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The *in vivo* characterization of the DNA repair gene apn-1 in the model organism *Caenorhabditis elegans*

par
Chadi Zakaria

Biologie Moléculaire
Faculté de Médecine

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The *in vivo* characterization of the DNA repair gene apn-1 in the model organism *Caenorhabditis elegans*

présenté par :

Chadi Zakaria

a été évalué par un jury composé des personnes suivantes :

président-rapporteur : El Bachir Affar

directeur de recherche : Dindial Ramotar

membre du jury : Darel Hunting

Résumé

Les sites apuriniques/apyrimidinique (AP) représentent une forme de dommage à l'ADN hautement mutagène et ce type de dommage peut survenir spontanément ou être induit par une variété d'agents. Afin de préserver la stabilité génomique, deux familles d'endonucléases de type AP, endo-IV et exo-III, sont nécessaires pour contrecarrer les effets mutagènes des sites AP. Malgré l'identification de membres des deux familles dans plusieurs organismes unicellulaire tels que *E.coli* et *S. cerevisiae*, aucun membre de la famille endo-IV n'a été identifié chez les organismes multicellulaires à l'exception de *C. elegans* et de *C. briggsae*. Nous avons donc décidé d'investiguer l'importance biologique de APN-1 chez *C. elegans* par l'utilisation d'une approche de knockdown du gène. Dans notre étude, nous avons montré que le knockdown du gène apn-1 chez *C. elegans*, en utilisant des ARN d'interférence (ARNi), cause une accumulation de mutations spontanées et induites par des drogues résultant en un délai de l'éclosion des œufs ainsi que par une diminution de la survie et de la longévité des vers adultes. De plus, nous avons montré que cette accumulation de mutations mène à un délai dans la progression du cycle cellulaire durant l'embryogénèse, représentant possiblement une explication du délai dans l'éclosion des œufs. Nous avons montré qu'il y avait une augmentation du niveau de mutations dans la gorge des vers, sans toutefois pouvoir confirmer la distribution de APN-1 qui possède une étiquette GFP. Les animaux transgéniques APN-1-GFP n'exprimaient pas suffisamment de la protéine de fusion pour permettre une visualisation à l'aide d'un microscope à fluorescence, mais la protéine a été détectée par immunobuvardage de type western. Les animaux transgéniques APN-1-GFP étaient instables et avaient des phénotypes concordants avec les défauts génétiques. En conclusion, il semble que *C. elegans* aie évolué afin de retenir un niveau de base de APN-1 jouant ainsi un rôle versatile afin de maintenir l'intégrité génétique d'autant plus que cet organisme semble manquer plusieurs enzymes de la voie de réparation par excision de base.

Mots clés : *C. elegans*, Endo IV, réparation par excision de base, site AP, endonucléase de type AP, stress oxidatif, agents causant des dommages à l'ADN, réparation de l'ADN.

Abstract

Apurinic/apyrimidinic (AP) sites are a form of highly mutagenic DNA damage that arise either spontaneously or by a variety of DNA damaging agents. To preserve genomic stability two AP endonuclease families, endo-IV and exo-III, evolved to counteract the mutagenic effect of AP sites. While members of both families were identified in multiple unicellular organisms, notably *E. coli* and *S. cerevisiae*, no members of the endo-IV family were identified in multicellular ones, with the exception of *C. elegans* and its close relatives, particularly *C. briggsae.* We set out to investigate the biological importance of APN-1 in *C. elegans* using gene knockdown approach. In our study, we showed that the knockdown of *C. elegans* apn-1 gene, using RNAi causes the accumulation of spontaneous and drug induced mutations, resulting in a delay in egg hatching, decreased survival and longevity. Furthermore, we have showed that the accumulated mutations lead to delays in cell cycle progression during early embryogenesis, thus providing a possible explanation for the observed delay in hatching. Although we showed increased mutations in the gut of the worm, we were unable to confirm APN-1 distribution tagged with GFP. The transgenic APN-1-GFP animal did not express enough of this fusion protein to be visualized by fluorescent microscopy, although it was detected by Western blot analysis. The transgenic animals over-expressing APN-1-GFP were unstable and showed phenotypes consistent with genetic defects. In conclusion, it would seem that *C. elegans* has evolved to retain a balanced level of APN-1, which plays a versatile role in maintaining genetic integrity, since this organism lacks a full complement of the enzymes in the base-excision repair pathway.

Key words: *C. elegans*, Endo IV, base excision repair, AP site, AP endonuclease, oxidative stress, DNA damaging agents, DNA repair

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List of abreviations

A	Adenine
AID	Activation-Induced cytidine Deaminase
AP site	apurinic-apyrimidinic site
APOBEC1	Apolipoprotein B mRNA Editing Catalytic subunit 1
Asn	Asparagine
Asp	Aspartic Acid
AT	Ataxia Telangiectasia
ATP	Adenosine-5'-triphosphate
BER	Base Excision Repair
BLM	Bleomycin
BRCA1	breast cancer 1, early onset
C	Cytosine
C. elegans	*Caenorhabditis elegans*
cDNA	Complementary DNA
CIA	Chloroform Isoamyl Alcohol
CIP	Calf Intestinal Phosphatases
CS	Cockayne Syndrome
Cys	Cysteine
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dRP	Deoxyribosephosphate
DSB	Double Strand Break
dsDNA	Double Stranded DNA
dsRNA	Double Stranded RNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Endo IV	Endonuclease IV
EtBr	Ethidium Bromide
EXO III	Exonuclease III
G	Guanine
GFP	Green Fluorescent Protein
GG-NER	Global Genomic nucleotide excision repair
Glu	Glutamic Acid
H_2O_2	Hydrogen Peroxide
His	Histidine
HR	Homologous Recombination
IPTG	Isopropyl β-D-1-thiogalactopyranoside

kb	Kilo bases
kDa	Kilo Dalton
LB	Luria Broth
LP	Long Patch
MCS	Multiple Cloning Site
Mg2+	Magnesium
MGMT	Methyl Guanine Methyl Transferase
MMS	Methyl Methane Sulfonate
mRNA	Messenger RNA
N	Nitrogen
N^3-meA	N^3-methyladenine
N^7-meG	N^7-methylguanine
NaOAc	Sodium Acetate
NER	Nucleotide Excision Repair
NGM	Nematode Growth Media
NHEJ	Non-Homologous End Joining
NIR	Nucleotide Incision Repair
NLS	Nuclear Localization Signal
Ogg1	8-oxoguanine DNA glycosylase
OH	Hydroxyl
PCNA	Proliferating Cell Nuclear Antigen
PMSF	phenylmethanesulphonylfluoride
q-RT-PCR	Quantitative Reverse transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S. cerevisiae	*Saccharomyces cerevisiae*
SAM	S-adenosylmethionine
SDS	Sodium Dodecyl Sulfate
SP	Short Patch
SSB	Single Strand Break
ssDNA	Single Strand DNA
SV40	Simian virus 40
T	Thymine
t-BH	*tert*-butyl hydroperoxide
TC-NER	Transcription Coupled Repair
U	Uracil

UV	UltraViolet
V(D)J	Variable Diversity Joining
XP	Xeroderma Pigmentosum
β-gal	β-galactosidase
8oxoG	8-oxo-7,8-dihydrodeoxyguanosine

For mum and dad, who sacrificed their own life so I would have a good one. For those who never stopped offering me their unconditional love throughout the years, and their relentless support throughout the course of my studies and till now.

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Chapter 1 - Introduction

1. General Review

A cell, the main constituent of any living organism, is constantly subjected to insults from the surrounding environment as well as from byproducts of its own metabolism. These attacks can affect any macromolecule within the cell, from proteins and lipids to nucleic acids and may therefore hinder the cell's capacity to grow normally and thrive [1-3]. While damage to lipids and proteins is restricted to the affected molecules and their function which can be replaced, damage to DNA represents a more widespread disastrous consequence as gene mutations may lead to the constant production of aberrant products with sometimes no or improper functions. As a result unrepaired DNA damage was found to cause apoptosis, cell cycle arrest, permanent mutational changes, and, in the worst scenario, a mutation that could lead to the formation of malignant tumors; hence the importance of the presence of multiple DNA repair mechanisms to ensure genomic stability [4-7]. As such, heritable mutations in multiple DNA repair genes have already been associated with cancer-prone phenotypes such is the case of xeroderma pigmentosum (XP), cockayne syndrome (CS), ataxia telangiectasia (AT) breast cancer 1, early onset (BRCA1) patients among others [8-14].

Despite the relatively extensive knowledge that we possess regarding DNA repair, more detailed aspects of the pathways that govern this process and their inter-connectivity are yet to be discovered, thus the necessity of studying DNA repair and its relation to human syndromes notably cancer and other degenerative diseases.

2. DNA damage

In order to further understand the importance of DNA repair and its relation to human diseases, it would be essential to understand first the factors that contribute to the creation of the wide variety of DNA damages. For this purpose, the current section of the introduction is dedicated to discuss in detail the multiple sources as well as the types of DNA damages that can occur in the genome of a living cell.

2.1. Sources of DNA damage

The cell's normal metabolic activity leads to the formation of reactive oxygen species (ROS) that are highly reactive due to the presence of unpaired valence shell electrons [15]. These small molecules that comprise oxygen ions, free radicals as well as peroxides originate primarily from cell respiration during electron transport reactions that occur in the mitochondria [15]. ROS molecules may also derive from a multitude of other endogenous or exogenous sources such as ultra violet (UV) light, ionizing radiation, gamma ray, metal-catalyzed reactions, neutrophils and macrophages during inflammation and the atmosphere where they are present as pollutants [15].

ROS molecules present a beneficial role at low levels where they are implicated in cell signaling [15, 16]. It is only when a substantial increase in ROS production occurs, such in the conditions mentioned above, that significant damage takes place in the cell and jeopardizes its ability to normally grow and survive [15]. These conditions are known as oxidative stress and can generate as many as 10,000 to 20,000 oxidative DNA lesions/cell/day that include apurinic/apyrimidinic (AP) sites and oxidized bases [17].

Other types of endogenously created DNA lesions are those created through programmed DNA breaks, such as V(D)J recombination, immunoglobulin

class switching and topoisomerase breakage of DNA covalent links for example [18, 19].

By contrast, multiple exogenous sources can account for DNA damage. Of these are multiple human-made chemicals such as methyl methane sulfonate (MMS), Radiations (UV, X-rays and gamma rays), viruses and other natural factors are also known to directly cause an important amount of DNA damage.

2.2. Types of DNA damage

The term DNA damage points to any type of aberrant changes to DNA integrity and these modifications can occur at any nucleotide or at any position of the DNA backbone. As previously discussed, the origin of these damages can be highly diverse. The variety of the factors causing DNA damage is the basis of the diversity in the type of the modifications observed. Base deamination, methylation, and oxidation, AP sites, single strand breaks, double strand breaks and inter-strand and intra-strand crosslinks are all examples of damages that can occur in the genome of a given cell.

2.2.1. Bulky lesions and inter/intra-strand crosslinks

Sunlight is composed of 3 main constituents: infrared, visible and ultra-violet light (UV). UV light is composed of 3 components: UVC (100 nm to 280 nm), UVB (280 nm to 315 nm) and UVA (315 nm to 400 nm). While UVC is largely blocked by the earth's atmosphere, UVA and UVB have the ability to reach the surface of the earth. UVA photons generally increase ROS production that in turn causes damage to DNA and other macromolecules. On the other hand, UVB photons attack directly biological macromolecules such as the DNA, to cause bulky lesions and inter/intra-strand crosslinking [20]. The best known bulky

lesions created by the effect of UVB are cis-syn cyclobutane pyrimidine dimers and pyrimidine (6-4) –pyrimidone photoproduct, which connect adjacent pyrimidine molecules present in the same or different strands of the DNA to create a distortion in the DNA structure [21].
Similarly, since DNA naturally interacts with proteins, UVB was also found to induce the formation of DNA-protein crosslinks [20].

