1 localize to nuclear speckles, structures that are present between chromatin domains enriched with RNA processing and transcription proteins [187].

For these reasons, we investigated the cellular distribution of the presumably oxidized and inactive APE1 in GAPDH knock down cells. We found that APE1 forms foci-like structures in GAPDH deficient cells.

Objective 3: To identify the redox-sensitive cysteine(s) of APE1 that is reduced by GAPDH

APE1 has seven cysteine residues at positions 65, 93, 99, 138, 208, 296 and 310, some of which were shown to be important for its redox activity (Cys 65). Since GAPDH was shown to reduce APE1, we expect that this reduction occurs at the level of one or more of these cysteines, causing a reduction of a disulfide bridge(s) inducing a conformational change in APE1 structure which allows it to be active and capable of cleaving AP sites. To identify the cysteine residue (s) involved we mutated each of the 7 cysteine to alanine, which cannot form disulfide bridges and assayed for the activity of these mutants in vitro and in the cells. We specifically asked whether one or more cysteine mutant would maintain its AP endonuclease activity under oxidative conditions in the absence of GAPDH and show no enhancement in AP endonuclease activity or redox state when GAPDH is added. Hence the expression of the candidate mutant(s) should rescue the defect in proliferation seen in GAPDH knock down cells.

Chapter 2: Materials and Methods

1. Expression and Purification of His-APE1 variants

His-APE1 was cloned in pET14-b vector under a T7 promoter and expressed in BL21 (DE3) pLysS strain and purified using Talon Metal Affinity column (Clonetech) according to manufacturer instructions with some modifications. Briefly, BL21 pLysS *E.coli* cells expressing His-APE1 (wild type or cysteine or active site mutants) were streaked out on Luria Broth (LB) agar plates containing Ampicillin (100 µg/mL), a single colony was inoculated in 20 mL of LB containing Ampicillin (100 µg/mL) for overnight incubation at 37˚C with shaking (180 rpm). Next morning, 10mL of the overnight culture was subcultured in 1L of LB and grown up to O.D= 0.5. IPTG (0.4mM) was added to the culture and left for two hours at 37˚C with shaking. Cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4˚C. Cell pellet was resuspended in the washing buffer (50 mM sodium phosphate pH 7.0, 300mM NaCl) containing protease inhibitors (Invitrogen) and is subjected to sonication for short bursts on ice at 50% amplitude (Branson Digital Sonifier ®) to lyse the cells. Cell lysate was then centrifuged at 13,000 rpm for 20 minutes at 4˚C. The purification steps were done at room temperature. The supernatant was loaded onto a column made of 1 ml of Talon metal affinity resin (Clonetech) and the flow through was collected and passed again on the column to maximize the amount of binding proteins. The column was washed with

the washing buffer containing protease inhibitors. His-tagged bound proteins were eluted by elution buffer (50mM sodium phosphate pH 7.0, 300nM NaCl, 150mM Imidazole), and collected as fractions of 500 µL each. Protein concentration and purity were estimated by Bradford assay and SDS-PAGE subsequently. The purified proteins were dialyzed against 1L of the storage buffer (20mM sodium phosphate pH 7.0, 100mM NaCl, 1mM EDTA, 10% Glycerol) at 4˚C.

2. Expression and purification of GST-GAPDH

GST-GAPDH was cloned in pGEX-4T-1 (Amersham Biosciences) and expressed in DH5α. Cells were cultured and proteins were extracted as previously described in section 1 of Materials and Methods except for protein extracts that were loaded on glutathione-Sepharose 4B mini columns (GE Healthcare). The column was washed with the wash buffer (50 mM Tris-HCl pH 7.0, 300mM NaCl). And the proteins were eluted with 50mM Tris-HCl pH 9.0, 20mM reduced glutathione.

3. Silver staining

Immediately after electrophoresis, the SDS-PAGE was fixed in a solution of 50% Methanol, 10% acetic acid for 30 minutes. The gel was then washed three times in 15% methanol for 10 minutes each with gentle shaking. The gel was then transferred to 7.5% Glutaraldehyde solution and incubated at 37˚C for 30 minutes, with gentle shaking.

Another washing step with 15% methanol and distilled water subsequently for 15 minutes each, for three times. The Gel was then stained with a silver nitrate ($AgNO_3$) for 10 minutes with gentle shaking. To remove excess silver nitrate, the gel is washed with water until the color of the solution becomes clear. To develop the gel, developing solution (0.005% citric acid, 0.2% formaldehyde) was added to the gel with gentle shaking, the staining should take place in the first 10 to 20 minutes of the incubation. A stop solution (5% acetic acid) was added to stop further staining.

4. Western Blot Analysis

Western blot analysis was performed according to standard protocols. For purified His-APE1 and GST-GAPDH *in vitro* assays, purified proteins concentrations were determined using the Bradford analysis. Proteins were loaded onto a 10% SDS polyacrylamide gel, which was run at 100 V. The proteins were then transferred to a nitrocellulose membrane at 100 V for 1 hour. The membrane was blocked with 5% of skim milk in TBSET (6.05 g Tris-Base, 43.83 g NaCl, 1.85 g of EDTA and 500 µl tween 20 in 500 ml of distilled water and then probed overnight at 4°C with mouse monoclonal anti-His (Santa Cruz biotechnology) antibody at a concentration of 1/2500 in 5% milk. Three successive washes (10 min each) with TBSET were then performed to wash all excess of the primary antibody. After the last wash, the membrane was incubated for 1 hour with the anti-mouse polyclonal antibody and was followed by 3 washes of 10 min each with TBSET. Finally the membrane was incubated for 1 min in chemiluminescence

reagent and developed using a FujiFilm Intelligent Dark Box and images were acquired with a LAS-3000 camera and the Image Reader LAS-3000 Lite software.

5. Site-Directed Mutagenesis

Site-directed mutagenesis (QuikChange® lightning kit, Stratagene) was used according to the manufacturer's instructions to mutate APE1 seven cysteines and the active site residues to alanine using native His-APE1 in pET-14b as the template. The primers corresponding to every mutant are listed in **table II**. Briefly, prior to PCR, sample reactions were prepared as follows:

- 100 ng of the dsDNA template containing plasmid
- 125 ng of each of the mutagenic primers, forward and reverse
- dNTP mix
- reaction buffers
- 1 µl of QuickChange® Lightning enzyme

Each reaction is then cycled using the following parameters:

Segment	Cycle(s)	Temperature (°C)	Time
1	**1**	**95**	**2 min**
2	**18**	**95**	**20 sec**
		60	**10 sec**
		68	**2min 30sec**
3	**1**	**68**	**5 min**

The PCR products are digested with *Dpn* I restriction enzyme to degrade the template plasmid. XL10-Gold® Ultracompetent cells (Stratagene) are transformed with 2 μL of the *Dpn* I-treated PCR product. Then transformed cells are grown in NZY^+ broth for 2 hours at 37°C with shaking (225rpm). 100 μL of the grown culture are plated on LB-ampicillin agar plates and incubated at 37°C overnight. Next morning, several single colonies are picked and grown in LB-ampicillin broth followed by plasmid extraction following the manufacturere's protocol (Qiagen Qiaprep® spin Miniprep kit). The extracted plasmids were verified for the presence of the desired point mutation by DNA sequencing analysis.

The primers used for sequencing of APE1 created variants are as follows:

APE1-C**65**A variant: 5'-GAAGAGTAAGACGGCCGCAAAG-3'

APE1-C**93**A and C**99**A variant: 5'-CAGATCAGAAAACCTCACCCAG-3'

APE1-C**138**A: 5'- CAGAGAACAAACTACCAGCTG-3'

APE1-C**208**A: 5'- GCTGGTAACAGCATATGTACCT-3'

APE1-C**296**A and C**310**A:5'- CACCTCTACCCCAACACAC-3'

A control reaction provided by the manufacturer was used to verify the efficiency of mutant plasmid generation.

6. Bacterial Transformation

For plasmid amplification and cloning purposes we prepared bacterial competent cells as follows: 1 mL of an overnight culture was subcultured in 100 mL for 4 hours at 37°C. The obtained bacterial pellet was incubated with 100 mM Calcium Chloride ($CaCl_2$) on ice for at least one hour. To 50 µL of prepared competent bacteria, 1 µg of the desired plasmid DNA was added and left to incubate on ice for 30 minutes. Then the bacteria-DNA mixture was incubated at 42°C for 90 seconds for heat shock. 1mL of LB broth was added to the transformation reaction and left to incubate at 37°C with shaking for 2-4 hours. The transformed bacteria were then plated on selective LB agar plates (containing either ampicillin (100 µg/ml) for pET-14b vector, or Kanamycin (50 µg/ml) for pEYFP-N1 vector).

APE1 variant	**Mutagenic Primers**
APE1-C**65**A	F: 5'-CCACACTCAAGATC**GCC**TCTTGGAATGTGGATG-3' R: 5'-CATCCACATTCCAAGAGGCGATCTTGAGTGTGG-3'
APE1-C**93**A	**F:** 5'-GCCCCAGATATACTG**GCC**CTTCAAGAGACC-3' **R:** 5'-GGTCTCTTGAAGGGCCAGTATATCTGGGGC-3'
APE1-C**99**A	**F:** 5'-GCCTTCAAGAGACCAAA**GCC**TCAGAGAACAAAC-3' **R:** 5'-GTTTGTTCTCTGAGGCTTTGGTCTCTTGAAGGC-3'
APE1-C**138**A	**F:** 5'-GCTTTCCCGCCAG**GCC**CCACTCAAAGTTTCTTACG-3' **R:**5'-CGTAAGAAACTTTGAGTGGGGCCTGGCGGGAAAGC-3'
APE1-C**208**A	**F:** 5'-CCTTGTGCTG**GCC**GGAGACCTCAATGTG-3' **R:** 5'-CACATTGAGGTCTCCGGCCAGCACAAGG-3'
APE1-C**296**A	**F:** 5'-CTCTCTGTTACCTGCATTG**GCC**GACAGCAAGATCCG-3' **R:** 5'-CGGATCTTGCTGTCGGCCAATGCAGGTAACAGAGAG-3'
APE1-C**310**A	**F:** 5'- CCTCGGCAGTGATCAC**GCC**CCTATCACCCTATAC-3' **R:** 5'GTATAGGGTGATAGGGGCGTGATCACTGCCGAGG-3'
APE1-H**309**A	**F:**5'GGCCCTCGGCAGTGAT**GCC**TGTCCTATCACCCTATACC3' **R:**5'GGTATAGGGTGATAGGACAGGCATCACTGCCGAGGGCC3'
APE1-D**210**A	**F:** 5'- CCTTGTGCTGTGTGGA**GCC**CTCAATGTGGCACAT-3' **R:** 5'ATGTGCCACATTGAGGGCTCCACACAGCACAAGG-3'
APE1-D**308**A	**F:** 5'- GGCCCTCGGCAGT**GCC**CACTGTCCTATCACC-3' **R:** 5' GGTGATAGGACAGTGGGCACTGCCGAGGGCC-3'

Table I. Site-directed mutagenic primers used to create Cys to Ala, His to Ala, Asp to Ala point mutations. They were designed according to manufacturer's recommendation, (Stratagene).

7. Cloning of APE1 variants in pEYFP-N1

APE1 variants were cloned in pEYFP-N1 vector that has and enhanced yellow fluorescent protein (EYFP) for expression in mammalian cells. Briefly, APE1 wild type and variants were amplified by PCR from the pET-14b construct. Primers were designed in a way to introduce APE1 between *Hin*d III and *Bam*H I in fusion with the EYFP gene by removing APE1 stop codon from the reverse PCR primer. A Kozak sequence (underlined) was added upstream of the start codon in the forward primer to enhance the translation of the resulting mRNA. Two additional nucleotides were added to the reverse primer downstream of BamH I restriction site, to put the insert in frame with the EYFP gene.

Oligo sequence for APE1 variants cloning in pEYFP-N1:

F: 5'- ATACGATAGC-AAGCTT- GCCACCATGCCGAAGCGTGGGAAAAA-3'

R: 5'-AGGCTGAGAT-GGATCCAG–CAGTGCTAGGTATAGGGTGATAGG- 3'

PCR was conducted in the presence of *Pfu* DNA polymerase (Feldan-Bio). PCR parameters used are the following:

Segment	Cycle(s)	Temperature (°C)	Time
1	1	94	3 min
2	35	94	30 sec
		60	30 sec
		72	1min
3	1	72	10 min

PCR products were ran on a 1% agarose gel, the DNA bands corresponding to APE1 variants genes are cut and DNA purified using GeneClean method that consists of incubating the DNA to be purified with powdered suspension of glass, called glass milk, to which DNA binds in high salt concentration (6 M NaI). The agarose band was incubated at 55°C for 10 minutes with NaI solution to dissolve it. Then the melted solution was allowed to cool to room temperature for 5 minutes. 5 µL of the glassmilk suspension was added to the melted NaI- agarose solution containing the DNA and was incubated at room temperature for 5 minutes to allow the DNA binding to the glassmilk. Subsequently the glassmilk was pelleted by centrifugation and washed twice with NEW wash buffer (50% ethanol in TE pH 7.5, 100 mM NaCl). Elute the bound DNA from the glassmilk using 10 µL of water or Tris-EDTA (TE).

The purified PCR products of APE1 variants and the empty pEYFP-N1 vector were digested with the two restriction enzymes, *Hin*d III and *Bam*H I for 1 hour 30 minutes at 37°C to give compatible ends for ligation. The digestion products were purified by the

previously described GeneClean method. Ligation mixtures containing the digested PCR products and empty vector were incubated with T4 DNA ligase (New England BioLabs) overnight at 16°C (20 µL reaction).

Transformation of DH5α competent cells with 4 µL of the ligation reaction was conducted the next morning, using the previously described transformation protocol. Transformed bacteria were plated on LB-kanamycin agar plates for selection of the positive clones.

Single colonies were grown in LB-kanamycin broth and plasmid extraction was done according to the manufacturer instructions (Qiagen). Double digestion using *Hin*d III and *Bam*H I was done to verify the success of the cloning. The digestion product was assessed for the presence of the insert (APE1 variants) by electrophoresis on an agarose gel.