2.2.2. Modification of bases

In contrast to bulky DNA damages, single base modifications are also a well known type of aberrant change in the DNA. In fact, nitrogenous bases can be frequently modified by natural endogenous molecules as well as a variety of exogenous chemicals. Three main types of modifications are frequently observed: base deamination, base oxidation and base alkylation.

2.2.2.1. Deamination

Deamination is the process of eliminating of the amino group from a molecule. In DNA, the bases containing an amino group outside the cyclic structure of the base, such as cytosine, adenine and guanine, are susceptible to spontaneous deamination. The process of deamination leads to the formation of an alternate base that can cause, if unrepaired, multiple types of DNA damage such as transversion mutations and base mispairing. For example the deamination of cytosine leads to the formation of uracil, which normally is not present in DNA. If not removed, uracil has the ability to pair to adenine resulting in GC to AT transversion mutation after DNA replication [22]. This mutation constitutes a significant source of spontaneous mutations in *Escherichia coli* (*E. coli*) [23]. Another example is T-G mispairs that arise from the spontaneous deamination of methylated cytosine to generate thymine and ammonia [24, 25].

Deamination is not only a spontaneously occurring event; multiple factors, such as reactive nitrogen species [26] as well as several proteins such as Activation-induced cytidine deaminase (AID) and Apolipoprotein B mRNA editing catalytic subunit 1 (APOBEC1) dramatically enhance this process [27, 28].

2.2.2.2. Base oxidation

Base oxidation can originate from the incorporation of oxidized bases present in the nucleotide pool during replication, or it can directly occur at any base within the DNA sequence [29]. As mentioned previously, ROS, and more precisely hydroxyl radicals, are the main molecules accountable for oxidative damage that occurs in normal conditions but that is dramatically increased during oxidative stress [15]. Oxidation usually occurs on carbon 5 and 6 of pyrimidines and carbon 4 and 8 of purines [30]. Thymine glycol, 8-oxoguanine, 5-formyluracil, 5-hydroxyuracil and uracil glycol are all examples of oxidized bases [31]. These modifications, if not repaired, present a deleterious effect on a living cell as they may lead to replication fork arrest which can be lethal, many types of transversion mutations that are sometimes associated with cancer, as well as miss-incorporation of nucleotides opposite the oxidized bases [15, 29]. Oxidized bases can also be removed by DNA glycosylases to create AP sites, which are highly mutagenic as they lead to replication fork arrest and when bypassed, can lead to the incorporation of an incorrect nucleotide [32]. It is important to mention that oxidation can also directly affect the sugar moiety. This generally leads to the formation of oxidized AP sites and single strand break formation with 3’-blocking groups [33].

2.2.2.3. Base alkylation

Alkylation is another type of modification that affects DNA bases. As for oxidation, alkylation can arise due to the action of endogenous molecules such as S-adenosylmethionine (SAM) as well as exogenous sources such as nitrate and other man-made drugs, e.g. MMS [34]. The N^7-methylguanine (N^7-meG) and the N^3-methyladenine (N^3-meA) are the most common endogenously formed alkylated bases [34]. These 2 alkylated bases are not directly mutagenic. However, the action of DNA glycosylases leads to the formation of AP sites, which in turn are mutagenic if not repaired [34].

2.2.3. Spontaneous base loss

The N-glycosyl bond linking the base to the sugar phosphate backbone is relatively weak and therefore is susceptible to spontaneous hydrolysis that will lead to base loss and the formation of AP sites. Even though modified bases increase the fragility of the N-glycosyl bond and consequently increase base loss and AP site formation, spontaneous base loss still occurs *in vivo* at a physiologically relevant rate [35]. Depurination in *E. coli* is estimated to occur at the rate of 0.5 purine / chromosome / generation [36]. In mammalian cells, it is estimated to occur at a frequency ranging from 2,000 to 10,000 purines / generation [36]. However, it has been noted that the rate of depyrimidination is lower than the rate of depurination [35, 36]. This is due to the double ring structure of purines that makes them more susceptible to hydrolysis than pyrimidine.

2.2.4. Single strand breaks

Similarly to the other types of damages, the hydrolysis of the deoxyribose subunit of the DNA backbone to create single strand breaks (SSBs)

can result from various exogenous as well as endogenous sources. On one hand, the action of free radicals, ionizing radiation and antitumor drugs such as bleomycin can directly attack the DNA backbone to create SSBs with 5'-phosphate and 3'-phosphoglycolate or 3'-phosphate termini [37-39].
On the other hand, endogenous breakage of the oxidized sugar and abnormal activity of cellular enzymes such as DNA topoisomerase 1, account for endogenous SSB formation [40]. Moreover, modified bases, such as oxidized or alkylated bases, are usually removed by the action of multiple DNA glycosylases to create AP sites that can lead to the formation of SSBs through the activity of the AP endonucleases or AP lyases [40]. In this case, these SSBs acts as intermediates in the base excision repair pathway and thus are not a direct primary damage. Whereas AP endonucleases create a single strand break with a 3'-hydroxyl and a 5'-deoxyribose phosphate termini, AP lyases lead to the formation of 3'-α,β-unsaturated aldehyde and a 5'-phosphate termini through a β-elimination reaction [41]. All these 3'-blocking groups prevent the DNA polymerase from fulfilling its role and therefore needs to be processed to recreate the 3'-hydroxyl termini normally recognized by the polymerase for the subsequent repair steps.

2.2.5. DNA double strand breaks

Another type of damage that occurs in the DNA can take the form of double-strand break (DSB) where both strands of the DNA phosphodiester backbone are broken. Like many other types of damages, both endogenous as well as exogenous factors account for DSB formation. Replication, recombination, meiosis and endogenously produced ROS are all examples of endogenous sources while ionizing radiation, UV irradiation and multiple natural or man-made drugs are all examples of exogenous sources [42]. It is also important to note that DSBs can arise as a result of other damages localized closely in space on complementary strands of the DNA molecule. These can be SSB, AP sites or modified bases [43,

44]. Regardless of the cause of the DSB formation, these lesions are very dangerous as they may lead to chromosomal fusions and genome rearrangement [42].

2.2.6. Result of unrepaired damage

One of the main processes that should be achieved with high fidelity in the cell is DNA replication. On some occasions, the bypass of a modified base or an AP site leads to the incorporation of an incorrect base [45, 46]. Usually, cells have evolved a proofreading and error-checking mechanism to ensure correct replication of DNA [47].

However, these replication errors sometimes escape the cell's surveillance systems and create mismatched bases (association of incompatible nucleotides). On other occasions, strand misalignments causes small insertions or deletions causing a shift in the coding sequence of the gene to create what are referred to as frame-shift mutations [48].

3. DNA repair – Multiple pathways with one aim

The ultimate goal of any living cell in a multi-cellular organism is to ensure that it is fulfilling its role. To be able to do that, any cell has to maintain the stability of its genome to avoid the potential devastating effect of DNA damage. As briefly discussed previously, there are a multitude of types of DNA damage that arise as a result of a variety of endogenous as well as exogenous factors. The vast array of DNA damage requires the presence of highly complex mechanisms capable of specifically repairing each type of lesion. In fact, cells have evolved multiple mechanisms that can be grouped into 5 main pathways that are capable of repairing any type of change occurring in the genome as a result of an insult.

These mechanisms are direct reversal, double strand break repair, mismatch repair, nucleotide excision repair and the base excision repair.

After detection of the damage, multiple signaling pathways are triggered, leading to an appropriate cellular response [49]. The activation of cell cycle checkpoints causes a cell cycle arrest allowing time for the cell to repair the damage [49, 50]. At this point, the choice of the DNA repair pathway largely depends on the type of the damage encountered. However, some types of DNA damages can be repaired through multiple repair pathways, which ensure alternatives for the cell to perform the appropriate corrections [51].

Although DNA repair is usually the best option for the cell, the extent of the damage can sometimes be large enough to force the cell into an irreversible state of dormancy (senescence) or programmed cell death (apoptosis) [4, 49, 52]. One should also keep in mind that in certain conditions, the cell is able to bypass some lesions that usually stall replication, through the use of alternate polymerases that are error-prone and may incorporate wrong nucleotides opposite to the site of damage. The bypass of lesions is termed as damage tolerance [45, 46].

3.1. Double strand break repair

As previously described, DSB is a lesion in which two breaks on opposite DNA strands occurs. This type of damage is particularly hazardous as it can lead to a loss of information as well as genomic rearrangement which can be lethal [42]. Two distinct repair pathways exist to repair this kind of lesions: homologous recombination (HR) and non-homologous end joining (NHEJ) [42].

During HR, and after the occurrence of the DSB, a resection of the two strands of DNA occurs at the 5'-side of the break to generate 3'-overhangs. One of the two single stranded overhangs then invades the homologous chromosome. This allows the DNA polymerase to copy the information from the homologous chromosome to fill the gap and thus preserve fidelity during repair. The contact point between the DNA strands of the 2 chromosomes form what are called

holiday junctions that should be resolved to recreate the normal dsDNA. The way this junction is cut determines whether a crossover occurs or not [53].

As opposed to HR, the NHEJ pathway is error-prone as it directly connects separated mismatched or damaged DNA ends without the use of the homologous chromosome [54]. It is important to note here that the choice between the two sub pathways depends on the cell cycle stage and is regulated at the resection step [53, 55].

3.2. Mismatch repair

The DNA mismatch repair pathway is a conserved mechanism that allows the recognition and repair of mismatched bases as well as some insertions and deletions that occur during DNA replication or recombination [56, 57]. Since the above mentioned damages occur in the newly produced strand during DNA synthesis, the cell distinguishes first, in a process that is not yet very well understood, between the newly synthesized strand and the template strand. After damage and strand recognition, an incision is created that can be up to thousands of base pairs away from the site of damage. The resulting nicked strand is then degraded, the created gap is filled with the correct nucleotides and the process finalized by ligation [57, 58].

3.3. Nucleotide excision repair (NER)

Multiple forms of DNA damage lead to a distortion in the helical structure of the DNA such as thymine dimers and 6-4-photoproducts caused by multiple chemical drugs and UV radiation [20, 21]. These types of lesions are processed by the NER pathway through 4 main steps. The process starts with

lesion recognition that is followed by removal of the lesion along with a few nucleotides to create a small single stranded gap [59].

Within NER, one can distinguish two distinct sub-pathways that have been very well characterized: transcription coupled repair (TC-NER) and global genomic repair (GG-NER). TC-NER is activated when the RNA polymerase stalls as a result of a lesion. TC-NER is therefore associated with transcriptionally active genes while global genomic repair permits the repair of genes in regions of the genome that are not actively transcribed or the non-transcribed strands of transcribed regions[60]. These sub-pathways differ by the method of lesion recognition but share the same steps of damage processing [59]. NER is a particularly important DNA repair pathway as its inactivation is associated with multiple human diseases such as XP and CS [59].

3.4. Direct reversal

Direct reversal is considered to be the simplest pathway to repair damaged DNA as it encompasses a single step, does not require a template and does not involve any phosphodiester breakage. Another factor of simplicity is the low number of enzymes identified so far that are directly able to reverse lesions. Thymine dimers, methylated guanine as well as certain methylation of cytosines and adenines are all known to be directly reverted by the cell to their original state. In this context, the UV-induced abnormal bonding between adjacent thymine to form thymine dimers can be reversed through a photoreactivation process that involves an enzyme called photolyase of which the activation depends on exposure to UV light [61]. It is important to note though that humans do not possess photolyases and thus rely on other repair pathways, such as the NER pathway, to repair these types of lesions [61]. Likewise, methylated guanine can also be directly reversed to its original state by the enzyme methyl guanine methyl

transferase (MGMT) [62]. In the same way, methyladenine and methylcytosine were found to be reversed in *E. coli* through the action of AlkB [62, 63].

3.5. Base excision repair pathway

The base excision repair (BER) pathway is a vital and a highly conserved mechanism that allows the processing of a variety of non helix-distorting DNA lesions. These comprise deaminated, oxidized or alkylated bases, AP sites and SSBs with 3'-blocked termini [64, 65]. The repair of these lesions occurs through a sequential process involving a set of highly conserved enzymes.

The first step of this pathway involves the recognition and processing of a modified base, which is generally done through the activity of one of several DNA glycosylases [64]. DNA glycosylases can be divided into two groups: mono-functional and bi-functional glycosylases [66]. While the former is restricted to the removal of modified bases through hydrolysis of the N-glycosyl bond linking the base to the sugar, the latter is endowed with an additional AP lyase activity [66, 67]. This activity allows the direct processing of AP sites through a β-elimination reaction to generate single strand breaks with 3'-unsaturated aldehyde and 5'-phosphate termini [41]. Although these enzymes have certain substrate specificity, the recognition of multiple lesions by the same glycosylase, or the same lesion by multiple glycosylases still occurs, thus providing alternatives to repair damage [68]. However, despite the diversity of the substrates and their corresponding glycosylases, the resulting base removal induces the formation of an AP site.

The second step of the pathway involves an AP lyase as described above, a delta lyase or an AP endonuclease, all with the ability to process AP sites. While delta lyases create an incision at AP sites and process 3'-aldehyde to create 3'-phosphate termini, AP endonuclease enzymes incise DNA backbone at the 5'-side of AP sites to generate 3'-hydroxyl and 5'-deoxyribosephosphate (dRP) termini [41]. AP endonuclease also possesses a 3'-diesterase activity allowing the

removal of 3'-blocking groups such as those left by AP lyase and delta lyases [41]. It is essential to create the hydroxyl group at the 3'-terminus of the DNA break as it serves as a primer for DNA polymerase for the subsequent steps of the pathway. At this point, the pathway can diverge into either short patch (SP) or long patch (LP) BER.

During SP repair, the 5'-dRP is removed by the action of a dRP lyase, resulting in the formation of a 5'-phosphate terminus. The removal of the 5'-dRP is followed by the incorporation of a single nucleotide by DNA polymerase, using the 3'-OH as a primer, to fill the created gap. Finally, the DNA ligase comes into play to reconnect the separated DNA fragments [69].

During LP repair, modified AP sites (e.g. oxidized) lead to the formation of modified dRPs which cannot be removed by the dRP lyase activity. As a result, multiple nucleotides are incorporated at the 3'-side of the damage leading to the displacement of the strand containing the 5'-dRP, thus creating a flap, which is then removed to create a 5'-phosphate terminus. Finally, the presence of 5'-phosphate and 3'-OH termini is essential for efficient ligation by DNA ligase that will reconnect the separated DNA fragments [69].

4. AP endonucleases/3'-diesterases

The frequent occurrence of AP sites necessitates efficient processing as they are highly mutagenic. The AP endonucleases and the AP lyases are two classes of enzymes with the ability to process AP sites. The incision made by AP lyase lead to the formation of blocked 3'-ends which need 3'-diesterase activity to be removed, whereas the ends created by AP endonuclease (i.e. 3'-hydroxyl and 5'-deoxyribose phosphates) can be directly used by dRP lyase and polymerase for the following steps of BER [41]. In addition, AP endonuclease enzymes possess a 3'-diesterase activity allowing them to process 3'-blocking groups left by AP lyase. Thus, AP endonucleases are considered to be far more important then AP lyases in processing AP sites [41]. This has also been supported by findings

showing that endonuclease deficient yeast strains are more sensitive to MMS, but not AP lyase deficient yeast strains [70, 71].

To date, two families of AP endonucleases / 3'-diesterases, termed Endo IV and Exo III after the ancestral bacterial enzymes, have been well characterized [41]. Multiple studies led to the identification of members of the Exo III family in many organisms both unicellular and multicellular (prokaryotes and eukaryotes). In contrast, members of the Endo IV family were only found in unicellular organisms with the exception of *Caenorhabditis elegans* (*C. elegans*). Most of these enzymes possess, in addition to the AP endonuclease and 3'-diesterase activities, 3'- to 5'-exonuclease and RNase H activities [72].

Despite the presence of a certain degree of redundancy between the two families, as shown in multiple studies of cross-species complementation, some differences regarding levels of expression and substrate specificity do exist [41]. However, the key feature used to differentiate between the two families is their magnesium dependency. While members of the Endo IV family are Mg2+ independent (EDTA resistant), it was shown that Exo III family members are magnesium dependent and therefore EDTA sensitive [41].

Since this project investigates the *in vivo* characteristics of the Endo IV family member in *C. elegans*, the current section of the introduction will address the present state of knowledge regarding AP endonucleases / 3'-diesterases in *E. coli*, *Saccharomyces cerevisiae* (*S. cerevisiae*), humans and *C. elegans* with special attention accorded to Endo IV family members.

4.1. AP endonuclease/3'-diesterase enzymes in *E. coli*

The first indication regarding the presence of a specific class of enzymes capable of processing AP sites came from studies in *E. coli*. These studies showed that an enzyme, first described as a 3'→ 5' exonuclease with a 3'-phosphatase activity (named exonuclease III), also possess an endonuclease activity capable of generating single strand breaks through the hydrolysis of the phosphodiester bonds

of alkylated bases [73-76]. At this time, the second activity was thought to be generated from a different enzyme then exonuclease III, that was named endonuclease II [73, 74]. Later, it was found that the same mutation commonly disrupts exonuclease III and endonuclease II activities [77]. It wasn't until 1976 that these enzymatic activities were attributed to the same enzyme. This discovery occured when it was found that the purification of exonuclease III always resulted in the co-purification of endonuclease II activity [78]. Moreover, denaturing polyacrylamide gel using the purified exonuclease III always showed a single band suggesting that one protein is responsible for both activities [78].
The hunt for the second AP endonuclease enzyme was initiated by the finding that exonuclease III mutants still possess a low AP endonuclease activity (~10%) [79, 80]. The endonuclease IV was isolated from crude extracts deriving from E. coli mutants lacking the exonuclease III enzyme.

4.1.1. Characteristics of *E. coli* exonuclease III

Exonuclease III is the major AP endonuclease enzyme in *E. coli* as it accounts for 90% of the total AP endonuclease activity. It is encoded by the xth gene, and is predicted to produce a ~31 kDa protein [81]. It has been shown that exonuclease III requires magnesium for its activity and therefore is EDTA sensitive [41]. Moreover, exonuclease III was found to be heat sensitive as it degrades rapidly upon incubation of the purified protein at moderately high temperatures [79, 80]. Studies of this enzyme established that it possesses 4 different catalytic activities. It is a 3' to 5' exonuclease with an activity specific for dsDNA allowing it to degrade blunt ends, 5'-overhangs or nicks to produce segments of ssDNA [72]. However, this activity was not observed on 3'-overhangs that are over 4 bases long similarly to what is observed on ssDNA [72]. Exonuclease III is also a phosphatase allowing the removal of 3'phosphate to generate a 3'-OH terminus and an RNase H that allows the degradation of RNA in DNA-RNA hybrids [72]. Finally, and most importantly, exonuclease III is an

apurinic/apyrimidinic endonuclease that has the ability to nick phosphodiester bond at AP sites as a step in BER [72].

4.1.2. Characteristics of *E. coli* endonuclease IV

The endonuclease IV, product of the *E. coli nfo* gene, is a predicted 31 kDa protein that was discovered as a second and minor AP endonuclease enzyme in *E. coli.* Multiple studies have shown that this enzyme can incise the phosphodiester bond at 5' of AP sites and certain oxidized nucleotides (Nucleotide incision repair pathway NIR), but not other types of damages (e.g. bulky lesions), to create single strand breaks with 3'-OH terminus that is involved in subsequent steps of the BER pathway [79]. In addition, this enzyme was found to possess a 3'-diesterase activity allowing it to remove 3'-blocking groups at single strand breaks such as those resulting from the activity of AP lyase [82]. In recent studies, it was discovered that purified endonuclease IV also possess a 3'→5' exonuclease activity with a preference for 3'-recessed ends of dsDNA [83]. By contrast to the AP endonuclease and 3'-diesterase activities, the 3'→5' exonuclease activity was found to be inhibited in the presence of the metal chelator EDTA and reducing conditions [83].

As mentioned earlier, endonuclease IV is considered to be the minor AP endonuclease in *E. coli* and it was found to constitute 10% of the total AP endonuclease activity in bacterial cells [80]. However, it was demonstrated that this enzyme can be induced as much as 20-fold by superoxide anion generators such as paraquat [84]. As such, the level of endonuclease IV becomes comparable to that of exonuclease III [84] but its 3'-diesterase activity always remains lower than that of exonuclease III [85].

4.1.3. Importance of the AP endonuclease/3'-diesterase enzymes in *E. coli*– mutational analysis

The *in vivo* relevance of AP endonuclease/3'-diesterase enzymes in *E. coli* was investigated using mutants defective in either of the two families or both. These studies revealed an important degree of overlap between the two families [86]. Studies of the *xth* mutant, lacking the major AP endonuclease, was found to be very sensitive to MMS [77] whereas the *nfo* mutant, lacking the minor AP endonuclease, seems only slightly sensitive to the same drug [86]. These observations are consistent with the expression level of each family, allowing exonuclease III to highly complement the absence of endonuclease IV. The DNA damaging agent MMS mainly alkylates purines by methylating guanine at position N-7 and adenine at position N-3, among others [87, 88]. Another type of DNA damaging agent to which the *xth* mutant is sensitive to is hydrogen peroxide (H_2O_2). This oxidant causes base oxidation as well as single strand breaks with 3'-blocked termini which cannot be processed by DNA polymerases since they require a 3'-OH terminus to initiate DNA repair synthesis [89, 90]. By contrast, the *nfo* mutant shows no apparent sensitivity to H_2O_2 [86].

The fact that the double knockout *xth nfo* shows a higher sensitivity to the above-mentioned DNA damaging agents suggests that endonuclease IV acts *in vivo* as a backup enzyme in conditions of stress [86]. However, certain studies have demonstrated that the overlapping function of the two enzymes is not entirely accurate. In fact, it was found that some lesions repaired by endonuclease IV are not processed by exonuclease III. This was illustrated by the fact that the *nfo* mutant shows a very high sensitivity to the DNA damaging agent bleomycin (BLM) as well as *tert*-butyl hydroperoxide (t-BH) whereas the *xth* mutant seems only slightly affected by t-BH and not at all by BLM [86]. Furthermore, the double mutant *xth nfo* did not show any apparent increase in sensitivity to either of the two DNA damaging agents when compared to the single mutants [86]. BLM is an anti-tumor drug known to produce oxidized AP sites, single strand breaks with 3'-

blocked ends as well as double strand breaks [91], while t-BH is an oxidant of which the resulting DNA damage is not very well understood.
Furthermore, endonuclease IV but not exonuclease III was found to directly incise at the 5'-side of various oxidized bases, independently of DNA glycosylases, to create 3'-hydroxyl and 5'-phosphate termini, in the NIR pathway [92].

4.2. AP endonuclease/3'-diesterase enzymes in *S. cerevisiae*

Shortly after the discovery of AP endonuclease enzymes in *E. coli*, evidence of the presence of their homologues came to light in the first eukaryotic organism, *S. cerevisiae*. These enzymes, Apn1 and Apn2, were found to have similar characteristics to their homologues in *E. coli*, endonuclease IV and exonuclease III, respectively. However, some differences are still noticeable.