8. Cloning of native Apn1 and its inactive mutant E158G in pEYFP-N1

Apn1 was amplified from p3xFLAGCMV10-Apn1 (a gift from Dr Bruce Demple, Harvard Medical School) as described in section 7. Apn1-E158A was amplified from pYES 2.0 vector. Primers used for cloning in pEYFP-N1 vector are listed below. It was cloned in fusion with EYFP gene. The cloning procedure including the PCR, the digestion, ligation and verification of the cloning were done as described in section 7 for APE1 variants cloning.

Primers used for Apn1 and its variant Apn1-E158G cloning in pEYFP-N1:

F: 5'-AATCGATGCT-AAGCTT-GCCACCATGCCTTCGACACCTAGC-3'

R: 5'-AGGCTGAGAT-GGATCCAG- TTCTTTCTTAGTCTTCCTCTTCTT-3'

9. HCT116 Cell culture

Human colon carcinoma HCT116 cell line was kindly provided by Dr.Elliot Drobetsky (University of Montreal). They were culture in Dulbecco's Modified Eagle Medium (DMEM) (Wisent Inc.) complemented with 10% of fetal bovine serum (FBS) (Wisent Inc.) and 0.1mg/ml penicillin, and 0.1 mg/ml streptomycin. Cells were incubated at 37°C and 5% CO_2. The media was changed every 48 hours.

10. GAPDH Knockdown by siRNA

Human Silencer GAPDH siRNA (Ambion) that targets the 5' medial region of GAPDH mRNA sequence was used to knock down the expression of GAPDH in HCT116 cells. The cells were cultured in Petri plates and transfected with 75 nM of GAPDH siRNA using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) according to the manufacturer's protocol. Negative control (75 nM) that has no homology to any known gene was also included in the transfection. The plates were incubated at 37°C in 5% CO2

for 4 h, after which the complete growth medium was added, and the cells were proceed for subsequent assays.

11. Protein extraction form HCT116

HCT116 adherent cells are collected from the Petri plates using a cell scraper and not trypsin, to avoid any possible protein degradation. Cell were washed with cold PBS and only the pellet was frozen at -80°C if the protein extraction was not done on the same day. For protein extraction, 200 µL of RIPA buffer (NaCl, EDTA, Tris Base, 1% Deoxycholic acid, 1% Nonidet p-40 in millipore water) supplemented with protease inhibitors and then added to the pelleted cells and incubated on ice for 1 hour. The lysed cells are centrifuged at 12,000 rpm at 4°C for 30 minutes. The supernatant was then stored at -80°C until used. Protein concentration was estimated using Bradford method.

12. Western blot to verify Knock down of GAPDH

For the verification of GAPDH Knock down in HCT116 cells, total cell extracts were quantified using Bradford protein estimation method, and equal amounts were loaded on a 10% SDS-PAGE and proceeded for western blot analysis as described in section 4 except for the use of the monoclonal mouse anti-GAPDH followed by probing with anti-mouse antibody. The membrane was probed with anti-tubulin antibody as a loading control.

13. HCT116 plasmid DNA transfection

Transfection of HCT116 with 1-2 µg of pEYFP-N1 plasmid carrying APE1 different variants was done as previously described in section 10 using lipofectamine 2000 and Opti-MEM media. The expression of APE1-EYFP proteins was detected after 4 hours of the transfection under a fluorescent microscope.

14. Establishing Stable Clones expressing APE1 variants, Apn1 and Apn1-E158G

HCT116 were transfected as described in section 10. 48 hours after transfection, G418 (Neomycin) (Wisent Inc.) was added to the media of the transfected cells at a concentration of 150 µg/mL for selection of the positive clones expressing the APE1-EYFP or Apn1-EYFP that has a neomycin resistant gene as a marker for selection. A serial selection of positive cells was done by increasing concentrations of G418 (150, 200,250, 350, and 400 µg/mL) over three weeks, the approximate time needed for single colonies of transfected cells expressing differential amounts of the protein of interest. Cell sorting (FACS Aria III, BD) was used to pick single cells that were then cultured in 200 µL of media containing 200 µg/mL of G418, and then subcultured in 1ml, 2ml, 5ml and 10 mL successively. G418 at 200 µg/mL was used for maintenance of the stably

transfected cells in culture. The efficiency of the transfection was assessed under the microscope and by western blot analysis.

15. Cell Cycle analysis

For cell cycle analysis of GAPDH knocked down cells, control siRNA cells, APE1-EYFP,Apn1- EYFP expression cells, cells were transfected 36 hours prior to nocodazole (200 ng/mL) (Sigma) treatment to arrest cells in G2/M phase. The collection of the sample starts after nocodazole treatment by the addition of trypsin (Wisent). Collected cells were then washed with PBS/1% EDTA to prevent clumping of the cells. Cells are stored in 75% Ethanol in PBS in FACs tubes at -20°C until the FACs analysis was done. During cell collection for cell cycle analysis a fraction of the collected cells was kept at -80°C for western blot analysis. Samples were collected as follows:

-Sample 0: cells not treated with nocodazole after 36 hours of transfection.

-Sample 12 hours, 24 hours and 36 hours: cells collected after 12, 24 and 36 hours of nocodazole treatment.

Before flow cytometry analysis, cell suspensions were centrifuged and washed with PBS to remove ethanol. Then they were resuspended in 500 µL of PBS with 0.05 µg/µL of propidium iodide to stain the DNA. The collected samples are subject to FACs analysis on the FACScan machine (Becton Dickinson) according the standard protocols. Data were analyzed using Cell Quest Pro allias software.

16. Indirect immunofluorescence

For monitoring APE1 localization in GAPDH knock down HCT116, 750,000 cells were plated. 24 hours cell were transfected with the corresponding siRNA as section 10. 24 hours after transfection, 125,000 cells were transferred to a sterilized cover slip in a 6-well plate. Cells were left for 48 hours to allow them to adhere to the glass surface of the coverslip.

Fixation and permeabilization: remove the media from the 6-well plates and wash the cells with 2mL of PBS. Cells were then incubated with 1 mL of 3% Paraform aldehyde (PFA) (Sigma) for 20 minutes. The PFA was removed and the cells are incubated with 2 mL of permeabilization buffer (PBS, 0.5% NP-40, 1mM Sodium azide) for 30 minutes. The cells are then washed with washing buffer (PBS, 0.1% NP-40, 1mM Sodium azide) and the blocking solution (10% FBS) for 1 hour.

Primary antibody: The primary antibody mouse anti-APE1 (cedarlane) 1:150 dilution was prepared in blocking solution. The coverslips holding the cells were then removed from the 6-well plate and 50 μL of primary antibody was added onto the cells and covered with a parafilm of the size of the coverslip. The primary antibody was left to incubate for 3 hours at room temperature.

The cells were then washed with washing buffer for 3 times/15 minutes each.

Secondary antibody: The secondary antibody, goat anti-mouse coupled to AlexaFluor (AlexaFluor 488, Invitrogen) was used at 1:500 dilution and was added to the cells as described for the primary antibody.

The cells were then washed with washing buffer for 3 times/15 minutes each. Then the cells were washed twice with PBS.

Mounting: the coverslips were rinsed with milliQ water, and dried gently using a paper towel. 15 μL of the mounting media (Vector laboratories, Inc. Burlingame, CA) containing DAPI was put on the slide and the coverslip was placed on top of it, with the cells facing down. The slides were then ready for microscopy. The slides were subsequently examined under a Leica, DMRE, Wetzlar fluorescence microscope supplemented with a camera. Exposure times varied from 300-400 ms. Slides were stored in the dark.

Chapter 3: Results

1. GAPDH knockdown in HCT116 causes G1 arrest

To determine whether GAPDH is required for APE1-dependent DNA repair function, we used siRNA knockdown to reduce its expression in HCT116 cells. As a negative control we used a scrambled control siRNA that has no significant homology to any known gene sequence. The knockdown efficiency was verified by western blot using anti-GAPDH antibody (**Figure 11**).

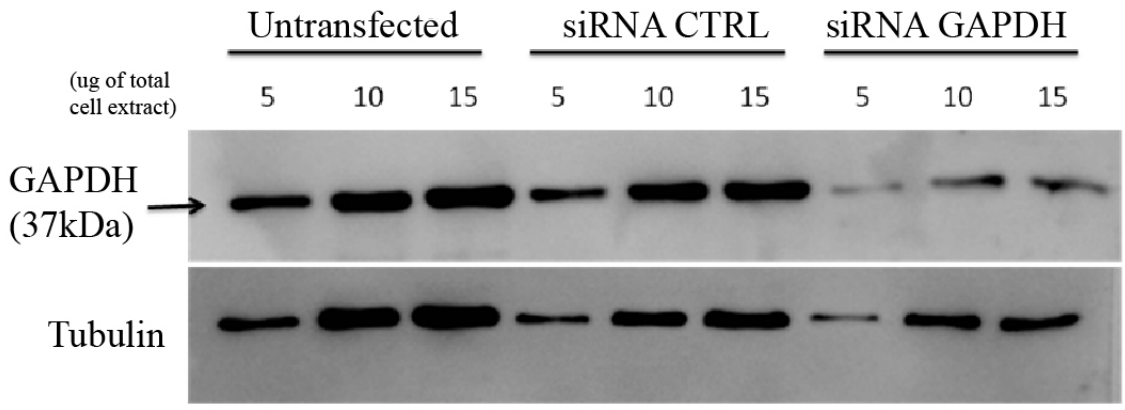

Figure 11. Verification of GAPDH knockdown in HCT116. HCT116 cells were incubated with 75 nM of either control siRNA or GAPDH siRNA, 36 hrs later total cell extracts were analyzed for GAPDH by western blot.

Since GAPDH knockdown HCT116 cells showed proliferative defects when challenged with DNA damaging agents [156], we sought to determine whether they also show cell cycle defects in the absence of exogenous DNA damage.

For cell cycle analysis, control siRNA and GAPDH knockdown cells were untreated and treated with nocodazole, 36 hours after the knockdown, to arrest cells in G2/M, then samples were collected after 12, 24 and 36 hours and processed for flow cytometry analysis.

After 36 hours of nocodazole treatment, around 70% of the control siRNA cells progressed to G2/M, whereas in GAPDH knockdown cells, only 37% were in G2/M phase (**Figure 12**). One possible explanation for this result is that the arrest is caused by the accumulation of unrepaired AP sites, which was previously observed in these cells [156].

To test this hypothesis, we expressed the major yeast AP endonuclease Apn1 in GAPDH knockdown cells. Unlike APE1, Apn1 does not require GAPDH for optimal activity since it does not contain redox sensitive cysteine residues as in the case of APE1 [84]. Apn1 would therefore be expected to rescue the GAPDH G1 arrest only if it is caused by accumulation of AP sites.

HCT116 cells were transiently co-transfected with Flag-Apn1 expressing plasmid along with GAPDH siRNA. Indeed, the transient expression of Apn1 in GAPDH deficient cells was able to release the G1 arrest (**Figure 12**). For instance, after 36 hours of nocodazole treatment, 55% of the cells progressed to G2/M compared to 37% of GAPDH knockdown cells not expressing Apn1 (**Figure 12**).

These results suggest that accumulated DNA damage underlies the observed cell cycle progression defects. It is most probably due to defective APE1 proteins in the absence of adequate amount of GAPDH for their activation.

To validate the complementation effect of Apn1, we next attempted to establish stable HCT116 clones expressing it. The selection was done using G418 over 3 weeks. Then single colonies were picked and subcultured. Protein extracts of different colonies were subject to western blot analysis using a Flag antibody. Unfortunately, we were unable to detect Apn1 expression. Another attempt to create Apn1-expressing stable clones was done with a different construct that expresses Apn1-EYFP as shown in **Figure 21** for APE1 variants. A fluorescent tag would make the selection process easier by allowing monitoring of expression using microscopy and giving the advantage of the use of cell sorting as another mean of selection. Similarly, we created a construct that expresses APN1-E158G-YFP, the AP endonuclease-deficient mutant of Apn1[188]. G418 was used for the selection as described in section 14 of chapter 2. The expression of the YFP-tagged Apn1 and Apn1-E158G in these stably transfected cell lines was monitored under a fluorescent microscope (data not shown). The expression of both proteins was seen as short as 6 hours after transfection by microscopy. Unfortunately, 48 hours later the number of cells showing fluorescent proteins start to decrease from the cell culture. Four days later, no cells were showing any expression of the EYFP-tagged proteins as compared to the empty vector that showed a very good expression of the EYFP. One possible explanation is that, as a foreign protein, Apn1 might need some posttranslational modification by yeast proteins (e.g. chaperones, etc…) that are not present in human cells for its proper folding.

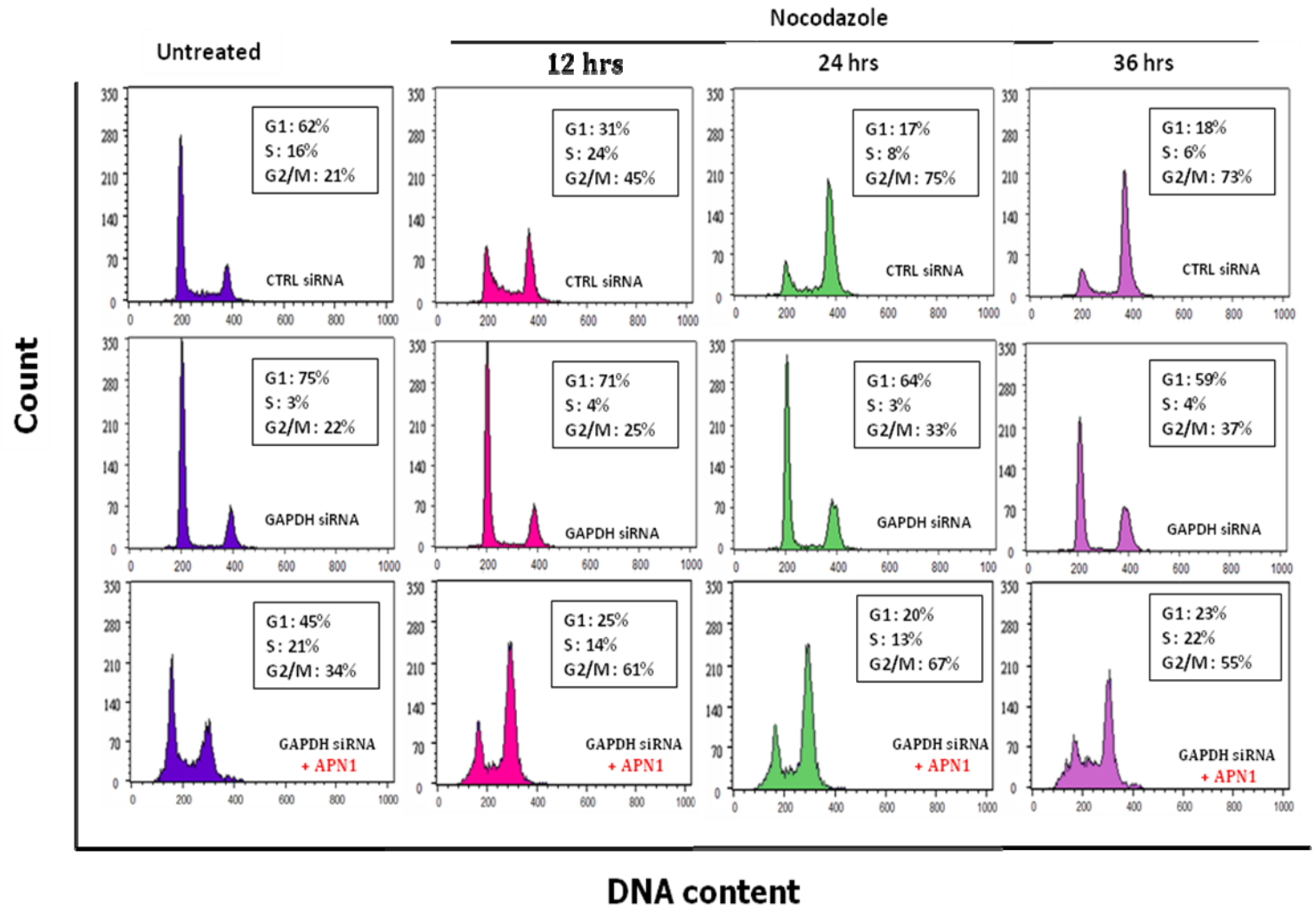

Figure 12. GAPDH knock down causes a G1 arrest. GAPDH knockdown cells transfected with Apn1 showed normal cell cycle progression, a result obtained in 3 independent experiments.