4.2.1. Characteristics of *S. cerevisiae* Apn1

The discovery of AP endonuclease enzymes in *E. coli* initiated efforts to find their homologues in higher organisms. The first hint of the presence of these enzymes in yeast cells came in 1978, when three AP endonuclease activities were partially purified by Paul R. Armel and Susan S. Wallace [93]. However, the actual major AP endonuclease enzyme in yeast was only purified and partially characterized in 1988 [94, 95].This 40.5 kDa monomer metalloenzyme was found to share significant amino-acid identity with its *E .coli* homologue, endonuclease IV, and was found to possess a similar zinc cluster in the active site [94, 96].
A couple of years later, the APN1 gene encoding the major yeast AP endonuclease enzyme was identified [97]. Disruption studies of this gene confirmed that its protein product accounts for 97% of the total cellular AP endonuclease as well as 3'-diesterase activities [97]. Further studies confirmed that, similar to its *E. coli* counterpart, the Apn1 protein possess a 3'→5' exonuclease activity on duplex

DNA with preference for 3'-recessed DNA ends [98]. This activity was shown to permit the removal of 8-oxo-7,8-dihydrodeoxyguanosine (8oxoG) resulting from either the misincorporation of 8oxoGTP by DNA polymerase or the direct oxidation of guanines [98]. Although the processing of the 8oxoG lesions mainly occurs through the activity of 8-oxo-dGuo DNA glycosylase, OGG1 [99], the 3'→5' exonuclease activity of Apn1 was found to constitute an alternative pathway for the repair of such lesions. Actually, the knockout of both ogg1 and apn1 was found to increase the rate of spontaneous mutations. These mutations are mainly due to G.C to T.A transversions which are normally observed in the Ogg1 single mutant [98]. Furthermore, Apn1 was shown to take part in the NIR pathway [100]. Similarly to *E. coli* endonuclease IV, Apn1 was not found to require magnesium for its activity and thus is EDTA resistant [41].

The study of the structure of the Apn1 protein revealed the presence of a long C-terminal region rich in basic amino acids that is not present in its *E. coli* homologue endonuclease IV [97]. This extension contains two main lysine/arginine clusters that serve as a nuclear localization signal (NLS) [101]. Removal of cluster one was not found to affect the enzyme's stability and activity as this protein was still able to complement AP endonuclease deficient *E. coli*; instead it abolished its nuclear localization. Furthermore, the replacement of cluster 1 with the simian virus 40 (SV40) NLS was also associated with defective nuclear localization indicating the presence of highly specific transport machinery for the transport of Apn1. In fact, the C-terminal region was found to act as a bipartite NLS as neither cluster 1 nor cluster 2 alone can target the protein to the nucleus [101].

In addition to its role in the nuclear localization of the protein, the C-terminal region was also found to target the enzyme to the mitochondria through its interaction with Pir1, a previously identified cell wall protein. The absence of Pir1 was found to correlate with an accumulation of the protein in the nucleus and the cytoplasm and a parallel reduced level of Apn1 in the mitochondria [102].

Other structural studies pointed out two conserved amino acids (Glu 158 and Asp 192) the substitution of which disrupts the biological function of the protein, suggesting that these amino acids might constitute the actual active site [103].

4.2.2. Characteristics of *S. cerevisiae* Apn2

The fact that apn1 knockout (Δ) yeast cells respond similarly to wild type yeast cells to certain DNA damaging agents such as BLM, raised the question of whether another enzyme was present to counter this type of lesions [104]. The effort of two independent groups led to the simultaneous identification of the Apn2 protein, the homologue of *E. coli* exonuclease III [88, 105]. By contrast to its homologue in *E. coli*, the Apn2 protein was found to possess AP endonuclease, 3'-diesterase and 3'→5' exonuclease activities that do not require magnesium and that are maintained even after 24 hours of incubation with EDTA [70]. The transcription of the gene was found to be induced up to six-fold by treatment with MMS [105] and its 3'-diesterase and 3'→5' exonuclease activities stimulated by proliferating cell nuclear antigen (PCNA) [106].

Structural studies revealed the presence of a large C-terminal extension that is not present in exonuclease III [105]. When deleted, the resulting truncated protein was found to retain its AP endonuclease activity *in vitro* but severely lack the ability to complement the AP endonuclease deficiency of the apn1 apn2 double mutant cells *in vivo* [70] suggesting that this portion of the protein might be responsible for the proper localization of the protein.

4.2.3. Importance of AP endonuclease/3'-diesterase enzymes in S. cerevisiae – mutational analysis

A mutational analysis revealed the major role of Apn1 in the BER pathway as compared to the lesser role of Apn2. In fact, the deletion of apn1 was found to increase 6- to12-fold the rate of spontaneous mutations in the cell, in contrast to apn2 where no increase was detected [104]. Furthermore, treatment of the apn1 mutant with DNA damaging agents such as MMS, H2O2 and t-BH, revealed an accumulation of DNA lesions that correlated with a hypersensitivity to the mentioned drugs [70, 104]. However, similar investigations using the apn2 mutant however, did not reveal any increased sensitivity [70, 105]. Interestingly, the apn1 apn2 double mutant was found to be even more sensitive than the apn1 single mutant to the same DNA damaging agents [70]. These results point to the fact that Apn1 provides the main AP endonuclease activity in the cell whereas Apn2 seems to be acting as a backup enzyme for Apn1. The study of the response of these mutants to BLM revealed that only the double mutant, but none of the single mutants, is sensitive to the drug, reflecting the inability of any of the two enzymes alone to process the damages created by the action of BLM [104, 105].

4.3. AP endonuclease/3'-diesterase enzymes in humans

Similarly to other organisms, human cells were found to contain AP endonuclease enzymes. However, the proteins found in human cells, APE1 and APE2, both belong to the Exo III family. To date, no homologues of the Endo IV family of the AP endonucleases/3'-diesterases have been identified in human cells.

4.3.1. General characteristics of human APE1

The human AP endonuclease enzyme, APE1, was purified for the first time from HeLa cells [107] and later cloned and sequenced from cDNA expression

libraries obtained from human placenta and melanoma cells [108, 109]. The APE1 gene was found to encode a 318 amino acid protein with a molecular weight of 37 kDa [108, 109].

Functional analysis revealed that the enzyme, similarly to *E. coli* exonuclease III, possesses a strong AP endonuclease activity [110]. However, unlike the other Exo III family members, the 3'-diesterase activity of APE1 was found to be relatively weak [110-112]. Many studies of the 3'→5' exonuclease activity of APE1 led to multiple publications with conflicting results. While some found that APE1 presented no exonuclease activity against blunt ended dsDNA [110, 113], others demonstrated that this exonuclease activity was structure specific and presented a preference for 3'-mismatched nucleotides [114-116]. The fact that this exonuclease activity processes only one to two nucleotides on duplex DNA suggests that this function might be providing a proofreading role to compensate the absence of proofreading by the error-prone polymerase β [116-118]. Additionally, RNase H activity has been also associated with APE1 [113, 119].

While the APE1 enzyme has been found to possess a strong affinity for AP sites and thus requires no magnesium to bind the DNA substrate, Mg^{2+} was found to be essential for the enzymatic activities of the protein as well as for its dissociation form the incised AP sites [120].

Mutational analysis revealed the presence of a common catalytic site for the different DNA repair activities of the protein, as the same substitution was found to alter all of these activities [113, 121]. Specific amino acid substitutions revealed the function of multiple key amino acid residues in the protein. While Glu 95 was established to be implicated in metal binding [113, 121-123], Asn 212 and Asp 219 were found to be implicated in AP site recognition and binding [113, 124]. Moreover, Asp 210 was found to be responsible for phosphodiester bond cleavage and separation of APE1 from the DNA substrate [125]. The His 309 residue was found to likely be the catalytic residue of the protein as its mutation leads to a reduction of all of the activities of the enzyme [121]

In addition to its direct role in DNA repair, APE1 was found to coordinate the whole BER pathway in human cells. In fact, it has been shown that APE1 interacts with multiple DNA glycosylases to stimulate their binding to DNA (i.e. adenine DNA glycosylase hMYH) [126] or to promote their dissociation from DNA (i.e. TDG, Ogg1…) [127-129]. Furthermore, studies have demonstrated the physical interaction of APE1 with DNA polymerase β, facilitating its access to AP sites and accelerating the removal of 5'-dRP resulting from the incised AP site [130]. APE1 was also found to interact with FEN1, PCNA and DNA ligase I, and to stimulate their activity [131, 132].

Very interestingly, APE1 was also found to be implicated in other processes that are not directly related to DNA repair. As such, it has been shown that APE1 is a redox factor and can activate and stimulate the binding of a number of transcription factors to DNA [133-135]. A mutational analysis identified Cys 65 as the redox active site of the protein [136].

While APE1 shares no sequence identity with Apn1 or endo IV, it was found to possess NIR activity, indicating that this enzyme has evolve with a broad substrate specificity and may compensate for the lack of endo IV-like enzyme in human cells [137].

4.3.2. General characteristics of human APE2

The second AP endonuclease enzyme identified in humans, APE2, is a 518 amino acid protein with a calculated molecular weight of 57.3 kDa [138]. This enzyme was found to possess a weak AP endonuclease activity that is about seven fold higher than its 3'-diesterase activity [110]. Structural studies revealed the absence of the redox domain that is present in APE1 [138]. However, APE2 was found to possess a long C-terminal domain that was shown to contain a PCNA binding motif, similar to the one present in *S. cerevisiae* Apn2, suggesting a possible role in LB BER [139]. Multiple reports also suggested a possible role for the C-terminal domain of APE2 in localizing the protein to the nucleus [138].

Interestingly, in addition to its nuclear localization, APE2 was found to be localized to the mitochondria through a potential mitochondrial localization signal in its N-terminal portion, suggesting a possible role in the maintenance of the mitochondrial DNA's integrity [139].

4.3.3. The importance of the BER and the AP endonuclease enzymes in mammalian cells

As is the case in different biological processes, BER can be compromised at the level of its operating enzymes. In fact, it has been shown that mutations in multiple genes of this pathway, including AP endonucleases / 3'-diesterases, can be associated with many human diseases such as neurodegenerative disorders and cancer [140-143]. Additional reasons fueling our interest in further studying the families of AP endonucleases / 3'-diesterases came from multiple observations that AP endonuclease knockout mice were found to be embryonic lethal and the knockdown of *in vitro* human cancer cell lines were found to arrest cell proliferation and activate apoptosis [144, 145].

5. *Caenorhabditis elegans*

5.1. General information

C. elegans is a free-living animal that is normally present in the soil and other bacteria-rich environments. It is a multi-cellular organism of the nematode phylum that has been very widely used in research since 1974 [146]. Multiple studies led to the portrayal of the complete cell lineage as well as the complete description of the anatomy of the whole animal [147]. The adult worm contains 1000 somatic cells, possesses a mouth, a pharynx, an intestine, a gonad, an excretory system, muscles an epithelial system and a cuticle made of collagen (wormatlas). It is also known to be the simplest animal with a central nervous

system [148]. In nature, one can find self-fertilizing hermaphrodites and males but no females [149]. The sexual organs differ between males and hermaphrodites as males have a single lobed gonade, a ductus deferens and a copulatory apparatus in the tail for mating while hermaphrodites, on the other hand, have two gonades, oviducts, a spermatheca, and a single uterus (wormatlas).

The reason behind the success of using *C. elegans* in research comes from the fact that it is easy to manipulate and can be easily grown for low cost in the laboratory on the bacterium *E. coli* [149]. It has a relatively short life span and can survive freezing for many years, which allows long-time storage of multiple strains. This organism is also transparent, allowing easy visualization of internal development and organs, as well as easy visualization of fluorescently tagged proteins and different types of staining [149]. Another strong point in the favor of the use of *C. elegans* in research is the relatively small genome in a small yet relatively complex metazoan. In fact, *C. elegans* was the first multi-cellular organism to have its genome sequenced and published in 1998 [150], with the last gap sequenced and published in 2002. The 100 million base pair genome contains an approximate 20, 000 genes that are represented on five pairs of autosomes and one pair of sexual chromosomes. The sexual orientation of the offspring is based on the XO sex-determination system, where XX signifies hermaphrodites and XO represents the rare males. Males are infrequently produced (0.1%) by spontaneous meiotic non disjunction in the hermaphrodites where one of the daughter cells inherits 2 X chromosomes while the other inherits none [151]. The production frequency of males increases by up to 50% upon introduction of males into a hermaphrodite population as male sperm preferentially fecund oocytes [149]. The complexity of this animal is exemplified by a diversity of intricate behavior including locomotion, foraging, feeding, defecation, egg laying, dauer larva formation, sensory response to touch, smell, taste, temperature, male mating, social behavior, learning and memory [152-156].

All the above mentioned characteristics render this organism a model of choice in multiple studies related to cellular processes such as cell fate

determination and differentiation as well as apoptosis. Genomics, aging and multiple studies concerning human nervous-based diseases such as Alzheimers and Parkinsons also used *C. elegans* as a model organism [157-160].

Similarly, this organism provides a big advantage to study DNA repair then yeast and bacterial cells do. This is mainly due to the complex behavior that this model possess, notably, egg hatching, speed of growth and individual survival, that can be studied upon a loss of a DNA repair protein and which cannot be assessed in other simpler organisms such as yeast and bacterial cells.

5.2. Life cycle

The *C. elegans* life cycle comprises six stages of development, starting with embryonic development that occurs in part inside the hermaphrodite, while the second part takes place in the outside environment in the form of laid eggs [149]. Each egg gives rise to an L1 larva which then develops quickly through a series of four molts passing from L1 to adult [149]. Under starvation conditions and overcrowding, the L2 larva can enter an alternative 3rd stage called the dauer larva that is characterized by a thick cuticle, a sealed mouth and a very low metabolism, allowing it to survive for months under these conditions, whereas all the adult worms die off. The dauers resume development by entering the L4 stage upon re-introduction to a food source [149].

Since *C. elegans* is a cold-blooded animal, the temperature of the environment largely influences the speed of development and the time intervals between the molts. Incubation temperatures usually used in the laboratory are 16, 20 and 25˚C, where 16˚C allows the slowest growth rate and 25˚C, the fastest. In fact, it was found that growth at 25˚C is 2.1 times faster than it is at 16˚C and 1.3 times faster than it is at 20˚C [149]. Incubation of plates at lower temperatures, such as 6-8˚C, result in growth arrest and can be used to temporarily arrest development for up to 15 hours [149]. The incubation temperature also affects the

overall longevity of the worm as *C. elegans* live longer at lower temperatures than in warmer environments.

5.3. RNA interference in *C. elegans*

Gene silencing through RNA interference (RNAi) has lately become an important tool to study gene function through analysis of the consequences of the decrease of its expression. However, prior to the detailed characterization of the technology, mis-conceptions and confusion regarding RNAi existed in the scientific community.

Early observations linking RNA molecules to gene silencing came in the early 1980's, when small RNA molecules (~100 nucleotides) in *E. coli* were found to bind complementary mRNAs and inhibit their translation [161]. This same translation regulation was also found to occur in eukaryotes, such as *C. elegans* [162-166], among others [167]. In early experiments, anti-sense RNA [168] and, surprisingly, sense RNA [99, 169] were both found to lead to gene silencing. Later, experiments conducted by Fire and Mello revealed that only the injection of double-stranded RNA (dsRNA), and not antisense nor sense RNA alone could lead to efficient gene silencing [170]. The earlier studies showing an effect with either sense or antisense RNA were explained by the fact that the RNA preparations were probably not totally pure and contained a small amount of dsRNA [171]. Within this study, Fire and Mello demonstrated that the observed gene silencing was specific for the mRNA homologous to the injected dsRNA [172]. The sequence of the dsRNA should correspond to that of the mature mRNA and not introns or promoter sequences, indicating that the silencing observed is post-transcriptional [172]. It was also shown that the targeted mRNA was degraded and only a few dsRNA molecules were sufficient to fully silence a given gene. More importantly, the silencing effect was found to spread from one cell to another and was found to be transmitted to the progeny [170].

Further characterization of the RNAi mechanism showed that the long dsRNA is first cut into short pieces (~25 nucleotides long) by a ribonuclease III-like nuclease named Dicer [173]. The short antisense strand is then loaded into a large complex called RISC (RNA-induced silencing complex), that is targeted to cleave the mRNA through the antisense small interfering RNA (siRNA) [174]. In fact, the RISC complex was found to contain a protein from the argonaute family that is thought to act as an endonuclease to cleave the mRNA [174]. Interestingly, the RNAi mechanism was found to be amplified in certain organisms such as worms and fungi, through the action of an RNA-dependent RNA polymerase (RdRP) that generates and/or amplifies siRNA [175].

In *C. elegans*, RNAi technique has been widely used to study gene function. Multiple techniques were tested in order to deliver the dsRNA that is essential for the knockdown of gene expression. These include injecting the dsRNA into the worm, soaking the worms in a solution containing the dsRNA or feeding the worms bacteria genetically designed to express the dsRNA of the targeted gene.

5.4. AP endonuclease/3'-diesterase enzymes in *C. elegans*

Early discoveries of AP endonuclease enzymes indicated the presence of two families, Endo IV and Exo III, in unicellular organisms whereas only members of the Exo III family were identified in multicellular beings. This reality soon changed with the identification of both families in the first multi-cellular organism, *C. elegans*. However, to date, very little is known about the *in vivo* characteristics of these DNA repair enzymes.

5.4.1. Known characteristics of *C. elegans* apn-1 and Ceexo-III

Previous studies in *E. coli* and *S. cerevisiae* have demonstrated the importance of the Endo IV and Exo III family members in repairing AP sites and single strand breaks with 3'-blocked termini. These characteristics led to the assumption that proteins of these families might be conserved in higher organisms. The high homology seen between *E. coli* endonuclease IV and *S. cerevisiae* Apn1 was used to isolate the cDNA of the *C. elegans* apn-1 gene using a λ phage cDNA library prepared from *C. elegans* [176]. The isolated cDNA was expected to encode a 278 amino acid protein with a calculated molecular weight of 30 kDa. Sequence analysis revealed that this protein shares 40.4% identity with ScApn1 and 44.9% identity with *E. coli* endonuclease IV [176]. Furthermore, these results were reinforced with the finding that crude extracts from *C. elegans* embryos possess AP endonuclease activity [177]. However, it was not sure whether this activity belonged to the product of the isolated apn-1 gene or to another AP endonuclease enzyme present in this organism. A few years later, cross species complementation studies using a *C. elegans* cDNA library to complement the yeast mutant lacking both Apn1 and Apn2 led to the isolation of two genes, apn-1 and exo-3, encoding the homologues of the Endo IV and Exo III families respectively [178]. Similarly to the high level of shared identity found between apn-1 and its homologues in *S. cerevisiae* and *E. coli*, the exo-3 gene was found to share 27% identity with *E. coli xth* and 45 % identity with *H. sapiens* Ape1 [178].

Expression of either APN-1 or EXO-3 reduced the spontaneous mutation rate exhibited by the yeast apn1 apn2 double mutant. Furthermore, the hypersensitivity of this yeast mutant to multiple DNA damaging agents such as H_2O_2 and MMS was alleviated by the introduction of either apn-1 or exo-3. However, while complementation using exo-3 restored full resistance to BLM, complementation using the apn-1 gene conferred only partial resistance to this drug, reflecting a certain degree of substrate recognition specificity between the

two enzymes [178]. These observations were supported by *in vitro* studies, where crude extracts from the yeast double mutant, transformed with either apn-1 or exo-3 were found to contain AP endonuclease activity, reflecting that the product of both genes are endowed with this activity [178].

Further characterization of *C. elegans* EXO-3 led to the identification of two point mutations, D190A and H279A, which disrupt the AP endonuclease and 3'-diesterase activities of the protein, revealing the importance of these residues for the activity of this enzyme. Another amino acid substitution, E68A, was found to retain AP endonuclease and 3'-diesterase activities but severely lack the ability to protect the yeast mutant against DNA damaging agents such as MMS, H_2O_2 and BLM. These amino acids correspond to residues E96, D219 and H309 that are critical for the function of the human Ape1 enzyme [178]. Additional investigation of the role of Glu68 revealed that this amino acid residue is important for the binding of Mg^{2+} to the EXO-3 protein as is the case with its corresponding amino acid, Glu96, in the human Ape1 enzyme. Furthermore, the E68 residue of EXO-3 was found to facilitate the release of the EXO-3 protein from the DNA to allow subsequent repair components to complete the pathway [179].
It is also important to note that, in contrast to E. coli exonuclease III and S. cerevisiae Apn2, C. *elegans* EXO-3 was found to possess a very low 3'→5' exonuclease activity [178, 179].

6. Research project and summary of results

The characterization of AP endonuclease enzymes in many models, notably humans, enhanced our understanding of the importance of the BER pathway in protecting cells from specific types of DNA damage. The search for these enzymes in multiple organisms, led to the identification of the Endo IV family in prokaryotes and some eukaryotes, but not in high eukaryotes such as humans, whereas the Exo III family was found in all organisms. This suggests a probable loss of the Endo IV family function through an evolutionary process.

Until the discovery of the *C. elegans* AP endonuclease enzymes, no members of the Endo IV family were identified in multi-cellular organisms, rising questions regarding the relevance of this family in *C. elegans* and providing a tool that might explain its absence in higher organisms. We therefore hypothesized that APN-1 is important to preserve the genomic integrity of *C. elegans*.

Previous characterization of the C. elegans exo-3 and apn-1 genes through cross species complementation demonstrated the presence of AP endonuclease and 3'-diesterase activities associated with the products of both genes [178]. The *C. elegans* genes were also found to complement yeast mutants deficient in AP endonuclease/3'-diesterase activity, allowing them to counteract damages caused by multiple DNA damaging agents such as H_2O_2 and MMS [178]. However, these findings do not totally reflect the *in vivo* function of these genes as the study was performed in a totally different organism than the natural host. Furthermore, the system used represents an over-expression system and therefore, might lead to an inaccurate assumption of the *in vivo* role of these enzymes.

Thus, we sought to characterize the apn-1 gene in *C. elegans*. We conducted RNAi experiments to decrease the expression of this gene and follow its consequences on different aspect of the animal's life. A transgenic worm, over-expressing the full length GFP tagged APN-1 protein, was prepared to be used for protein localization and the verification of knockdown efficiency. Western blot analysis using crude extracts obtained from the transgenic animals demonstrated the efficiency of the knockdown system used. Similarly, qRT-PCR studies using RNA extracts from the *C. elegans* parental N2 strains further verified the knockdown efficiency in the parental strain. Additional analysis, using the β-galactosidase reporter assay, revealed elevated mutation levels as a result of apn-1 knockdown. This was found to correlate with a delay in embryonic development and a decrease in the egg hatching percentage. Interestingly, cell cycle studies revealed that the delay observed during embryonic development may be partially due to the slow progression of the cell cycle that is resulting from the knockdown of the apn-1 gene and that is increased by DNA damaging agents.

Surprisingly however, the knockdown of apn-1 was not found to decrease the longevity of the worms. Nevertheless, when challenged with DNA damaging agents that cause AP sites, (t-BH and MMS), the worms' lifespan was decreased. Our study therefore provides solid evidence that APN-1 can function in vivo to maintain the integrity of the genome.

Chapter 2 – Materials and methods

1. *C. elegans* Strains and maintenance of nematode cultures

C. elegans strains Bristol N2 (parent) and NL3400: *pkIs1604* [*rol-6*(*su1006*) *hsp16/2*::ATG(A)17*gfp/lacZ* (out of frame)] were obtained from the *C. elegans* stock center (University of Minnesota, USA). *C. elegans* strain expressing full length GFP tagged APN-1 protein (APN-1-GFP strain) was prepared by Dr. XiaoMing Yang and Dr. Jean-Claude Labbé as described below (page 39-41). Worm stocks were grown and maintained on 9 cm NGM agar plates at 16^{o}C as described [149]. NGM media was prepared by adding 3 g of NaCl, 2,5 g of peptone and 17 g of agar to 975 ml of water, autoclaved, cooled down to 55^{o}C and then the following were added in order: 1 ml of cholesterol (5 mg/ml in 100% ethanol), 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, and 25 ml of 1 M KH_2PO_4 adjusted to pH 6.0 by the addition of KOH.