Interestingly, we observed that cells start to die few hours after transfection with Apn1 plasmid, which could explain the gradual decrease in the number of cells expressing Apn1 and hence the failure in establishing stable clones.

2. siRNA mediated GAPDH silencing triggers the formation of APE1 aggregates

Upon oxidative stress, some BER enzymes including OGG1 and APE1 localize to nuclear speckles, structures that are present between chromatin domains enriched with RNA processing and transcription proteins [187]. To verify whether GAPDH cellular level affects APE1 localization, we carried out an indirect-immunofluorescence experiment using APE1 antibody in GAPDH knockdown and control siRNA cells. While normal nuclear distribution was observed in control siRNA cells, in GAPDH knockdown cells we detected foci in the nucleus of the majority of the cellular population **(Figure 13)**. The nature of the detected structures needs further investigations; we speculate that they could be stalled DNA repair foci of inactive APE1 on DNA lesions or it could just be an aggregation due to misfolding of the oxidized proteins in the absence of enough GAPDH to reduce them.

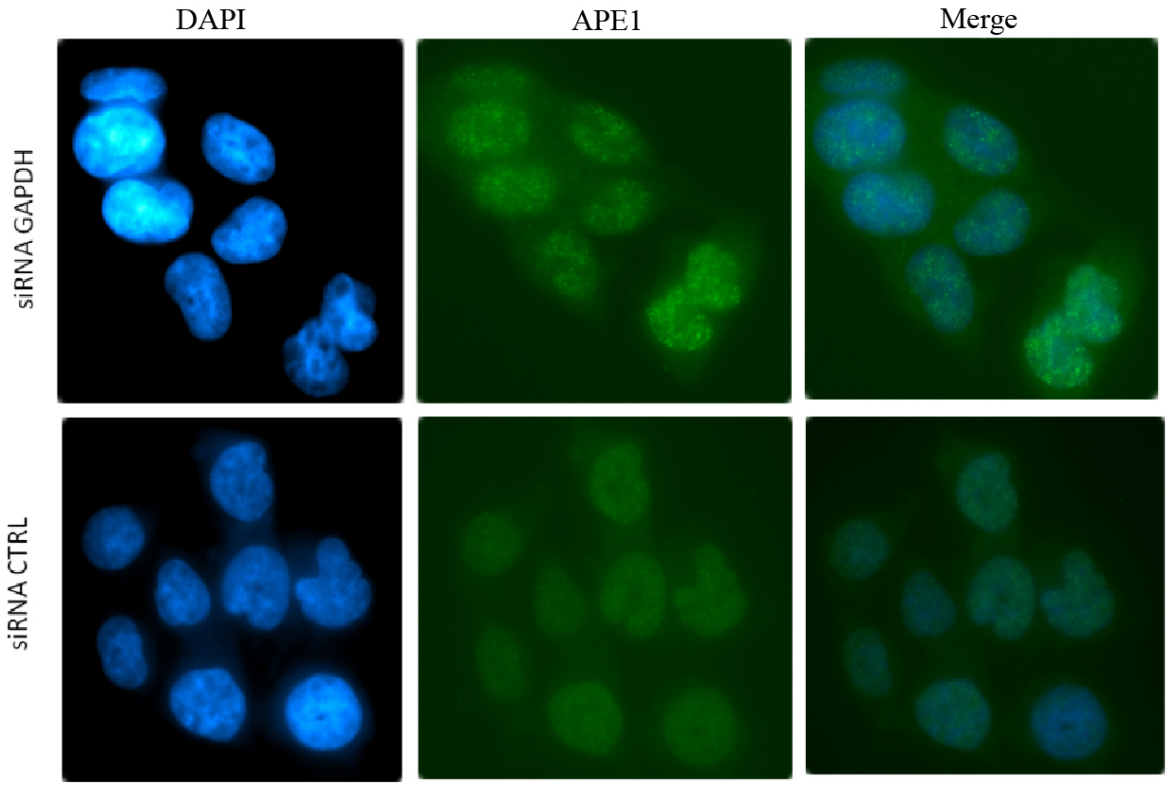

Figure 13. APE1 nuclear aggregates in GAPDH knockdown cells. HCT116 cells were transfected with GAPDH siRNA or Control siRNA, 48 hours later cells were fixed and permeabilized for indirect-immunofluorescence microscopy. DAPI was used for DNA staining.

3. Identification of the cysteine residue(s) in APE1 that are reduced by GAPDH

We next sought to identify the cysteine residue(s) that are involved in the reduction of APE1 by GAPDH. The conservation of cysteine residues between APE1 and bacterial Exo III is limited to two residues, Cys208 and Cys310. On the other hand, all seven of the

cysteines present in human APE1 are conserved in other vertebrates except for Cys138 and Cys65 [189]. However, none of these cysteines are considered part of the catalytic active site of the protein. Nonetheless, oxidation and reduction of these cysteines could still affect the catalytic activity by inducing a conformational change in the protein, affecting the active site. We mutated each of the seven cysteine to alanine in an expression vector that includes a His tag. Mutants created include: C65A, C93A, C99A, C138A, C208A, C296A and C310A (**Figure 14**). As controls, active site mutants, D210A, D308A and H309A were also created. **Table II** summarizes the current knowledge of the function of these mutants and **Figures 15** and **16** show the position of cysteine residues in the APE1 protein.

The created cysteine mutants are going to be the subject of an *in vitro* redox state characterization as well as of a complementation experiments in HCT116 cells to identify the residue(s) that are affected by GAPDH reduction of APE1.

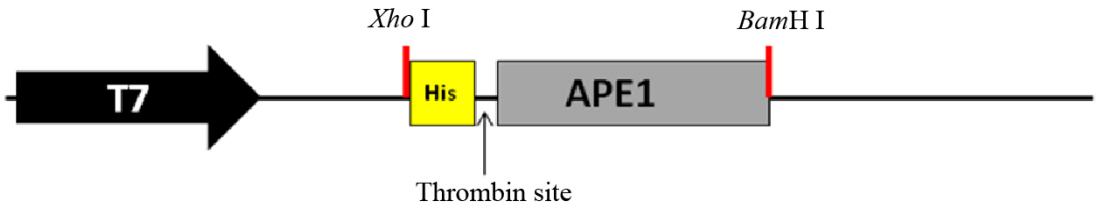

Figure 14. His-APE1 cloning sites in pET-14b. APE1 and its variants were cloned between X*ho* I and B*amH* 1 restriction sites, expressed as His-fusion proteins under the control of T7 promoter. pET-14b has an ampicillin resistance gene.

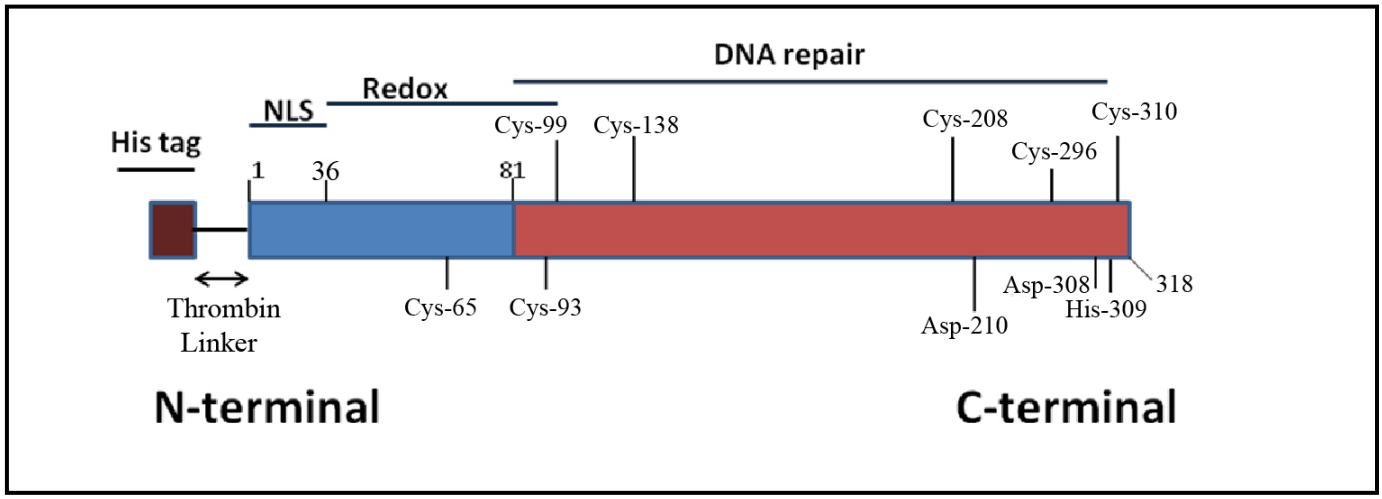

Figure 15. APE1 Linear map showing Cysteine residues and the three active site residues mutated to Alanine. DNA repair and redox domains are indicated [124, 155].

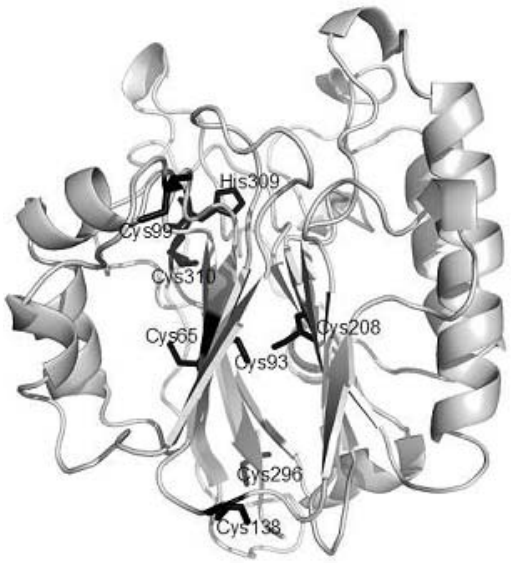

Figure 16. Positions of Cysteine residues within APE1 positioned relative to the active site H309 [190].

APE1 variants	Consequence(s) on APE1 function
C65A	**Loss of redox function**
C93A	**Loss of redox function**
C99A	**Loss of affinity to DNA at high [Mg^{2+}]**
C138A	**?**
C208A	**?**
C296A	**?**
C310A	**DNA repair Efficient**
H309A	**Low affinity to DNA, No incision activity**
D210A	**Low incision activity**
D308A	**Less binding to DNA, Less incision**

Table II. Effects of the created mutations on APE1 redox and DNA repair functions [105, 125, 138, 191, 192].

We next purified the His-tagged APE1, native (**Figure 17A**) and cysteines mutants (**Figure 17B**) .Bacterial protein extract were loaded onto a column with cobalt-coupled beads, where the imidazole ring of the Histidine residues of adjacent polyhistidine-tag binds with high affinity to the cobalt ion. After several steps of washing to eliminate impurities, His-APE1 was eluted by competition with a buffer containing a high concentration of imidazole. Eluted proteins were collected by fractions of 500 µL/fraction

(**figure 17A**). Next, we purified GST-tagged GAPDH (**Figure 18**) proteins from E.coli using glutathione sepharose affinity purification.