The *E. coli* AP endonuclease deficient strain BW528 (Δ (*xth-pnc*), *nfo1::kan*) was chosen instead of OP50 to avoid any contamination of the protein or the RNA extracts with the bacterial homologue of *C. elegans* AP endonuclease enzymes [177].

Bacterial stocks were prepared by growing the BW528 strain overnight in 5 ml of liquid Luria Broth (LB) media at 37^{o}C (LB: 16 g tryptone, 10 g yeast extract, 5 g NaCl per litre of water). This was followed by sub-culturing the cells for an additional 8 hours at 37^{o}C in 50 ml of LB. The bacteria were then harvested by centrifugation, re-suspended in an isotonic solution (M9 buffer) and glycerol (ratio 1:1) and stored at –80^{o}C. The M9 buffer was prepared by dissolving 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl and 0,25 g of $MgSO_4 \cdot 7H_2O$ in 1 L of H_2O). Prior to plating, the bacterial stock was diluted in M9: glycerol to an O.D. of 0,1.

The absorption was measured using a spectrophotometer. BW528 strain was spread onto the NGM plates to provide a nutrient source for the worms.

A total of 20 nematodes were then transferred to each plate previously spread with the *E. coli* BW528 strain and incubated at 16 ^{o}C. In order to maintain these cultures at 16 ^{o}C, a transfer to freshly prepared and seeded plates must occur once every 4-5 days.

2. RNAi knockdown feeding system

Delivery of the double stranded RNA (dsRNA) that is responsible for the knockdown was performed through a feeding system where bacteria expressing the dsRNA were used as a food source for the worms [180]. In fact, dsRNA has the ability to cross cell membranes and propagate throughout the whole body of the worm, causing a systemic knockdown of the target gene simply through feeding.

For this purpose, bacterial strain HT115 (F-, mcrA, mcrB, IN(rrnD-rrnE)1,rnc14::Tn10(DE3 lysogen: lavUV5 promoter -T7 polymerase IPTG-inducible, RNAse III minus) lacking the dsRNA-specific RNase, was transformed with L4440 vector to stably produce dsRNA of the apn-1 gene. L4440 vector has a multiple cloning site (MCS) flanked by two T7 promoters [180]. Since these promoters present opposite orientations, transcription in both directions of the insert through the T7 promoters leads to the formation of a dsRNA that has an identical sequence to the DNA fragment inserted in the MCS [180]. Moreover, the HT115 strain has a T7 RNA polymerase gene that is inducible by Isopropyl β-D-1-thiogalactopyranoside (IPTG) allowing indirect induction of dsRNA production [180]. These characteristics of HT115 strain and the L4440 vector provide a great system to reach our goal. The bacterial strain and the L4440 vector were obtained from Dr. Simon Bulton.

2.1. L4440-apn-1 Plasmid preparation (prepared by Dr. Andrea Shatilla)

The preparation of the L4440-apn-1 plasmid started with the amplification of a ~900bp piece of the apn-1 gene (nucleotides 46-949) through a PCR reaction. The 5'-end of these primers were designed to be cut with the same restriction enzymes as those used to linearize the L4440 vector, Kpn I and BamH I. The amplification products as well as the L4440 vehicle were treated with the above mentioned restriction enzymes for two hours at 37^{o}C to create compatible ends for ligation. Prior to ligation, the vector was treated with 0.5 µl of calf intestinal phosphatases (CIP) for 30 min at 37^{o}C to remove 5'-phosphate and therefore prevent the vector from re-circularizing. Heat inactivation of CIP was performed by incubating the sample at 65^{o}C for 15 min. The ligation reaction was incubated overnight at 16^{o}C, transformed into competent DH5α, plated on LB agar petri dishes and grown overnight at 37 ^{o}C. Single colonies were picked up and plasmid DNA was prepared from each colony using QIAprep spin Miniprep Kit from QIAGEN. Plasmid DNA was digested using Kpn I, BamH I, and the combination of Kpn I and BamH I in order to verify correct ligation. Digestion of the correct plasmid with either Kpn I or BamH I are supposed to yield a single band with a molecular weight higher than the linearized L4440 vector (3.7 kb vs 2.8 kb, respectively), used as a control. On the other hand, the double digestion is supposed to produce 2 distinct bands: one of 2.8 kb and the other of 0.9 kb, corresponding to the separated vector and insert, respectively. The digestion product was visualized on a 1% DNA agarose gel stained with Ethidium Bromide (EtBr). One of the colonies presenting the above described digestion pattern was inoculated into 2 ml of liquid LB supplemented with 100 µg/ml of ampicilin and incubated overnight at 37^{o}C. Plasmid DNA was then extracted using QIAprep Spin Miniprep kit (Qiagen). Purified plasmid was used to transform competent HT115 bacterial cells using standard transformation procedures. Bacterial cultures

and stocks were prepared by inoculating a single colony of the transformed bacteria into 2 ml of liquid LB supplemented with ampicillin (100 µg⁄ml) and incubated overnight at 37^{o}C. This was followed by sub-culturing the cells for an additional 8 h in 50 ml of LB with ampicillin after which cells were harvested by centrifugation (4000 rpm for 10 min), re-suspended in M9:glycerol (ratio 1:1) aliquoted and stored at -80^{o}C.

The Taq polymerase and the 10x PCR buffer were from Feldan while the CIP, T4 DNA ligase and the ligase buffer were purchased from New England Bio labs.

2.2. Preparation of knockdown plates

Knockdown plates were prepared as described previously [180]. The NGM plates used for the knockdown of apn-1 had the same composition as described previously with the addition of 25 µg⁄ml of ampicillin and IPTG to a final concentration of 1 mM. IPTG induces the T7 RNA polymerase in the bacterial strain that in turn, binds to the T7 promoter flanking the multiple cloning site in the L4440 vector to enhance the dsRNA production. The addition of ampicillin helps prevent the bacteria from losing the plasmid.

Prior to plating, the HT115 strain containing the empty L4440 vector or the plasmid expressing the dsRNA for apn-1 were inoculated into 2 ml of liquid LB supplemented with ampicillin to a final concentration of 100 µg⁄ml. The cultures were then shaken for 4-5 hours at 37^{o}C to reach log-phase and 15-20µl were spread onto each IPTG and ampicillin containing plate and incubated overnight at room temperature. After the overnight incubation, a total number of 20 L4-adult worms were transferred to each plate, which were then incubated at 16^{o}C for 4-5 days.

3. Verification of the knockdown efficiency through RT-PCR

RNA interference is known to induce the degradation of the target messenger RNA [174]. Thus, a good way to verify the knockdown efficiency in the parental strain is to look at the level of the mRNA before and after the knockdown.

3.1. RNA extraction

Worm cultures were prepared as described previously. About 5 plates, containing the *C. elegans* N2 strain of the control RNAi and 5 of the apn-1 RNAi were collected and washed 2-3 times with M9 buffer to remove all traces of the feeding bacteria. This was done by centrifugation at 1000 rpm for 4-5 min. After the last wash, the worms were transferred into 1.5 ml eppendorf tubes, the buffer was removed completely and an equal volume of lysis buffer was added. The lysis buffer was composed of 50 µl of 10% SDS, 50 µl of β-mercaptoethanol, 10 µl of 1 M Tris HCl pH 8.0, 25 µl of proteinase K and 20 µl of 0,5 M EDTA PH 7-8 in a total volume of 1 ml.

The tubes containing the worms and the lysis buffer were then incubated at 42ºC for 15-30 min while re-suspending the precipitated worms once every 5 min. After the incubation, 1 ml of trizol (Invitrogen) was added and mixed with the content of the tube. Three to 5 freeze-thaw cycles in liquid nitrogen were performed to further release the RNA from the cells, after which 200 µl of chloroform was added and mixed by vortexing for 3-5 min. Tubes were allowed to sit for 3 min at room temperature then a centrifugation at 12, 000 xg for 15 min was performed in an eppendorf micro-centrifuge at 4ºC. After centrifugation the top layer was transferred to new 1.5 ml eppendorf tubes and RNA was precipitated

by addition of an equal volume of isopropanol, mixing and allowing tubes to sit at room temperature for 10 min. After centrifuging at 12, 000 xg for 15 min at 4ºC, the RNA pellets were washed with 75% ethanol in RNase free water and centrifuged at 7500 xg for 5 min. Finally, the pellets were air-dried briefly and re-suspended in RNase free water. RNase free water was prepared by adding 500 µl of Diethyl pyrocarbonate (DEPC) to 500 ml of water.

The RNA samples were then treated with DNase to avoid the DNA co-amplification with the target cDNA during the PCR reaction. This was done using a Ribo Pure-yeast kit from Ambion. To each RNA sample, 10X DNase 1 buffer and 8 units of DNase 1 were added and mixed. The tubes were then incubated at 37ºC for 30 min after which the enzyme was inactivated by the addition of inactivation reagent. The contents of the tube were mixed by vortexing and allowed to sit for 5 min at room temperature. A quick centrifugation for 2-3 min at 13, 000 rpm allows the precipitation of the inactivation reagent. The solution containing the RNA was then transferred into a new tube which was frozen at -80 ºC until needed.

3.2. Genomic DNA extraction

The genomic DNA was extracted using the Jorgensen lab's protocol and was used as a positive control for the RT-PCR. Four to five plates containing the parental N2 strain grown on the *E. coli* BW528 were washed 2-3 times by centrifugation to eliminate all traces of bacteria. After the last centrifugation, the worm pellet was re-suspended in TEN solution containing Tris, EDTA and NaCl pH 7.5 to a final concentration of 20 mM, 50 mM and 100 mM respectively. The worms were then centrifuged at 1000 rpm for 1 min, the supernatant removed and the worm pellet was again re-suspended in an additional 500µl of TEN and then transferred into a 1.5 ml eppendorf tube. To the content of the tube, 25 µl of 10% SDS and 2.5 µl of 20 mg/ml proteinase K were added, mixed well, and incubated

at 55 ºC for one hour. Settled worms were re-suspended once every 10 min. After the first hour of incubation, an additional 2.5 µl of proteinase K was added and the tube was re-incubated for a second hour at 55 ºC. After the second incubation, a phenol extraction was performed followed by a phenol/CIA and finally a CIA extraction (CIA=24:1 chloroform: isoamyl alcohol). After each extraction, the top layer was transferred into a fresh tube that was kept on ice. To precipitate the DNA, 45 µl of 3 M sodium acetate (NaOAc) and 0.8 ml of 100% ethanol were added and mixed well with the content of the tube. The DNA precipitates almost immediately. A short spin (2-5 sec) is long enough to pull down the DNA. At this point the ethanol was drained and then the pellet was washed with 70% ice cold ethanol. The wash was eliminated and the pellet was allowed to dry completely. The dried pellet was re-suspended in 500 µl of TEN. Since this re-suspension appears to be difficult, the tubes were kept overnight at 4 ºC. An RNase treatment was then performed to purify the DNA preparation. To do this, 2µl of 10 mg/ml RNase A was added and the mixture was incubated at 37 ºC for 1 hour. This incubation was followed by a phenol, then a phenol/CIA and finally a CIA extraction. The DNA was precipitated by the addition of 45 µl of NaOAc and 800 µl of 100% ethanol. The pellet was washed with 70% ethanol, drained and allowed to completely dry. Finally, the dried pellet was re-suspended in 100 µl of TE

3.3. Reverse-transcriptase (RT)-PCR and q-RT-PCR reactions

RT-PCR was conducted according to standard protocols. The reverse transcription part of the reaction was performed using either oligo (dT) primers (Amersham Pharmacia Biotech) or the reverse transcription kit with random primers (Invitrogen). The PCR reaction was performed using standard Taq polymerase and 10x PCR buffer that were purchased from Feldan. Twenty cycle

PCR reactions were performed and the resulting products were visualized using an agarose gel stained with EtBr.

q-RT-PCR reactions were performed using 7500 Real Time PCR systems from Applied Biosystems. Briefly cDNA from the RT step of the reaction was mixed with PerfeCTa SYBR green supermix low ROX (2X) from Quanta Biosciences and primers to a final concentration of 5 μM. The obtained results were assessed using Microsoft excel software.