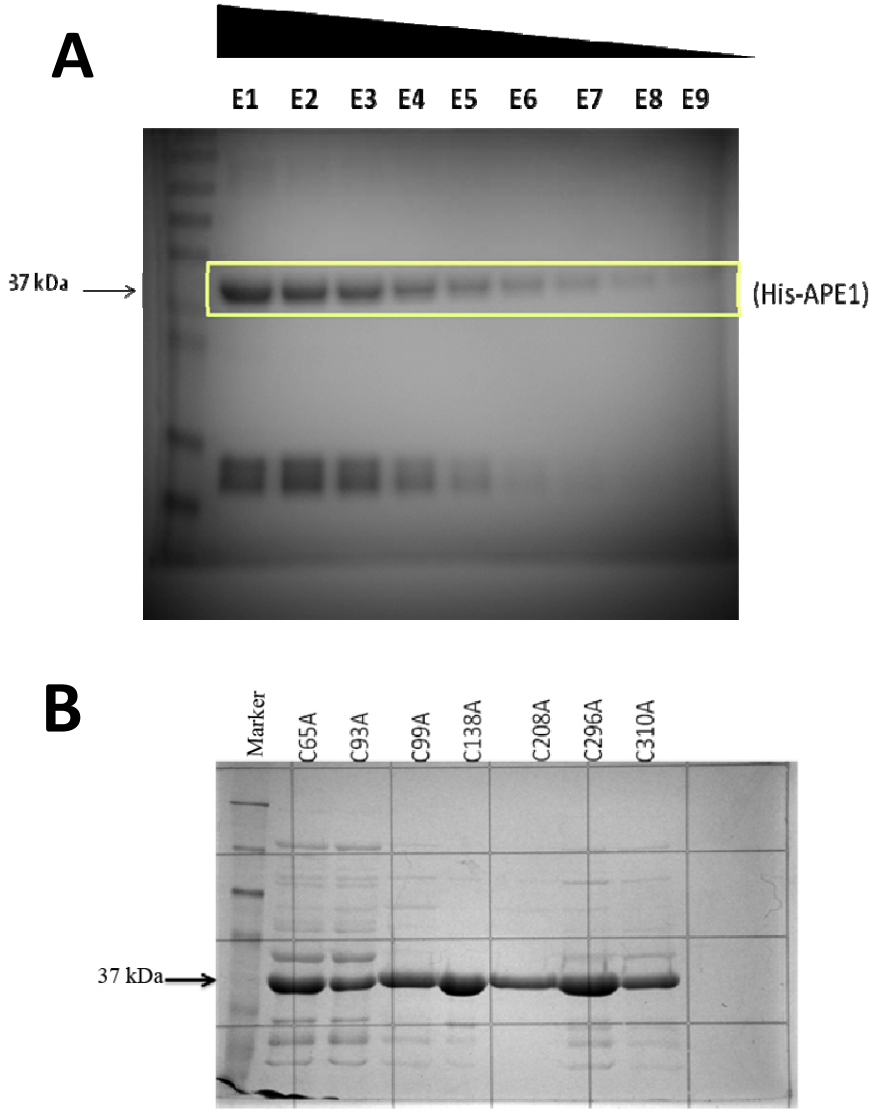

Figure 17. Purification of His-APE1, native and cysteine mutants. A. Wildtype His-APE1 purified protein elution fractions. Elution fractions were loaded in same order of elution on a SDS-PAGE (12%) under reducing conditions (6.25% β-mercaptoethanol). The gel was stained with Coomasie blue. His-APE1 was detected at the expected size (37kDa). The observed low molecular weight fragments were eliminated after the dialysis step. B. Purification of APE1 cysteine mutants**.** One elution fraction of each purified protein was loaded on a SDS-PAGE (10%) under reducing conditions. The gel was stained with Coomasie blue.

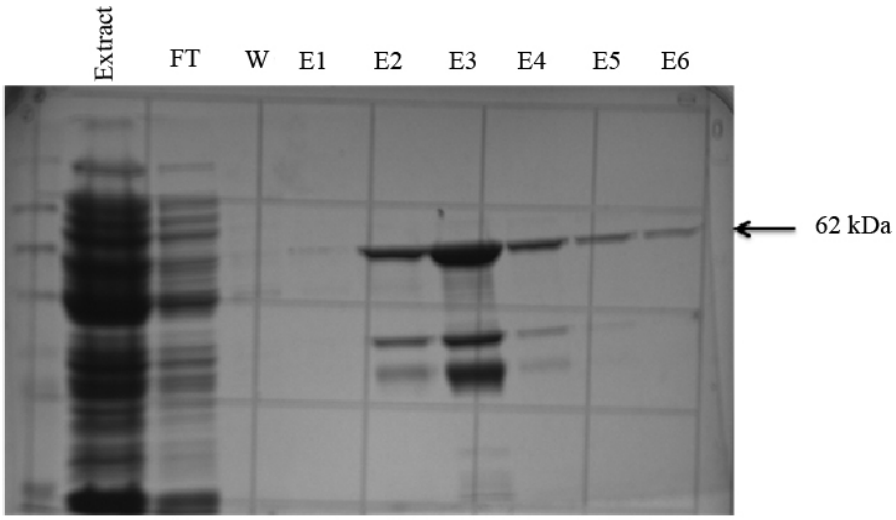

Figure 18. GST-GAPDH purification. Extract: total bacterial protein extract. FT: flow through. W: washing buffer fraction.E1→E5: elution fractions.

4. *In vitro* reduction of APE1 by GAPDH

After creating and purifying APE1 cysteine mutants, we sought to choose an adequate assay to identify candidate cysteine (s) involved in the reduction of APE1 by GAPDH. We decided to use silver staining assay which is used to detect proteins in a gel. This protein detection technique is about 50 times more sensitive than the conventional Coomasie Brilliant blue staining in detecting trace amounts of proteins in a SDS-PAGE [193].

The use of this technique to evaluate the redox state of proteins assay was validated before [156] as an enhanced protein staining was observed under reducing conditions compared to non-reducing conditions.

Under reducing gel migration conditions (i.e. addition of 2-mercaptoethanol in the protein loading buffer), we observed that purified wild-type APE1 is more intensely stained with silver nitrate compared to non-reducing conditions (**Figure 19**, lanes 1 and 2). Similarly, when His-APE1 was incubated for 10 min with GAPDH under non-reducing conditions, an enhanced staining of APE1 was noticed (**Figure 19**, lane 4). This indicates that GAPDH is reducing APE1.

The incubation of APE1 with both GAPDH and 2-mercaptoethanol had no additional effect on the staining of APE1 by silver nitrate, indicating that GAPDH is able to fully reduce APE1 (data not shown).

It is noteworthy that when APE1 migrates in the absence of a reducing agent (**Figure 19**, Lane 2), it cannot be detected by silver stain. This triggered us to monitor APE1 redox state by other means than the silver staining, that is by western blotting [156].
APE1 was incubated with and without 2-mercaptoethanol (**Figure 20**, lanes 1 and 2) or with GAPDH for different time periods (**Figure 20**, lane 3, 4 and 5). Under non-reducing conditions and in the absence of GAPDH, APE1 forms high molecular weight species (~175 kDa) that most probably correspond to oxidized aggregated proteins (**Figure 20**, lane 1) that were not detected by silver staining (**Figure 19**, lane 2). When GAPDH was added to the reaction, APE1 can be detected at its predicted molecular weight of 37 kDa with the anti-His antibody, similarly to what was observed when APE1 was loaded under reducing conditions (**Figure 20**, lane 2).

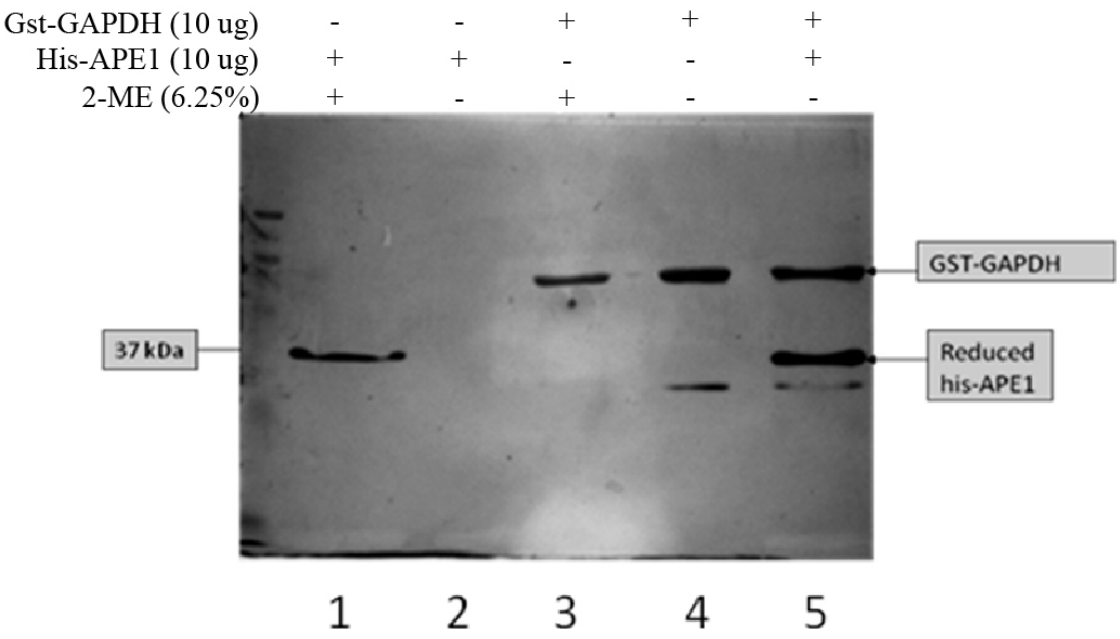

Figure 19. *In vitro* reduction of His-APE1 by GST-GAPDH. Purified His-APE1 and GST-GAPDH were incubated for 10 min at 37°C; the reaction was stopped on ice and by the addition of the protein loading buffer. Samples were run on a SDS-PAGE (12%) and the gel was stained with silver nitrate as described in chapter 2, section 3. The lower band in lanes 4 and 5 is an impurity (GST as seen by western blot using anti-GST).

We next attempted to test the purified APE1 cysteine mutants under the same assay conditions. We anticipate that GAPDH might cause a conformational change in APE1 structure by reducing an intramolecular disulfide bridge(s) between cysteine residues that forms as a result of oxidation of the protein. Therefore, APE1 cysteine mutants will be assayed for their redox state to identify which mutant(s) will have enhanced silver staining. This mutant is expected to be more reduced, independently of GAPDH compared to the native APE1, as a Cys to Ala mutation will simulate the reduced state of the substituted cysteine since Alanine cannot be engaged in disulfide bridge formation upon oxidation. This experiment is still in progress.

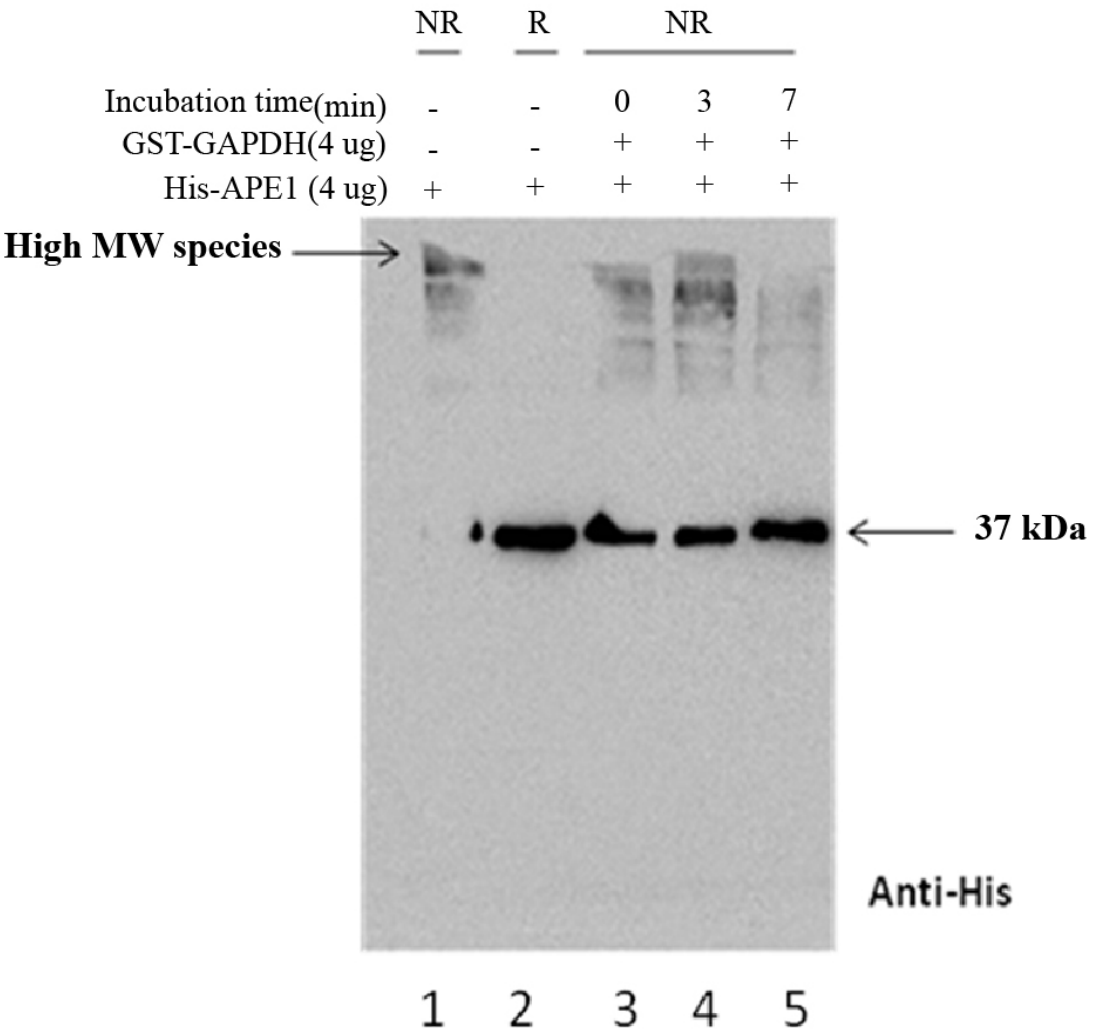

Figure 20. GAPDH reduction of APE1 is a fast reaction. His-APE1 and GST-GAPDH were incubated for at 37°C for different periods of time (up till 45 min, not shown). Samples were run on a SDS-PAGE (12%) followed by western blot (anti-his antibody). The reduced APE1 is seen at 37 kDa. In lane 2, 2-mercaptoethanol was the reducing agent used. MW: molecular weight.

5. Cloning of APE1 variants in pEYFP-N1 vector for expression in mammalian cells

In addition to the *in vitro* characterization of APE1 cysteine mutants redox state in relation to GAPDH, we sought to express theses variants in mammalian cells. Our model predicts that APE1 cysteine mutant that cannot be oxidized will rescue the cell cycle defects seen in GAPDH deficient cells, as this mutant APE1 will remain active independent of GAPDH. For expression in mammalian cells, APE1 variant were cloned in pEYFP-N1 (**figure 21**).