The PCR primers for the apn-1 gene are:

RRT apn-1 F: 5'-TAGTTCTCGAGACAATGGCTGG-3'
RRT apn-1 R: 5'-CCAGCAAAAATGTGACACGTG-3'

The PCR primers for the loading control rpb-12 (RNA polymerase II (B) subunit – Sequence name: F23B2.13

RRT F23B2.13 F: 5'-CAGGTCAAGCTCATCTCAAGTCA-3'
RRT F23B2.13 R: 5'-GCGTCGTACACCATCAACTTTC-3'

The PCR primers for the unc-22 gene:

RRT unc-22 F: 5'-GATCAAAAGATCAGGATTGAAAGC-3'
RRTunc-22 R: 5'-GAATGTTTGTCGCTTCCAGAAG-3'

4. Construction of the APN-1-GFP expressing plasmid, microinjection and generation of transgenic worms

Given that all the experiments performed in this study were based on the knockdown of the *C. elegans* apn-1 gene, the verification of the efficiency of the knockdown system on the protein levels had to be verified. Taking into consideration that the endogenous level of expression of the studied protein is very low for any western blot or activity detection, we used the engineered transgenic *C. elegans* line over-expressing a C-terminal GFP-tagged APN-1 protein to assess knockdown efficiency.

4.1. Preparation of the pPD95.70-APN-1-GFP plasmid (Prepared by Dr. Xiaoming Yang)

The full length apn-1 gene was amplified using cosmid TO50H plasmid and a PCR touchdown program with pfu DNA polymerase (Fermentas). The PCR fragments obtained this way contained sequences overlapping with sequences in the vector pPD95.70 that contains a GFP gene following the apn-1 promoter region. The pPD95.70 vector was digested with restriction enzymes SalI and Sac I. The full length apn-1 product was co-transformed along with the linearized vector into *S. cerevisiae* YW465. The colonies were selected on –URA plates and the positive colonies were confirmed by Western blot analysis and multiple restriction digestions for verification.

The oligos used for the PCR reaction were:

Ceapn1-UTR2500-F1:

CACGAAAGAATCGTCGACTCAAGAAGCAACGCTAGCTG

and oligo **Ceapn1-GFP-R1**

AAACAAGAAGAGCTCTTTCTTTTATCCATATTGT

4.2. Generation of transgenic nematodes (prepared by Dr. Jean-claude Labbe)

Worms were transformed by microinjection as described previously [181].Plasmid pPD95.70, containing the wild-type apn-1 gene fused to GFP under the control of the apn-1 promoter, was coinjected into the cytoplasmic syncytium of the gonad in wild-type worms along with the transformation marker pRF4 [containing a dominant mutation in the collagen gene, rol-6(su1006)] at concentrations of ~1 µg/ml and 50 µg/ml, respectively. pRF4 carries the dominant mutation Su1006 in the rol-6 collagen gene, which causes animals to roll and

move in circles, acting as a marker for the transformation. The high number of copies of the plasmid led to the slight over-expression of APN-1-GFP. Microinjection of adult hemaphrodites was performed using a zeiss axiovert 10 micrsocope. The recovered transgenic worms were called the APN-1-GFP strain and were used for the assessment of knockdown efficiency.

5. Visualization of APN-1-GFP levels

Transgenic worms over-expressing the APN-1 protein fused to GFP at the C-terminus were used to assess knockdown efficiency by fluorescent microscopy. Briefly, the FL strain was grown for 4 days on NGM media plates containing 1 mM IPTG and 25 μg⁄ml ampicilin and seeded with the appropriate *E. coli* strains (control and apn-1 RNAi). To be certain that the chosen worms were indeed transgenic and over-expressed the GFP tagged protein, and since we had previously observed that up to 40% of the transgenic worms were lost with each generation, only those that still display the transformation marker were picked and examined under the fluorescence microscope. For this purpose, 1 drop of M9 buffer was placed on a microscope slide and approximately 10 worms were then transferred onto the slide and covered with a cover slip, after which the slide was directly examined under a Leica, DMRE, Wetzlar fluorescence microscope supplemented with a camera.

6. Assessment of knockdown efficiency: Western blot analysis

An alternative way to assess knockdown efficiency was to prepare crude extracts of the worms and perform a western blot analysis using the anti-GFP

antibody. While GFP visualization by microscopy is easier and faster, this method can provide a more quantitative assessment of the knockdown level.

6.1. Preparation of total protein extract

After 4 to 5 days of RNAi feeding period, 5 apn-1 RNAi plates and 5 control plates were flooded with sterile M9 buffer to collect the worms in 50 ml falcon tubes. The worms were then washed three times each with 40 ml of sterile M9 followed by centrifugation at 1000 rpm for 1 min. After the last wash, the M9 solution was removed and the pellet was re-suspended in extraction buffer (100 mM TRIS-HCl pH 6,8, 15 % glycerol, 1 mM PMSF and 1 x inhibitor cocktail). Total protein extracts were then prepared from the re-suspended worms through sonication using Branson Sonifier 250 set at a duty of 50% and an output of 5. The sonication process was performed in 15 cylces of 10 sec on ice.

6.2. Western blot analysis

Western blot analysis was performed according to standard protocols. Total protein concentrations were determined using the Bradford analysis. Increasing quantities (10-20-40-80 µg) of the total protein extracts obtained from the control and apn-1 RNAi worms 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 with a mouse-anti-GFP monoclonal antibody (Santa Cruz Biotechnology) 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 anti-GFP antibody from the membrane. 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 supplemented with a LAS-3000 camera and the Image Reader LAS-3000 Lite software.

7. Detection of the AP endonuclease activity

The detection of AP endonuclease activity in crude extracts obtained from a yeast mutant lacking both AP endonuclease enzymes and transformed with the *C. elegans* apn-1 gene led us to assume that the undetectable AP endonuclease activity in crude extract derived from the *C. elegans* parental N2 strain is due to a very low expression level of the protein. Thus we decided to investigate whether the transgenic worm, expected to over-express APN-1, would express the protein at an enzymatically detectable level.

7.1. Preparation of oligonucleotide substrates

The double stranded substrate used in the AP endonuclease assay was prepared as previously described [177, 178]. Briefly, a synthetic 42 mer oligonucleotide with a uracil at position 21 was purchased from GIBCO, BRL or BioCorp (5'-GCTGCATGCCTGCAGGTCGA**U**TCTAGAGGATCCCGGGTACCT-3'). 100 ng of this oligonucleotide was 5'-radiolabelled with 50 μCi of [γ-^{32}P] ATP (6000 Ci/mmol ; Amersham) using T_4 polynucleotide kinase (Promega) as previously described [177]. The ethanol-precipitated and gel-purified labelled oligonucleotides were annealed with an equimolar concentration of the complementary strand

(3’-CGACGTACGGACGTCCAGCTGAGATCTCCTAGGGCCCATGGA-5’) to create a double stranded 5’-labelled substrate with a uracil at position 21.
The AP site was then generated through the incubation of 32 ng of the 5’-labelled double stranded oligonucleotide with 2 units of *E. coli* Uracil N-glycosylase Ung (New England Biolabs). The buffer used for this reaction (buffer D) contained 30 mM Hepes–KOH (pH 7.6), 50 mM NaCl and 2 mM EDTA. The reaction mixture was incubated at 37˚C for 30 min and the enzyme was then heat inactivated by incubating at 65˚C for 10 min [178].

7.2. Enzyme assays

The AP endonuclease assay was performed by incubating increasing quantities (500 ng-1-10-20 μg) of crude extracts obtained from the FL strain with 0.8 ng of the previously described AP substrate. The buffer used for this reaction contained 100 mM Tris-HCL (pH 7.0) and 5 mM $MgCl_2$ in a total volume of 12.5 μl. The reaction mixture was incubated at 37ºC for 30min after which it was stopped by the addition of 5 μl formamide loading buffer (76% formamide, 0.3% bromophenol blue, 0.3% xylene cyanole, 10 mM EDTA). The reaction mixtures were then heated for 3-5 min at 65 ◦C in order to dissociate the labeled oligonucleotides from the DNA-binding proteins. To visualise the digestion product, a 10% poly-acrylamide-7 M urea gel was prepared. After the separation of the reaction product, the gel was developed by autoradiography or phosphoimager [178].

8. Assessment of the mutation frequency through the detection of β-galactosidase Activity

Staining was performed by a modification of the protocol used by Fire et al [182]. The control RNAi and the apn-1 RNAi-treated plates containing the *C.*

elegans NL3400 strain were washed with sterile M9 buffer to collect the worms. The worms were then washed 2-3 times with 40 ml of sterile M9 buffer to eliminate the bacteria. These washes were done by centrifugation at 1000 rpm for 1 min. Following the last wash, the M9 buffer was removed completely. The worms were then vacuum-dried for 20 min. It appears to be crucial to use completely a dried worm pellet for β-galactosidase activity detection. To make holes in the cuticle, 35 µl of ice cold acetone was added to the dry worm pellet and allowed to completely air dry on the bench (~45 min). This step was repeated once more with another 35 µl of ice cold acetone. To the completely dried worm pellet, 200 µl of freshly prepared oxidation buffer was added and mixed by pipetting. The oxidation buffer consisted of 0,2 M sodium phosphate buffer, pH 7,5, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM $MgCl_2$, 0,004% sodium dodecyl sulfate, 7,5 µg/ml kanamycin and 0,04% X-gal. The worms were gently mixed with the oxidation buffer and the tubes were covered with aluminium foil and incubated overnight at 37^{o}C. For microscopy, the entire worm mixture was mixed by pipetting and one drop was placed on a microscope slide and gently sealed with a cover slip. All excess fluid was dried with a paper towel, and the microscope slide was directly examined under a microscope (Leica, DMRE, Wetzlar, Germany) at 250 magnification using immersion oil (Cargille Lab Inc, Cedar Grove, NJ, USA).

Worms were counted from the microscopy images considering the worms displaying high number of blue patches to be mutation carriers (accumulated high levels of mutations that brought the LacZ gene back into frame) and those with few or no patches to not be carrying significant mutations.

9. Egg-hatching assay: Test for sensitivity to DNA damaging agents

In order to verify whether the knockdown of the DNA repair gene apn-1 had any effect on the embryonic development of the nematode *C. elegans*, we developed a protocol in the lab to directly examine this possibility. This protocol can be divided into 3 steps: worm culture synchronization, drug plate preparation, and worm transfer and incubation.

9.1. Worm culture synchronization

An average of 30-40 young adult worms were transferred from the control or RNAi treated plates to new 9 cm NGM with IPTG and ampicilin plates seeded with either the control or knockdown HT115 strain. These worms were allowed to lay eggs for a period of 4-5 hours after which they were removed using a heated platinum wire attached to a pasteur pipette. The plates containing the eggs were then incubated at 16 ^{o}C for 3.5 days, resulting in a population of synchronized worms at the L4 stage.

9.2. Drug plate preparation

The egg hatching assay was always done in quadruplicates for each drug tested. The drugs tested in this study were H_2O_2, t-BH and MMS. Other drugs such as 4-NQO were tried and then omitted from this study due to stability issues. Cisplatin was also omitted due to an unexplained reactivity of this drug in *C. elegans*. UVC treatment was chosen as an alternative to cisplatin since it does not cause any damage that is repaired by apn-1.