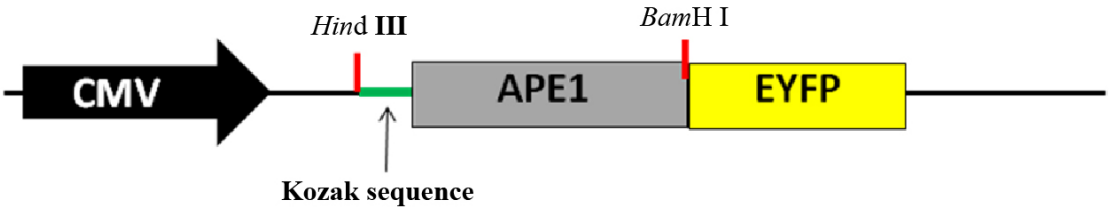

Figure 21. pAPE1-EYFP construct for expression in HCT116. APE1 variants were cloned in pEYFP-N1 vector. APE1 stop codon was removed to allow the expression of APE1-EYFP fusion protein. In addition, a Kozak sequence (GCC ACC) was added to APE1 PCR primers to enhance the translation of corresponding mRNAs and hence the protein expression. The EYFP gene contains four amino acid substitutions which makes the emission spectrum in the green-yellow region.

6. Establishing stable clones expressing APE1 cysteine variants

HCT116 were transfected with APE1-EYFP expression constructs and subjected to G418 serial selection to generate stable clones. The expression of APE1 variants in the established stable clones is monitored by fluorescent microscopy and by western blotting (in progress). All of the APE1-EYFP variants were localized in the nucleus (data now shown).

Chapter 4: Discussion

The diverse functions of APE1 as a DNA repair enzyme and a redox factor render it an essential gene for cell survival. Studying the consequences of APE1 activation by GAPDH, another essential gene, is of great importance to unravel a new posttranslational modification affecting APE1 activity as well as the indirect involvement of GAPDH in maintaining the genomic stability.

In the present study we investigated the consequences of GAPDH knockdown on the cell cycle progression and we reported that cells in which GAPDH is knocked-down arrest in G1 phase. APE1 subcellular localization study showed that APE1 forms foci-like structures in the nucleus of cells knocked down for GAPDH.

Since GAPDH activation of APE1 is achieved by reduction, we sought to search for the cysteine residue(s) that are possibly targeted by GAPDH. APE1 cysteine mutants were created by site-directed mutagenesis and purified using affinity chromatography. *In vitro* attempts to identify the redox-sensitive cysteine (s) target(s) of GAPDH reduction did not succeed so far due to the quality of the purified proteins. A construct for expression of EYFP-tagged APE1 variants, including cysteine and active site mutants, in mammalian cells was successfully established and the expression was seen under the microscope.

1. GAPDH deficiency causes G1 arrest

GAPDH is an essential gene, hence a knockdown instead of a knockout approach was used to evaluate GAPDH role in activating APE1 in mammalian cells. siRNA knockdown of GAPDH caused a defect in cell cycle progression, which was not surprising since a decrease in cell proliferation was also seen in GAPDH knockdown cells (Ramotar Lab, unpublished data). Moreover, GAPDH deficiency caused an increase in the formation of spontaneous AP sites and showed a decrease in the AP endonuclease activity of the cells [156]. These data suggest that the delay seen in the cell cycle is most probably due to an accumulation of AP sites which is known to activate checkpoints, delaying cell cycle progression to allow repair [194]. Checkpoints signalling pathways activate damage-specific DNA repair mechanisms, in this case the BER, before the transition from G1 to S phase and prior to DNA synthesis [195], as BER pathway is mostly active in G1 phase where oxidized bases and strand breaks are repaired [194].

The observed G1 arrest of GAPDH depleted cells might be occurring via the classical activation of p53 by phosphorylation on Ser-15, which was reported by another group that also showed that siRNA-mediated GAPDH knockdown accumulate in G1 phase [196]. It would be also interesting to examine checkpoints activation in knockdown cells by monitoring Chk1 and Chk2 phosphorylation state by western blot analysis.

2. Use of Apn1 for complementation assays

Given that APE1 is the major human AP endonuclease responsible for processing AP sites, the accumulation of oxidized inactive species of this protein in GAPDH knockdown cells explains the deficiency in AP endonuclease activity observed in these cells [156], which might also validate the observed cell cycle defects. Complementation strategy was used to verify that the lack of APE1 activity is the reason behind the G1 arrest. Overexpression of wild type APE1 in GAPDH depleted cells can be confusing since the ratio of the endogenous to the introduced APE1 in addition to the redox state of the total APE1 population were a concern. Consequently, the expression of the yeast Apn1 that is not redox sensitive was used to verify the implication of APE1 activity in the defective cell cycle, as this enzyme shares many functional features with APE1, most importantly the repair of AP sites. A direct measurement of AP sites frequency in GAPDH knockdown cells transfected with or without Apn1 plasmid can be done to further verify Apn1 rescuing effects. In fact, complementation experiments in which Apn1 was expressed in mammalian cells and compensated for a deficiency in APE1 activity were reported [121].

3. Apn1 expression in HCT116 cells is lethal

We noticed that stable and transient transfection of HCT116 cells with Flag- and EYFP-tagged Apn1 was causing many cells to die about 24 hours following transfection. The observed cell death, that probably explains the expression problems seen in Apn1

transfected cells, prompted us to think that Apn1 might be toxic for the cells. Importantly, Apn1 expression under the constitutive CMV promoter in GAPDH knockdown cells, where higher AP site frequency was observed, caused even an increased lethality. In fact, it was shown that the overexpression of Apn1 in a cells with high frequency of AP sites enhances cells sensitivity to MMS [197]. It seems that cells are better able to tolerate intact AP sites than the strand breaks generated by Apn1 incision.

Hence, during cell cycle analysis, the rescuing phenotype attributed by Apn1 in GAPDH knockdown cells might be due to surviving untransfected cells.

Interestingly, the expression of the enzymatically inactive variant of Apn1, E158G, did not increase cell death in GAPDH knockdown cells as compared to wild type Apn1. Therefore, we plan to design a survival assay (e.g. the clonogenic assay) where we monitor the effects of Apn1 and its inactive variant on the survival of GAPDH knockdown cells.

4. Apn1 stable clones

Considering the difficulties faced in transiently expressing Apn1 in HCT116, we tried to establish stable HCT116 cell lines expressing Flag-Apn1 similarly to what was done before [121]. After three weeks of selection with G418 and after isolating and growing 20 separate single colonies, western blot analysis with anti-Flag antibody failed to show any expression of Apn1. Another attempt to detect Apn1 expression using a serum containing Apn1 antibody prepared in the laboratory was unsuccessful.

Surprisingly, Fung and Demple (2005) [121] successfully established stable clones expressing Flag-Apn1 in HCT116 and that was proved by western blot analysis. Although we were using the same protocols and the same cell line, we were unable to reproduce their work.

Establishing stable clones using Apn1-EYFP and its variant Apn1- E158G-EYFP constructs was not very successful either. We detected a low level of expression corresponding to a low number of cells showing fluorescent proteins. Four days after starting the treatment with G418, and a total of six days after the transfection, almost none of the cells expressed the fluorescent Apn1 and its variant, most probably due to cell death. The pEYFP-N1 vector that we are using has an origin of replication that is induced by T antigen, hence expressing Apn1 in HEK 293T cells that have SV40 T antigen might be an alternative.

It is noteworthy that using the same construct and the same cell line, the expression of APE1- EYFP variants was successful and stable clones were established. As a foreign protein, Apn1 proteins might be unstable in mammalian cells context, which could lead to their degradation due to the absence of potentially needed yeast proteins for its proper folding. Moreover, the presence of a tag might also be interfering with the protein folding and stability. RT-PCR of RNA extracts from Apn1-transfected cells will provide an evidence of whether the expression problem is at the protein level or at the transcriptional level.

5. APE1 foci-like structure could be aggregates of oxidized species

Upon oxidative stress, as a DNA repair enzyme and a transcription regulator, APE1 translocates to the nucleus [104]. In fact it is considered mainly a nuclear protein, although many reports showed its cytoplasmic localization that was correlated to tumoregenesis [198]. It was shown to localize to the endoplasmic reticulum (ER) where it forms a complex with other proteins and might get cleaved at its Lys 31 giving rise to NΔ33APE1 form that was shown to localize to the mitochondria [185, 186].

It was not surprising that APE1 was localized to the nucleus in our indirect-immunofluorescence experiments, but what was striking is the foci-like structures seen only in GAPDH knockdown cells and not in the control cells. They could be either nuclear foci composed of recruited and inactive APE1 that retains the ability to be recruited to DNA damage sites or they simply represent aggregates of oxidized forms of APE1 in the absence of enough GAPDH to reduce the proteins.

A key experiment where we test if the observed APE1 structures co-localizes with XRCC1, a BER enzyme that usually co-localize with APE1 on DNA lesions [199], might favor the nuclear foci hypothesis. Additionally, the expression of Apn1 in these cells is expected to abolish the formation of these foci by competing with the inactive APE1 for recruitment and binding to DNA lesions. Alternatively, if what we observed was just protein aggregates, treatment of GAPDH knocked down cells with reducing agents such as the antioxidant N-acetyl cysteine (NAC) should abolish APE1 nuclear “aggregates” by conferring a reduced cellular environment that can compensate for the lack of GAPDH.

Furthermore, monitoring APE1 subcellular localization in each phase of the cell cycle of GAPDH knockdown cells will provide us with the status of APE1 in G1 where the cells are arresting: are these nuclear foci present mainly in G1? If these foci are present evenly in all cell cycle phases, they are most probably a result of aggregation of oxidized proteins.

6. Purified cysteine mutants aggregate

There are many possible reasons behind the problems encountered with the purified APE1 mutants. First, the purified proteins were aggregating, as we saw the non-homogeneity of the protein-containing solution by the naked eye. Attempts to change storage conditions (buffer, temperature) and to remove the observed white aggregates from the solution by high speed centrifugation, failed to resolve the quantification problem. The addition of DTT might have solved the problem but since we are interested in the redox state of these proteins, the addition of a strong reducing agent will interfere with our assays.

Second, purified proteins were extracted from 1L of bacterial culture, which yielded enormous amounts of proteins as visualized by SDS-PAGE. The high concentration of purified proteins can cause aggregation. We tried to dilute the protein suspensions but the aggregation problem was not solved. Starting with a smaller bacterial culture could be a better idea than diluting what is already aggregated.

Oxidized proteins tend to aggregate in solution due to many reasons but mainly because of the formation of inter-and intra-molecular disulfide bridges. However, it is surprising that cysteine mutants would aggregate taking into consideration that a lack in a cysteine residue presumably will result in less disulfide bridge formation, hence less aggregation.

Other groups have purified these variant with no mention of such problems [191]. However, to make cysteine mutants they used Cys to Ser mutation and they also used DTT in their purification buffers.

7. Expression of APE1 mutants in AP endonuclease deficient bacteria

To test the functional state of APE1 cysteine mutants *in vivo*, we tried to express them in a bacterial strain, BW528 [Δ(xth-pnc), nfo1::kan], lacking both Endo IV and Exo III AP endonucleases and look for complementation when challenged with DNA damaging drugs. Interestingly, the expression of wild type APE1 as well as its cysteine mutants in this strain was lethal compared to the empty vector. Lethality was more striking in wild type APE1, C65A, C93A and C99A transformed bacteria (data not shown). Considering that it lacks AP endonuclease activity, this strain is expected to accumulate a high level of unrepaired AP sites. If they are functional, the expression of APE1 and its variants in this strain will possibly catalyze the incision of these abundant AP sites, leading to DNA fragmentation which possibly explains the lethality, an observation reported in yeast where the overexpression of Apn1 is lethal [197]. Another observation in this context was in *C.elegans* where the knockdown of APN-1 suppressed lethality in strain where

depletion of dUTP nucleotidohydrolase (DUT-1) results in an increase of mis-incorporated uracil in the DNA, which will be removed by uracil-DNA glycosylase (UNG-1) leaving a lot of AP sites behind [95]. It is noteworthy that the transformation of BL21 *E.coli* strain that does not lack Exo III and Endo IV was successful.

8. Conclusion and future work

GAPDH was shown once again to be more than just a glycolytic enzyme. We revealed a novel role for this enzyme in DNA repair by maintaining APE1 activity. Depletion in GAPDH caused an increase in the mutagenic AP site lesions, arrested the cells in G1 phase, halted cell proliferation and caused APE1 to form nuclear foci-like structures that needs to be studied further. Therefore, by activating APE1, GAPDH is helping in maintaining the genomic integrity of the cells and promoting their survival under normal conditions, during oxidative stress and when challenged with DNA damaging agents. Moreover, GAPDH reduction of APE1 emphasizes the importance of APE1 posttranslational modifications in defining its functional pattern as a multifunctional protein. But more questions related to APE1-GAPDH interaction need to be addressed: which APE1 cysteine residue(s) is the target of reduction by GAPDH? Does this reduction reaction trigger a conformational change in APE1 structure rendering it active? In addition to its DNA repair function, does this interaction affect the redox function of APE1?

8.1 Study the AP endonuclease activity of APE1 variants

Although there are many reports where the activity of these variants was tested (see **table I** of chapter 3), some of these reports used Cys to Ser instead of Cys to Ala substitution and used different conditions under which the assays were performed. Therefore, in the process of identifying the cysteine residue(s) of APE1 that are reduced by GAPDH, it is of major importance to study the AP endonuclease activity of these mutants after oxidizing them with H_2O_2 using the AP endonuclease assay. This assay allows the detection of AP endonuclease activity using a 42 mer radiolabled oligonucleotide containing a centered AP site as described previously [96]. This substrate is then incubated with the enzyme that, when active, will cleave the AP site, leading to the formation of a 20 mer radiolabled fragment that migrates faster than the intact substrate on a 10% polyacrylamide/7 M urea gels. The mutant(s) that retain their AP endonuclease activity will be expected to play a complementing role in AP endonuclease deficient yeast cells for instance and in GAPDH deficient cells. This mutant will be a possible candidate of GAPDH reduction. On the other hand, the mutant(s) lacking the AP endonuclease activity might be crucial for APE1 catalytic activity and will need further investigation since no cysteine residue has been reported to participate in APE1 catalytic reaction. As controls, we are going to use APE1 active site mutants, H309A, D210A and D308 in addition to the wild type APE1 and Apn1. We are mostly interested in the C310A variant since it was shown that the oxidation of this protein by H_2O_2 did not affect its AP endonuclease activity [155].

8.2 Expression of APE1 cysteine mutants in GAPDH knockdown cells

The HCT116 stable clones expressing wild type APE1 and its cysteine mutants will be knocked down for GAPDH and will be assayed for cell cycle analysis, subcellular localization studies, and measurement of AP sites frequency and AP endonuclease activity of total cell extracts. We expect that at least one cysteine mutant will be active in the cells similarly to a wild type but that is not susceptible for redox inactivation. This variant is expected to release cells from the G1 arrest seen in GAPDH depleted cells. In addition, whether APE1 foci-like structures seen in GAPDH knocked down cells are protein aggregates or nuclear foci on DNA lesions, this variant is not expected to form such structures as we expect it to be reduced and active independent of GAPDH. We also anticipate that GAPDH knockdown cell expressing this variant will have less spontaneous AP sites and an enhanced AP endonuclease activity.

8.3 Expression of APE1 cysteine mutants in AP endonuclease deficient yeast strain

The AP endonuclease deficient *S. cerevisiae* YW778 strain in which APN1 and APN2 were deleted will be used as a host for APE1 cysteine mutants complementation studies. To clone these mutants in a yeast expression vector we are planning to use gap-repair

cloning. Next, the transformed YW778 strain will be subjected to survival assays using many DNA damaging agents such as MMS, H_2O_2 and bleomycin. UVC will be used as a negative control.

8.4 GAPDH-induced APE1 Conformational change

Most posttranslational modifications affect the activity of their targets proteins by acting on their conformation, such as hiding or exposing the active site, an interaction domain, a localization signal, etc… In proteins, thiol groups (-SH) present on cysteine residues are susceptible to oxidation that will engage two cysteines to form a disulfide bridge (R-S-S-R), a phenomenon known to be reversible by the addition of an adequate reducing agent. These intra-molecular changes are expected to cause conformational changes of the protein; especially if thiol groups are abundant as in the case of APE1 that has seven cysteine residues. We believe that this is the case of APE1 reduction by GAPDH. To test this hypothesis, we plan to use circular dichroism (CD) analysis to study purified APE1structural changes after its interaction with GAPDH, in addition to the proper controls, in the far UV range.

8.5 Study the effect of GAPDH on APE1 redox function

So far we know that GAPDH reduction of oxidized APE1 activates its DNA repair function [156], but no characterization of its redox function in relation to this reduction has been done yet. Thioredoxin was shown to reduce and enhance APE1 redox function

which in turn enhances the AP-1 DNA binding and p53 activation [153, 154]. To examine whether GAPDH is involved in the regulation of APE1 redox function, activity of the major transcription factors regulated by APE1 (AP-1, p53, Jun, Fos…) in GAPDH knockdown cells should be tested. For instance, to check AP-1 activity we plan to check the activity of the human collagenase I promoter in GAPDH knockdown cells, which contains an AP-1-binding site, as described previously[153]. It remains possible that thioredoxin and GAPDH may perform distinct redox functions on oxidized APE1. However, since Apn1 does not have a redox function, the effect of its expression in GAPDH knockdown cells is of great importance in differentiating between the redox and repair functions of APE1 that could be regulated by GAPDH.

9. Perspectives

Unrepaired AP sites lead to an increase in cytotoxicity, cancer or apoptosis [12]. As the major DNA repair enzyme that processes AP sites, APE1 plays an essential role in maintaining genomic integrity and promoting cell survival, hence the importance of the regulation of its activities in normal as well as in cancer cells. In our laboratory, we showed that GAPDH deficiency sensitizes the cells to bleomycin which induces DNA strand breaks with blocked 3'-termini processed by APE1. Consequently targeting its DNA repair function through GAPDH could be a novel mechanism for APE1 involvement in chemotherapeutic treatments. By preventing structural inactivation of APE1, we believe that GAPDH is acting to maintain the genetic stability which might explain GAPDH involvement in the pathology of many oxidative stress related

neurodegenerative diseases where an accumulation of mutations leading to apoptotic cell death was shown [200].

References

1. Kultz, D., *Molecular and evolutionary basis of the cellular stress response.* Annu Rev Physiol, 2005. 67: p. 225-57.
2. Marnett, L.J., *Oxyradicals and DNA damage.* Carcinogenesis, 2000. 21(3): p. 361-70.
3. Cooke, M.S., et al., *Oxidative DNA damage: mechanisms, mutation, and disease.* Faseb J, 2003. 17(10): p. 1195-214.
4. Valko, M., et al., *Free radicals, metals and antioxidants in oxidative stress-induced cancer.* Chem Biol Interact, 2006. 160(1): p. 1-40.
5. Cadenas, E., *Biochemistry of oxygen toxicity.* Annu Rev Biochem, 1989. 58: p. 79-110.
6. Poli, G., et al., *Oxidative stress and cell signalling.* Curr Med Chem, 2004. 11(9): p. 1163-82.
7. Dudley, D.D., et al., *Mechanism and control of V(D)J recombination versus class switch recombination: similarities and differences.* Adv Immunol, 2005. 86: p. 43-112.
8. Takimoto CH, C.E., *Principles of Oncologic Pharmacotherapy*. 11 ed. Cancer Management: A Multidisciplinary Approach. 2008.
9. Gates, K.S., *An overview of chemical processes that damage cellular DNA: spontaneous hydrolysis, alkylation, and reactions with radicals.* Chem Res Toxicol, 2009. 22(11): p. 1747-60.
10. Lindahl, T. and A. Andersson, *Rate of chain breakage at apurinic sites in double-stranded deoxyribonucleic acid.* Biochemistry, 1972. 11(19): p. 3618-23.
11. Lindahl, T. and B. Nyberg, *Rate of depurination of native deoxyribonucleic acid.* Biochemistry, 1972. 11(19): p. 3610-8.
12. Loeb, L.A. and B.D. Preston, *Mutagenesis by apurinic/apyrimidinic sites.* Annu Rev Genet, 1986. 20: p. 201-30.
13. Goodman, M.F., et al., *Nucleotide insertion and primer extension at abasic template sites in different sequence contexts.* Ann N Y Acad Sci, 1994. 726: p. 132-42; discussion 142-3.
14. Sung, J.S. and B. Demple, *Roles of base excision repair subpathways in correcting oxidized abasic sites in DNA.* Febs J, 2006. 273(8): p. 1620-9.
15. Aust, A.E. and J.F. Eveleigh, *Mechanisms of DNA oxidation.* Proc Soc Exp Biol Med, 1999. 222(3): p. 246-52.
16. Nakabeppu, Y., et al., *Biological significance of the defense mechanisms against oxidative damage in nucleic acids caused by reactive oxygen species: from mitochondria to nuclei.* Ann N Y Acad Sci, 2004. 1011: p. 101-11.
17. Ames, B.N., *Endogenous oxidative DNA damage, aging, and cancer.* Free Radic Res Commun, 1989. 7(3-6): p. 121-8.
18. Imlay, J.A. and S. Linn, *DNA damage and oxygen radical toxicity.* Science, 1988. 240(4857): p. 1302-9.
19. Xiao, W. and L. Samson, *In vivo evidence for endogenous DNA alkylation damage as a source of spontaneous mutation in eukaryotic cells.* Proc Natl Acad Sci U S A, 1993. 90(6): p. 2117-21.

20. **Sedgwick, B., *Nitrosated peptides and polyamines as endogenous mutagens in O6-alkylguanine-DNA alkyltransferase deficient cells.* Carcinogenesis, 1997. 18(8): p. 1561-7.**
21. **Lundin, C., et al., *Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks.* Nucleic Acids Res, 2005. 33(12): p. 3799-811.**
22. **Sedgwick, B., *Repairing DNA-methylation damage.* Nat Rev Mol Cell Biol, 2004. 5(2): p. 148-57.**
23. **Barnes, D.E. and T. Lindahl, *Repair and genetic consequences of endogenous DNA base damage in mammalian cells.* Annu Rev Genet, 2004. 38: p. 445-76.**
24. **Iyer, R.R., et al., *DNA mismatch repair: functions and mechanisms.* Chem Rev, 2006. 106(2): p. 302-23.**
25. **Lim, K.S., et al., *Potential artifacts in the measurement of DNA deamination.* Free Radic Biol Med, 2006. 40(11): p. 1939-48.**
26. **Coker, H.A. and S.K. Petersen-Mahrt, *The nuclear DNA deaminase AID functions distributively whereas cytoplasmic APOBEC3G has a processive mode of action.* DNA Repair (Amst), 2007. 6(2): p. 235-43.**
27. **Leibeling, D., P. Laspe, and S. Emmert, *Nucleotide excision repair and cancer.* J Mol Histol, 2006. 37(5-7): p. 225-38.**
28. **Demple, B. and M.S. DeMott, *Dynamics and diversions in base excision DNA repair of oxidized abasic lesions.* Oncogene, 2002. 21(58): p. 8926-34.**
29. **Hegde, M.L., T.K. Hazra, and S. Mitra, *Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells.* Cell Res, 2008. 18(1): p. 27-47.**
30. **Pogozelski, W.K. and T.D. Tullius, *Oxidative Strand Scission of Nucleic Acids: Routes Initiated by Hydrogen Abstraction from the Sugar Moiety.* Chem Rev, 1998. 98(3): p. 1089-1108.**
31. **Chen, J. and J. Stubbe, *Bleomycins: towards better therapeutics.* Nat Rev Cancer, 2005. 5(2): p. 102-12.**
32. **Caldecott, K.W., *Single-strand break repair and genetic disease.* Nat Rev Genet, 2008. 9(8): p. 619-31.**
33. **Shrivastav, M., L.P. De Haro, and J.A. Nickoloff, *Regulation of DNA double-strand break repair pathway choice.* Cell Res, 2008. 18(1): p. 134-47.**
34. **Sutherland, B.M., et al., *Clustered DNA damages induced in isolated DNA and in human cells by low doses of ionizing radiation.* Proc Natl Acad Sci U S A, 2000. 97(1): p. 103-8.**
35. **Sutherland, B.M., et al., *Clustered DNA damages induced by x rays in human cells.* Radiat Res, 2002. 157(6): p. 611-6.**
36. **McKinnon, P.J., *ATM and ataxia telangiectasia.* EMBO Rep, 2004. 5(8): p. 772-6.**
37. **Harper, J.W. and S.J. Elledge, *The DNA damage response: ten years after.* Mol Cell, 2007. 28(5): p. 739-45.**
38. **Weinert, T., *DNA damage checkpoints update: getting molecular.* Curr Opin Genet Dev, 1998. 8(2): p. 185-93.**
39. **Nurse, P., *Checkpoint pathways come of age.* Cell, 1997. 91(7): p. 865-7.**
40. **Morgan, D.O., *Principles of CDK regulation.* Nature, 1995. 374(6518): p. 131-4.**
41. **Bartek, J. and J. Lukas, *Chk1 and Chk2 kinases in checkpoint control and cancer.* Cancer Cell, 2003. 3(5): p. 421-9.**
42. **Kastan, M.B. and J. Bartek, *Cell-cycle checkpoints and cancer.* Nature, 2004. 432(7015): p. 316-23.**

43. McCullough, A.K., M.L. Dodson, and R.S. Lloyd, *Initiation of base excision repair: glycosylase mechanisms and structures.* Annu Rev Biochem, 1999. 68: p. 255-85.
44. Dianov, G.L. and J.L. Parsons, *Co-ordination of DNA single strand break repair.* DNA Repair (Amst), 2007. 6(4): p. 454-60.
45. Lindahl, T. and R.D. Wood, *Quality control by DNA repair.* Science, 1999. 286(5446): p. 1897-905.
46. Wood, R.D., *DNA repair in eukaryotes.* Annu Rev Biochem, 1996. 65: p. 135-67.
47. Zharkov, D.O., *Base excision DNA repair.* Cell Mol Life Sci, 2008. 65(10): p. 1544-65.
48. Hitomi, K., S. Iwai, and J.A. Tainer, *The intricate structural chemistry of base excision repair machinery: implications for DNA damage recognition, removal, and repair.* DNA Repair (Amst), 2007. 6(4): p. 410-28.
49. Lindahl, T., *An N-glycosidase from Escherichia coli that releases free uracil from DNA containing deaminated cytosine residues.* Proc Natl Acad Sci U S A, 1974. 71(9): p. 3649-53.
50. Lindahl, T., *Uracil-DNA glycosylase from Escherichia coli.* Methods Enzymol, 1980. 65(1): p. 284-90.
51. Friedberg, E.C., A.K. Ganesan, and K. Minton, *N-Glycosidase activity in extracts of Bacillus subtilis and its inhibition after infection with bacteriophage PBS2.* J Virol, 1975. 16(2): p. 315-21.
52. Olsen, L.C., et al., *Molecular cloning of human uracil-DNA glycosylase, a highly conserved DNA repair enzyme.* Embo J, 1989. 8(10): p. 3121-5.
53. Hoeijmakers, J.H., *Genome maintenance mechanisms for preventing cancer.* Nature, 2001. 411(6835): p. 366-74.
54. Khanna, K.K. and S.P. Jackson, *DNA double-strand breaks: signaling, repair and the cancer connection.* Nat Genet, 2001. 27(3): p. 247-54.
55. Svejstrup, J.Q., *Mechanisms of transcription-coupled DNA repair.* Nat Rev Mol Cell Biol, 2002. 3(1): p. 21-9.
56. Weiss, B., *Endonuclease II of Escherichia coli is exonuclease III.* J Biol Chem, 1976. 251(7): p. 1896-901.
57. Ljungquist, S., *A new endonuclease from Escherichia coli acting at apurinic sites in DNA.* J Biol Chem, 1977. 252(9): p. 2808-14.
58. Baute, J. and A. Depicker, *Base excision repair and its role in maintaining genome stability.* Crit Rev Biochem Mol Biol, 2008. 43(4): p. 239-76.
59. Krokan, H.E., R. Standal, and G. Slupphaug, *DNA glycosylases in the base excision repair of DNA.* Biochem J, 1997. 325 (Pt 1): p. 1-16.
60. Yamamoto, R., et al., *A novel monofunctional DNA glycosylase activity against thymine glycol in mouse cell nuclei.* J Radiat Res (Tokyo), 2008. 49(3): p. 249-59.
61. Ramotar, D., *The apurinic-apyrimidinic endonuclease IV family of DNA repair enzymes.* Biochem Cell Biol, 1997. 75(4): p. 327-36.
62. Scharer, O.D. and J. Jiricny, *Recent progress in the biology, chemistry and structural biology of DNA glycosylases.* Bioessays, 2001. 23(3): p. 270-81.
63. Unk, I., et al., *Apurinic endonuclease activity of yeast Apn2 protein.* J Biol Chem, 2000. 275(29): p. 22427-34.
64. Gellon, L., et al., *Intrinsic 5'-deoxyribose-5-phosphate lyase activity in Saccharomyces cerevisiae Trf4 protein with a possible role in base excision DNA repair.* DNA Repair (Amst), 2008. 7(2): p. 187-98.
65. Robertson, A.B., et al., *DNA repair in mammalian cells: Base excision repair: the long and short of it.* Cell Mol Life Sci, 2009. 66(6): p. 981-93.

66. Klungland, A. and T. Lindahl, *Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1).* Embo J, 1997. 16(11): p. 3341-8.
67. Fortini, P. and E. Dogliotti, *Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways.* DNA Repair (Amst), 2007. 6(4): p. 398-409.
68. Jones, S., et al., *Biallelic germline mutations in MYH predispose to multiple colorectal adenoma and somatic G:C-->T:A mutations.* Hum Mol Genet, 2002. 11(23): p. 2961-7.
69. Chevillard, S., et al., *Mutations in OGG1, a gene involved in the repair of oxidative DNA damage, are found in human lung and kidney tumours.* Oncogene, 1998. 16(23): p. 3083-6.
70. Fishel, M.L. and M.R. Kelley, *The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target.* Mol Aspects Med, 2007. 28(3-4): p. 375-95.
71. Demple, B. and L. Harrison, *Repair of oxidative damage to DNA: enzymology and biology.* Annu Rev Biochem, 1994. 63: p. 915-48.
72. Barzilay, G. and I.D. Hickson, *Structure and function of apurinic/apyrimidinic endonucleases.* Bioessays, 1995. 17(8): p. 713-9.
73. Friedberg, E.C. and D.A. Goldthwait, *Endonuclease II of E. coli. I. Isolation and purification.* Proc Natl Acad Sci U S A, 1969. 62(3): p. 934-40.
74. Friedberg, E.C., S.M. Hadi, and D.A. Goldthwait, *Endonuclease II of Escherichia coli. II. Enzyme properties and studies on the degradation of alkylated and native deoxyribonucleic acid.* J Biol Chem, 1969. 244(21): p. 5879-89.
75. Cunningham, R.P. and B. Weiss, *Endonuclease III (nth) mutants of Escherichia coli.* Proc Natl Acad Sci U S A, 1985. 82(2): p. 474-8.
76. Kim, J. and S. Linn, *The mechanisms of action of E. coli endonuclease III and T4 UV endonuclease (endonuclease V) at AP sites.* Nucleic Acids Res, 1988. 16(3): p. 1135-41.
77. Mol, C.D., et al., *DNA-bound structures and mutants reveal abasic DNA binding by APE1 and DNA repair coordination [corrected].* Nature, 2000. 403(6768): p. 451-6.
78. Levin, J.D., A.W. Johnson, and B. Demple, *Homogeneous Escherichia coli endonuclease IV. Characterization of an enzyme that recognizes oxidative damage in DNA.* J Biol Chem, 1988. 263(17): p. 8066-71.
79. Johnson, A.W. and B. Demple, *Yeast DNA diesterase for 3'-fragments of deoxyribose: purification and physical properties of a repair enzyme for oxidative DNA damage.* J Biol Chem, 1988. 263(34): p. 18009-16.
80. Sander, M. and S.M. Huang, *Characterization of the nuclease activity of Drosophila Rrp1 on phosphoglycolate- and phosphate-modified DNA 3'-termini.* Biochemistry, 1995. 34(4): p. 1267-74.
81. Mol, C.D., et al., *Structure and function of the multifunctional DNA-repair enzyme exonuclease III.* Nature, 1995. 374(6520): p. 381-6.
82. Ljungquist, S., T. Lindahl, and P. Howard-Flanders, *Methyl methane sulfonate-sensitive mutant of Escherichia coli deficient in an endonuclease specific for apurinic sites in deoxyribonucleic acid.* J Bacteriol, 1976. 126(2): p. 646-53.
83. Popoff, S.C., et al., *Yeast structural gene (APN1) for the major apurinic endonuclease: homology to Escherichia coli endonuclease IV.* Proc Natl Acad Sci U S A, 1990. 87(11): p. 4193-7.
84. Ramotar, D., et al., *Cellular role of yeast Apn1 apurinic endonuclease/3'-diesterase: repair of oxidative and alkylation DNA damage and control of spontaneous mutation.* Mol Cell Biol, 1991. 11(9): p. 4537-44.

85. Kunz, B.A., et al., *Specificity of the mutator caused by deletion of the yeast structural gene (APN1) for the major apurinic endonuclease.* Proc Natl Acad Sci U S A, 1994. 91(17): p. 8165-9.
86. Johnson, R.E., et al., *Identification of APN2, the Saccharomyces cerevisiae homolog of the major human AP endonuclease HAP1, and its role in the repair of abasic sites.* Genes Dev, 1998. 12(19): p. 3137-43.
87. Bennett, R.A., *The Saccharomyces cerevisiae ETH1 gene, an inducible homolog of exonuclease III that provides resistance to DNA-damaging agents and limits spontaneous mutagenesis.* Mol Cell Biol, 1999. 19(3): p. 1800-9.
88. Unk, I., et al., *Stimulation of 3'-->5' exonuclease and 3'-phosphodiesterase activities of yeast apn2 by proliferating cell nuclear antigen.* Mol Cell Biol, 2002. 22(18): p. 6480-6.
89. Seki, S., et al., *A mouse DNA repair enzyme (APEX nuclease) having exonuclease and apurinic/apyrimidinic endonuclease activities: purification and characterization.* Biochim Biophys Acta, 1991. 1079(1): p. 57-64.
90. Robson, C.N., et al., *Structure of the human DNA repair gene HAP1 and its localisation to chromosome 14q 11.2-12.* Nucleic Acids Res, 1992. 20(17): p. 4417-21.
91. Xanthoudakis, S. and T. Curran, *Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity.* Embo J, 1992. 11(2): p. 653-65.
92. Tsuchimoto, D., et al., *Human APE2 protein is mostly localized in the nuclei and to some extent in the mitochondria, while nuclear APE2 is partly associated with proliferating cell nuclear antigen.* Nucleic Acids Res, 2001. 29(11): p. 2349-60.
93. Chen, D.S., T. Herman, and B. Demple, *Two distinct human DNA diesterases that hydrolyze 3'-blocking deoxyribose fragments from oxidized DNA.* Nucleic Acids Res, 1991. 19(21): p. 5907-14.
94. Ribar, B., T. Izumi, and S. Mitra, *The major role of human AP-endonuclease homolog Apn2 in repair of abasic sites in Schizosaccharomyces pombe.* Nucleic Acids Res, 2004. 32(1): p. 115-26.
95. Zakaria, C., et al., *Caenorhabditis elegans APN-1 plays a vital role in maintaining genome stability.* DNA Repair (Amst). 9(2): p. 169-76.
96. Shatilla, A., et al., *Characterization of Caenorhabditis elegans exonuclease-3 and evidence that a Mg2+-dependent variant exhibits a distinct mode of action on damaged DNA.* Biochemistry, 2005. 44(38): p. 12835-48.
97. Daley, J.M., C. Zakaria, and D. Ramotar, *The endonuclease IV family of apurinic/apyrimidinic endonucleases.* Mutat Res.
98. Kane, C.M. and S. Linn, *Purification and characterization of an apurinic/apyrimidinic endonuclease from HeLa cells.* J Biol Chem, 1981. 256(7): p. 3405-14.
99. Demple, B., T. Herman, and D.S. Chen, *Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: definition of a family of DNA repair enzymes.* Proc Natl Acad Sci U S A, 1991. 88(24): p. 11450-4.
100. Robson, C.N. and I.D. Hickson, *Isolation of cDNA clones encoding a human apurinic/apyrimidinic endonuclease that corrects DNA repair and mutagenesis defects in E. coli xth (exonuclease III) mutants.* Nucleic Acids Res, 1991. 19(20): p. 5519-23.
101. Robson, C.N., et al., *Isolation of cDNA clones encoding an enzyme from bovine cells that repairs oxidative DNA damage in vitro: homology with bacterial repair enzymes.* Nucleic Acids Res, 1991. 19(5): p. 1087-92.
102. Seki, S., et al., *cDNA and deduced amino acid sequence of a mouse DNA repair enzyme (APEX nuclease) with significant homology to Escherichia coli exonuclease III.* J Biol Chem, 1991. 266(31): p. 20797-802.

103. **Wilson, D.M., 3rd and D. Barsky, *The major human abasic endonuclease: formation, consequences and repair of abasic lesions in DNA.* Mutat Res, 2001. 485(4): p. 283-307.**

104. **Ramana, C.V., et al., *Activation of apurinic/apyrimidinic endonuclease in human cells by reactive oxygen species and its correlation with their adaptive response to genotoxicity of free radicals.* Proc Natl Acad Sci U S A, 1998. 95(9): p. 5061-6.**

105. **Evans, A.R., M. Limp-Foster, and M.R. Kelley, *Going APE over ref-1.* Mutat Res, 2000. 461(2): p. 83-108.**

106. **Parsons, J.L., Dianova, II, and G.L. Dianov, *APE1 is the major 3'-phosphoglycolate activity in human cell extracts.* Nucleic Acids Res, 2004. 32(12): p. 3531-6.**

107. **Tom, S., et al., *Regulatory roles of p21 and apurinic/apyrimidinic endonuclease 1 in base excision repair.* J Biol Chem, 2001. 276(52): p. 48781-9.**

108. **Fan, J. and D.M. Wilson, 3rd, *Protein-protein interactions and posttranslational modifications in mammalian base excision repair.* Free Radic Biol Med, 2005. 38(9): p. 1121-38.**

109. **Mol, C.D., D.J. Hosfield, and J.A. Tainer, *Abasic site recognition by two apurinic/apyrimidinic endonuclease families in DNA base excision repair: the 3' ends justify the means.* Mutat Res, 2000. 460(3-4): p. 211-29.**

110. **Doetsch, P.W. and R.P. Cunningham, *The enzymology of apurinic/apyrimidinic endonucleases.* Mutat Res, 1990. 236(2-3): p. 173-201.**

111. **Mitra, S., et al., *Complexities of DNA base excision repair in mammalian cells.* Mol Cells, 1997. 7(3): p. 305-12.**

112. **Gros, L., et al., *The major human AP endonuclease (Ape1) is involved in the nucleotide incision repair pathway.* Nucleic Acids Res, 2004. 32(1): p. 73-81.**

113. **Ischenko, A.A. and M.K. Saparbaev, *Alternative nucleotide incision repair pathway for oxidative DNA damage.* Nature, 2002. 415(6868): p. 183-7.**

114. **Peddi, S.R., et al., *The human apurinic/apyrimidinic endonuclease-1 suppresses activation of poly(adp-ribose) polymerase-1 induced by DNA single strand breaks.* Toxicology, 2006. 224(1-2): p. 44-55.**

115. **Chou, K.M. and Y.C. Cheng, *An exonucleolytic activity of human apurinic/apyrimidinic endonuclease on 3' mispaired DNA.* Nature, 2002. 415(6872): p. 655-9.**

116. **Chou, K.M., M. Kukhanova, and Y.C. Cheng, *A novel action of human apurinic/apyrimidinic endonuclease: excision of L-configuration deoxyribonucleoside analogs from the 3' termini of DNA.* J Biol Chem, 2000. 275(40): p. 31009-15.**

117. **Hadi, M.Z., et al., *Determinants in nuclease specificity of Ape1 and Ape2, human homologues of Escherichia coli exonuclease III.* J Mol Biol, 2002. 316(3): p. 853-66.**

118. **Jiricny, J., *An APE that proofreads.* Nature, 2002. 415(6872): p. 593-4.**

119. **Barzilay, G., et al., *Site-directed mutagenesis of the human DNA repair enzyme HAP1: identification of residues important for AP endonuclease and RNase H activity.* Nucleic Acids Res, 1995. 23(9): p. 1544-50.**

120. **Ishchenko, A.A., et al., *The 3'->5' exonuclease of Apn1 provides an alternative pathway to repair 7,8-dihydro-8-oxodeoxyguanosine in Saccharomyces cerevisiae.* Mol Cell Biol, 2005. 25(15): p. 6380-90.**

121. **Fung, H. and B. Demple, *A vital role for Ape1/Ref1 protein in repairing spontaneous DNA damage in human cells.* Mol Cell, 2005. 17(3): p. 463-70.**

122. **Jayaraman, L., et al., *Identification of redox/repair protein Ref-1 as a potent activator of p53.* Genes Dev, 1997. 11(5): p. 558-70.**

123. **Prieto-Alamo, M.J. and F. Laval, *Overexpression of the human HAP1 protein sensitizes cells to the lethal effect of bioreductive drugs.* Carcinogenesis, 1999. 20(3): p. 415-9.**

124. **Xanthoudakis, S., G.G. Miao, and T. Curran, *The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains.* Proc Natl Acad Sci U S A, 1994. 91(1): p. 23-7.**
125. **Walker, L.J., et al., *Identification of residues in the human DNA repair enzyme HAP1 (Ref-1) that are essential for redox regulation of Jun DNA binding.* Mol Cell Biol, 1993. 13(9): p. 5370-6.**
126. **Gorman, M.A., et al., *The crystal structure of the human DNA repair endonuclease HAP1 suggests the recognition of extra-helical deoxyribose at DNA abasic sites.* Embo J, 1997. 16(21): p. 6548-58.**
127. **Beernink, P.T., et al., *Two divalent metal ions in the active site of a new crystal form of human apurinic/apyrimidinic endonuclease, Ape1: implications for the catalytic mechanism.* J Mol Biol, 2001. 307(4): p. 1023-34.**
128. **Ordway, J.M., D. Eberhart, and T. Curran, *Cysteine 64 of Ref-1 is not essential for redox regulation of AP-1 DNA binding.* Mol Cell Biol, 2003. 23(12): p. 4257-66.**
129. **Xanthoudakis, S., et al., *The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice.* Proc Natl Acad Sci U S A, 1996. 93(17): p. 8919-23.**
130. **Ludwig, D.L., et al., *A murine AP-endonuclease gene-targeted deficiency with post-implantation embryonic progression and ionizing radiation sensitivity.* Mutat Res, 1998. 409(1): p. 17-29.**
131. **Zou, G.M., et al., *Ape1 regulates hematopoietic differentiation of embryonic stem cells through its redox functional domain.* Blood, 2007. 109(5): p. 1917-22.**
132. **Izumi, T., et al., *Two essential but distinct functions of the mammalian abasic endonuclease.* Proc Natl Acad Sci U S A, 2005. 102(16): p. 5739-43.**
133. **Bobola, M.S., et al., *Apurinic/apyrimidinic endonuclease activity is associated with response to radiation and chemotherapy in medulloblastoma and primitive neuroectodermal tumors.* Clin Cancer Res, 2005. 11(20): p. 7405-14.**
134. **Madhusudan, S. and M.R. Middleton, *The emerging role of DNA repair proteins as predictive, prognostic and therapeutic targets in cancer.* Cancer Treat Rev, 2005. 31(8): p. 603-17.**
135. **Hiramoto, M., et al., *Nuclear targeted suppression of NF-kappa B activity by the novel quinone derivative E3330.* J Immunol, 1998. 160(2): p. 810-9.**
136. **Lau, J.P., et al., *Effects of gemcitabine on APE/ref-1 endonuclease activity in pancreatic cancer cells, and the therapeutic potential of antisense oligonucleotides.* Br J Cancer, 2004. 91(6): p. 1166-73.**
137. **Ono, Y., et al., *Stable expression in rat glioma cells of sense and antisense nucleic acids to a human multifunctional DNA repair enzyme, APEX nuclease.* Mutat Res, 1994. 315(1): p. 55-63.**
138. **Walker, L.J., et al., *A role for the human DNA repair enzyme HAP1 in cellular protection against DNA damaging agents and hypoxic stress.* Nucleic Acids Res, 1994. 22(23): p. 4884-9.**
139. **Robertson, K.A., et al., *Altered expression of Ape1/ref-1 in germ cell tumors and overexpression in NT2 cells confers resistance to bleomycin and radiation.* Cancer Res, 2001. 61(5): p. 2220-5.**
140. **Tell, G., et al., *An 'environment to nucleus' signaling system operates in B lymphocytes: redox status modulates BSAP/Pax-5 activation through Ref-1 nuclear translocation.* Nucleic Acids Res, 2000. 28(5): p. 1099-105.**

141. Gillardon, F., B. Bottiger, and K.A. Hossmann, *Expression of nuclear redox factor ref-1 in the rat hippocampus following global ischemia induced by cardiac arrest.* Brain Res Mol Brain Res, 1997. 52(2): p. 194-200.
142. Grosch, S. and B. Kaina, *Transcriptional activation of apurinic/apyrimidinic endonuclease (Ape, Ref-1) by oxidative stress requires CREB.* Biochem Biophys Res Commun, 1999. 261(3): p. 859-63.
143. Okazaki, T., et al., *A redox factor protein, ref1, is involved in negative gene regulation by extracellular calcium.* J Biol Chem, 1994. 269(45): p. 27855-62.
144. Tell, G., et al., *The many functions of APE1/Ref-1: not only a DNA repair enzyme.* Antioxid Redox Signal, 2009. 11(3): p. 601-20.
145. Barzilay, G., et al., *Identification of critical active-site residues in the multifunctional human DNA repair enzyme HAP1.* Nat Struct Biol, 1995. 2(7): p. 561-8.
146. Rothwell, D.G. and I.D. Hickson, *Asparagine 212 is essential for abasic site recognition by the human DNA repair endonuclease HAP1.* Nucleic Acids Res, 1996. 24(21): p. 4217-21.
147. Yacoub, A., M.R. Kelley, and W.A. Deutsch, *The DNA repair activity of human redox/repair protein APE/Ref-1 is inactivated by phosphorylation.* Cancer Res, 1997. 57(24): p. 5457-9.
148. Fritz, G. and B. Kaina, *Phosphorylation of the DNA repair protein APE/REF-1 by CKII affects redox regulation of AP-1.* Oncogene, 1999. 18(4): p. 1033-40.
149. Bhakat, K.K., et al., *Role of acetylated human AP-endonuclease (APE1/Ref-1) in regulation of the parathyroid hormone gene.* Embo J, 2003. 22(23): p. 6299-309.
150. Fantini, D., et al., *APE1/Ref-1 regulates PTEN expression mediated by Egr-1.* Free Radic Res, 2008. 42(1): p. 20-9.
151. Busso, C.S., T. Iwakuma, and T. Izumi, *Ubiquitination of mammalian AP endonuclease (APE1) regulated by the p53-MDM2 signaling pathway.* Oncogene, 2009. 28(13): p. 1616-25.
152. Qu, J., et al., *Nitric oxide controls nuclear export of APE1/Ref-1 through S-nitrosation of cysteines 93 and 310.* Nucleic Acids Res, 2007. 35(8): p. 2522-32.
153. Hirota, K., et al., *AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1.* Proc Natl Acad Sci U S A, 1997. 94(8): p. 3633-8.
154. Ueno, M., et al., *Thioredoxin-dependent redox regulation of p53-mediated p21 activation.* J Biol Chem, 1999. 274(50): p. 35809-15.
155. Kelley, M.R. and S.H. Parsons, *Redox regulation of the DNA repair function of the human AP endonuclease Ape1/ref-1.* Antioxid Redox Signal, 2001. 3(4): p. 671-83.
156. Azam, S., et al., *Human glyceraldehyde-3-phosphate dehydrogenase plays a direct role in reactivating oxidized forms of the DNA repair enzyme APE1.* J Biol Chem, 2008. 283(45): p. 30632-41.
157. Tatton, W.G., et al., *Glyceraldehyde-3-phosphate dehydrogenase in neurodegeneration and apoptosis signaling.* J Neural Transm Suppl, 2000(60): p. 77-100.
158. Ashmarina, L.I., et al., *Phosphorylation of D-glyceraldehyde-3-phosphate dehydrogenase by Ca2+/calmodulin-dependent protein kinase II.* FEBS Lett, 1988. 231(2): p. 413-6.
159. Duclos-Vallee, J.C., et al., *Phosphorylation of the hepatitis B virus core protein by glyceraldehyde-3-phosphate dehydrogenase protein kinase activity.* J Gen Virol, 1998. 79 (Pt 7): p. 1665-70.

160. **Reiss, N., et al., *Phosphatidylserine directs differential phosphorylation of actin and glyceraldehyde-3-phosphate dehydrogenase by protein kinase C: possible implications for regulation of actin polymerization.* Biochem Mol Biol Int, 1996. 40(6): p. 1191-200.**

161. **Reiss, N., et al., *Interaction of purified protein kinase C with key proteins of energy metabolism and cellular motility.* Biochem Mol Biol Int, 1996. 38(4): p. 711-9.**

162. **Reiss, N., H. Kanety, and J. Schlessinger, *Five enzymes of the glycolytic pathway serve as substrates for purified epidermal-growth-factor-receptor kinase.* Biochem J, 1986. 239(3): p. 691-7.**

163. **Kawamoto, R.M. and A.H. Caswell, *Autophosphorylation of glyceraldehydephosphate dehydrogenase and phosphorylation of protein from skeletal muscle microsomes.* Biochemistry, 1986. 25(3): p. 657-61.**

164. **Huitorel, P. and D. Pantaloni, *Bundling of microtubules by glyceraldehyde-3-phosphate dehydrogenase and its modulation by ATP.* Eur J Biochem, 1985. 150(2): p. 265-9.**

165. **Glaser, P.E. and R.W. Gross, *Rapid plasmenylethanolamine-selective fusion of membrane bilayers catalyzed by an isoform of glyceraldehyde-3-phosphate dehydrogenase: discrimination between glycolytic and fusogenic roles of individual isoforms.* Biochemistry, 1995. 34(38): p. 12193-203.**

166. **Robbins, A.R., R.D. Ward, and C. Oliver, *A mutation in glyceraldehyde 3-phosphate dehydrogenase alters endocytosis in CHO cells.* J Cell Biol, 1995. 130(5): p. 1093-104.**

167. **Singh, R. and M.R. Green, *Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase.* Science, 1993. 259(5093): p. 365-8.**

168. **Ikemoto, A., D.G. Bole, and T. Ueda, *Glycolysis and glutamate accumulation into synaptic vesicles. Role of glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase.* J Biol Chem, 2003. 278(8): p. 5929-40.**

169. **Morgenegg, G., et al., *Glyceraldehyde-3-phosphate dehydrogenase is a nonhistone protein and a possible activator of transcription in neurons.* J Neurochem, 1986. 47(1): p. 54-62.**

170. **Demarse, N.A., et al., *Direct binding of glyceraldehyde 3-phosphate dehydrogenase to telomeric DNA protects telomeres against chemotherapy-induced rapid degradation.* J Mol Biol, 2009. 394(4): p. 789-803.**

171. **Baxi, M.D. and J.K. Vishwanatha, *Uracil DNA-glycosylase/glyceraldehyde-3-phosphate dehydrogenase is an Ap4A binding protein.* Biochemistry, 1995. 34(30): p. 9700-7.**

172. **Meyer-Siegler, K., et al., *A human nuclear uracil DNA glycosylase is the 37-kDa subunit of glyceraldehyde-3-phosphate dehydrogenase.* Proc Natl Acad Sci U S A, 1991. 88(19): p. 8460-4.**

173. **Schulze, H., et al., *Rat brain glyceraldehyde-3-phosphate dehydrogenase interacts with the recombinant cytoplasmic domain of Alzheimer's beta-amyloid precursor protein.* J Neurochem, 1993. 60(5): p. 1915-22.**

174. **Burke, J.R., et al., *Huntingtin and DRPLA proteins selectively interact with the enzyme GAPDH.* Nat Med, 1996. 2(3): p. 347-50.**

175. **Kragten, E., et al., *Glyceraldehyde-3-phosphate dehydrogenase, the putative target of the antiapoptotic compounds CGP 3466 and R-(-)-deprenyl.* J Biol Chem, 1998. 273(10): p. 5821-8.**

176. **Brodie, A.E. and D.J. Reed, *Cellular recovery of glyceraldehyde-3-phosphate dehydrogenase activity and thiol status after exposure to hydroperoxides.* Arch Biochem Biophys, 1990. 276(1): p. 212-8.**

177. Arutyunova, E.I., et al., *Oxidation of glyceraldehyde-3-phosphate dehydrogenase enhances its binding to nucleic acids.* Biochem Biophys Res Commun, 2003. 307(3): p. 547-52.
178. Dastoor, Z. and J.L. Dreyer, *Potential role of nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase in apoptosis and oxidative stress.* J Cell Sci, 2001. 114(Pt 9): p. 1643-53.
179. Sen, N., et al., *Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis.* Nat Cell Biol, 2008. 10(7): p. 866-73.
180. Ismail, S.A. and H.W. Park, *Structural analysis of human liver glyceraldehyde-3-phosphate dehydrogenase.* Acta Crystallogr D Biol Crystallogr, 2005. 61(Pt 11): p. 1508-13.
181. Puglisi, F., et al., *Prognostic significance of Ape1/ref-1 subcellular localization in non-small cell lung carcinomas.* Anticancer Res, 2001. 21(6A): p. 4041-9.
182. Moore, D.H., et al., *Alterations in the expression of the DNA repair/redox enzyme APE/ref-1 in epithelial ovarian cancers.* Clin Cancer Res, 2000. 6(2): p. 602-9.
183. Russo, D., et al., *Apel/Ref-I expression and cellular localization in human thyroid carcinoma cell lines.* J Endocrinol Invest, 2001. 24(3): p. RC10-2.
184. Puglisi, F., et al., *Prognostic role of Ape/Ref-1 subcellular expression in stage I-III breast carcinomas.* Oncol Rep, 2002. 9(1): p. 11-7.
185. Chattopadhyay, R., et al., *Identification and characterization of mitochondrial abasic (AP)-endonuclease in mammalian cells.* Nucleic Acids Res, 2006. 34(7): p. 2067-76.
186. Jackson, E.B., et al., *Analysis of nuclear transport signals in the human apurinic/apyrimidinic endonuclease (APE1/Ref1).* Nucleic Acids Res, 2005. 33(10): p. 3303-12.
187. Campalans, A., et al., *UVA irradiation induces relocalisation of the DNA repair protein hOGG1 to nuclear speckles.* J Cell Sci, 2007. 120(Pt 1): p. 23-32.
188. Jilani, A., et al., *Characterization of two independent amino acid substitutions that disrupt the DNA repair functions of the yeast Apn1.* Biochemistry, 2003. 42(21): p. 6436-45.
189. Georgiadis, M.M., et al., *Evolution of the redox function in mammalian apurinic/apyrimidinic endonuclease.* Mutat Res, 2008. 643(1-2): p. 54-63.
190. Luo, M., et al., *Redox regulation of DNA repair: implications for human health and cancer therapeutic development.* Antioxid Redox Signal. 12(11): p. 1247-69.
191. Mantha, A.K., et al., *Unusual role of a cysteine residue in substrate binding and activity of human AP-endonuclease 1.* J Mol Biol, 2008. 379(1): p. 28-37.
192. Xi, T., I.M. Jones, and H.W. Mohrenweiser, *Many amino acid substitution variants identified in DNA repair genes during human population screenings are predicted to impact protein function.* Genomics, 2004. 83(6): p. 970-9.
193. Switzer, R.C., 3rd, C.R. Merril, and S. Shifrin, *A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels.* Anal Biochem, 1979. 98(1): p. 231-7.
194. Branzei, D. and M. Foiani, *Regulation of DNA repair throughout the cell cycle.* Nat Rev Mol Cell Biol, 2008. 9(4): p. 297-308.
195. Sancar, A., et al., *Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints.* Annu Rev Biochem, 2004. 73: p. 39-85.
196. Phadke, M.S., et al., *Glyceraldehyde 3-phosphate dehydrogenase depletion induces cell cycle arrest and resistance to antimetabolites in human carcinoma cell lines.* J Pharmacol Exp Ther, 2009. 331(1): p. 77-86.

197. **Wu, X. and Z. Wang, *Relationships between yeast Rad27 and Apn1 in response to apurinic/apyrimidinic (AP) sites in DNA.* Nucleic Acids Res, 1999. 27(4): p. 956-62.**

198. **Tell, G., et al., *The intracellular localization of APE1/Ref-1: more than a passive phenomenon?* Antioxid Redox Signal, 2005. 7(3-4): p. 367-84.**

199. **Campalans, A., et al., *XRCC1 interactions with multiple DNA glycosylases: a model for its recruitment to base excision repair.* DNA Repair (Amst), 2005. 4(7): p. 826-35.**

200. **Obulesu, M. and D.M. Rao, DNA damage and impairment of DNA repair in Alzheimer's disease. Int J Neurosci. 120(6): p. 397-403.**