Small, 3 cm plates, were used in this study instead of the larger 9 cm plates used previously. The drug concentrations chosen after standardization were 0.07% for MMS, 5 μM for t-BH, 0.001% for H_2O_2 and 150J/cm^2 for UVC. As in the preparation of the 9 cm knockdown plates, one drop of the log-phase bacterial culture was spread to form one thin line of bacteria on the plate. This facilitated the counting of the eggs and progeny that remained strictly on the bacterial line.

9.3. Worm transfer and incubations

Three L4-adult worms were transferred to each 3 cm plate. The plates were then incubated for 12-15 hours at 16 ^{o}C. After the first incubation, the adult worms were removed from the plates and the eggs were counted. The plates were then re-incubated and the progeny scored at two time points, namely 24 and 36 hours.

10. Longevity assay

Culture synchronization and the drug plate preparation were performed as described in the egg hatching assay. For this assay, 12 synchronized L4 worms were transferred to each 3 cm drug plate. Plates were then scored daily for any dead worms. A stimulation of the immotile nematodes with a platinum wire attached to a pastor pipette provided a proof of death. Those that failed to respond to this stimulation were considered dead. Three transfers were performed during this 1 month experiment. The first transfer was at t=0, the second was at day 3 and the last at day 8. These transfers helped avoid drug degradation, media desiccation and culture contamination with the progeny and thus, avoid confusion of the studied adult worms with the progeny.

11. Cell cycle length assessment

Cell cycle studies were performed with a modification of the protocol used by Labbe, J.C. et al. 2006[183]. Briefly, adult worms from the control and apn-1 RNAi plates were transferred to a drop of egg buffer [184] placed on a cover slide coated with 1% polylysine. Live embryos were obtained by cutting open the transferred adult gravid worms using two 25-gauge needles. The cover slip was placed on a microscope slide containing a 3% agarose pad in the middle and the edges were sealed by Vaseline to prevent the egg buffer from drying. Using an Olympus IX71 microscope with a 60X objective, supplemented with a Retiga 2000R from Qimaging, time-lapse images were taken with 10 sec intervals. Cell cycle length was measured by determining the time of each cytokinesis.

Chapter 3 – Results

1. Transgenic APN-1-GFP shows increased levels of AP endonuclease activity

As discussed in the introduction, one of the main advantages that *C. elegans* possesses over yeast and bacteria is its anatomical complexity, providing a way to assess if APN-1 presents tissue specific expression, and thus presenting a possible explanation for the unsuccessful identification of its homologue in humans to date. In order to investigate this, we created a transgenic animal expressing a GFP tagged APN-1 to follow the expression pattern of the protein in the body of the worms using fluorescence microscopy.

Briefly, apn-1 plus 2 kb upstream was amplified from the cosmid TOH50 and subcloned into the pPD95.70 vector such that APN-1-GFP expression is driven by its own promoter. As such, the expression level of the fusion protein can be controlled by the amount of the introduced plasmid. Unfortunately, the injection of the plasmid at any concentration higher than 1 ng/µl appeared to be toxic as no transgenic animals were isolated after multiple attempts. However, a successful line was isolated when the plasmid was injected at a concentration lower than 1 ng/µl.

Unfortunately, direct examination of these transgenic animals under fluorescent microscopy revealed no detectable levels of GFP when compared to the N2 parent; instead only autofluorescent granules normally present in the gut were detected (figure 1 A vs B). This observation indicates that the expression level of the fusion protein in the isolated transgenic line is not high enough to be detected by fluorescent microscopy and therefore is not adequate to directly study the distribution of the protein.

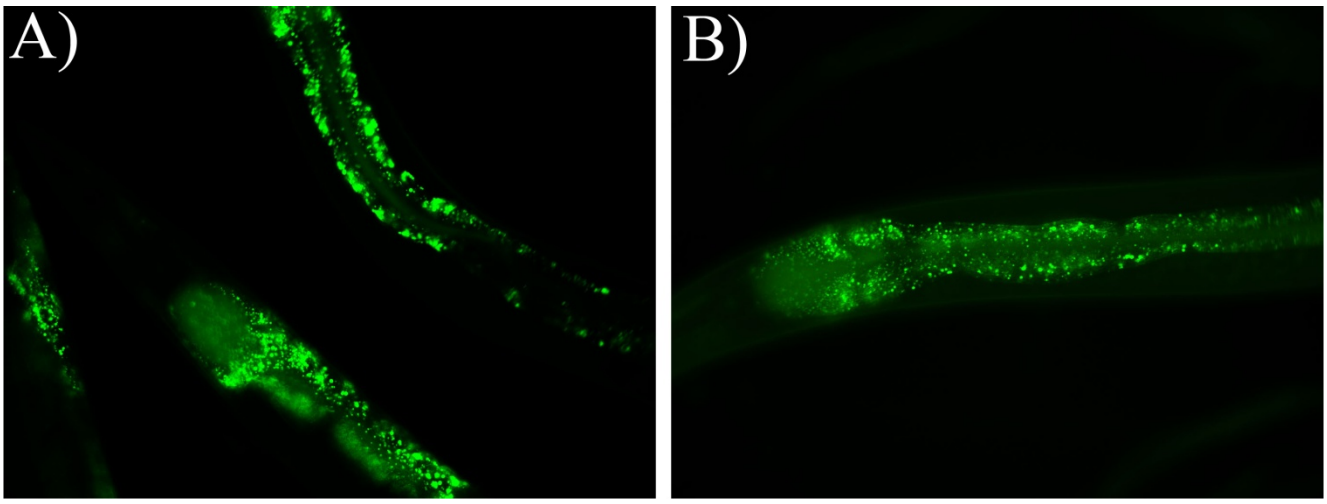


Figure 1. The APN-1-GFP transgenic line show no detectable GFP as compared to the parental N2 strain. Direct visualization of A) parental N2 Strain and B) APN-1-GFP transgenic worms under fluorescent microscopy.

Previous attempts failed to detect any Mg^{2+}-independent AP endonuclease activity, characteristic of the Endo IV family members, in crude extracts derived from the parental N2 worms suggesting no or a very low expression level of the APN-1 enzyme in *C. elegans* (figure 2 B lanes 8-15). Taking into consideration that the amount of the injected plasmid might lead to the overexpression of APN-1 in the isolated transgenic line, crude extracts from this line were assayed for AP endonuclease using the well established and highly sensitive AP endonuclease assay. This assay allows the detection of AP endonuclease activity using a 42 mer radiolabled oligonucleotide containing a centered AP site [177, 179]. The cleavage of the AP site by the action of the AP endonuclease enzymes leads to the formation of a 20 mer radiolabled fragment that migrates lower on a 10% polyacrylamide/7 M urea gels [179]. The assessment of the AP endonuclease activity in crude extracts derived from the transgenic line showed a concentration dependent Mg2+-independent AP endonuclease activity (figure 2A, lanes 4-7), likely reflecting a moderately elevated expression of APN-1 in the APN-1-GFP strain as compared to the parental N2 strain (figure 2 A vs B). The apparent normal growth of the transgenic worms suggests that the isolated transgenic line is capable of tolerating the slight over-expression of the APN-1-GFP fusion protein.

Furthermore, western blot analysis using the anti-GFP anti-body of total extracts derived from the transgenic line revealed the presence of a major ~75-kDa polypeptide that corresponds to the expected size of the full length APN-1-GFP fusion protein (figure 3). GFP fragments were also noticeable, suggesting that the GFP tag might not be very stable.

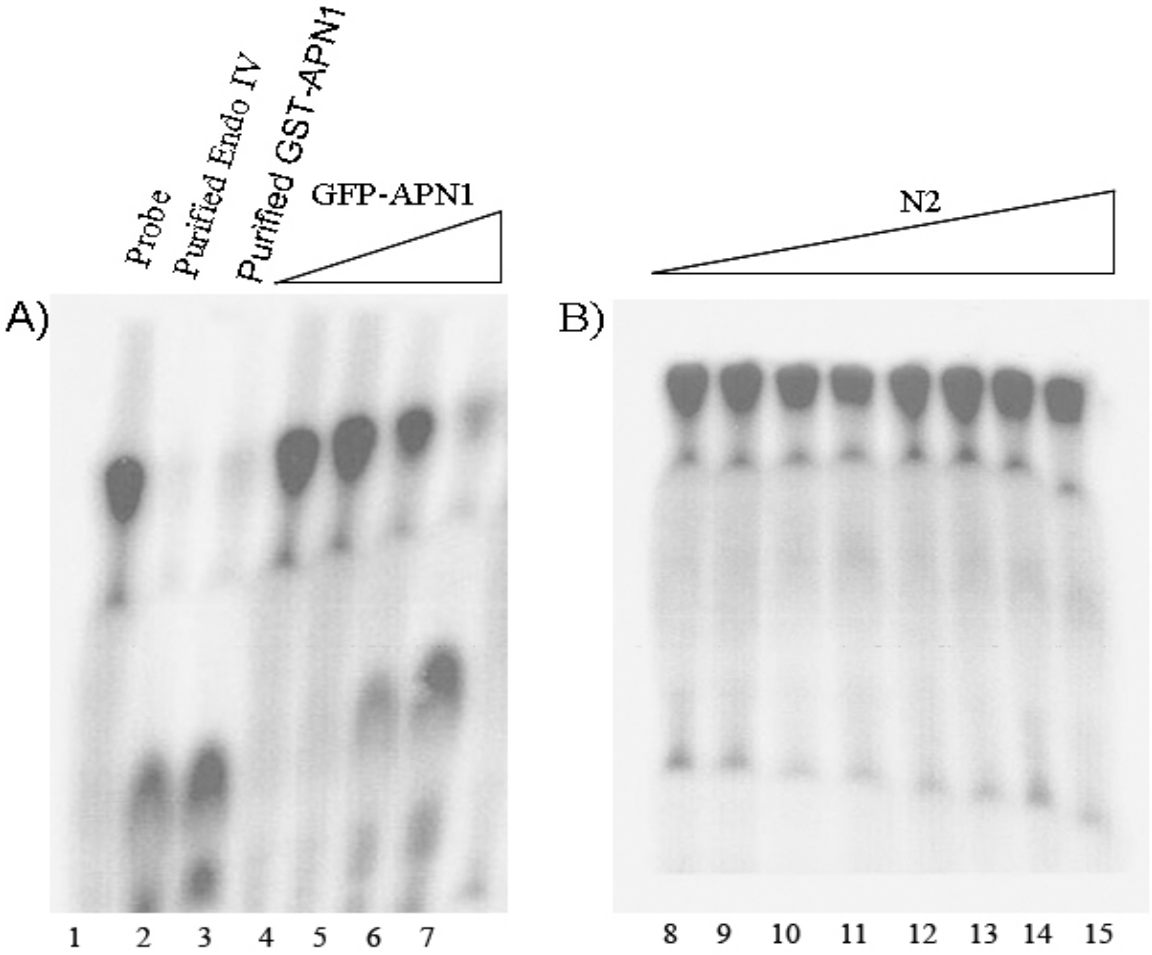


Figure 2. A) Crude extracts derived from the APN-1-GFP transgenic animals possess AP endonuclease activity.

B) Crude extracts derived from the parental N2 strain are devoid of AP endonuclease activity.

Crude extracts were prepared from the indicated *C. elegans* strains and monitored for AP endonuclease activity using a [32-P] labeled 42-mer oligonucleotide substrate containing a single AP site at position 21. A) lane 1, substrate alone, lanes 2 and 3 substrate with either purified endo IV or purified GST-APN1(yeast), lanes 4-7, extracts from the transgenic APN-1-GFP, B) lanes 8-15, increasing quantities of crude extracts derived from N2 strain

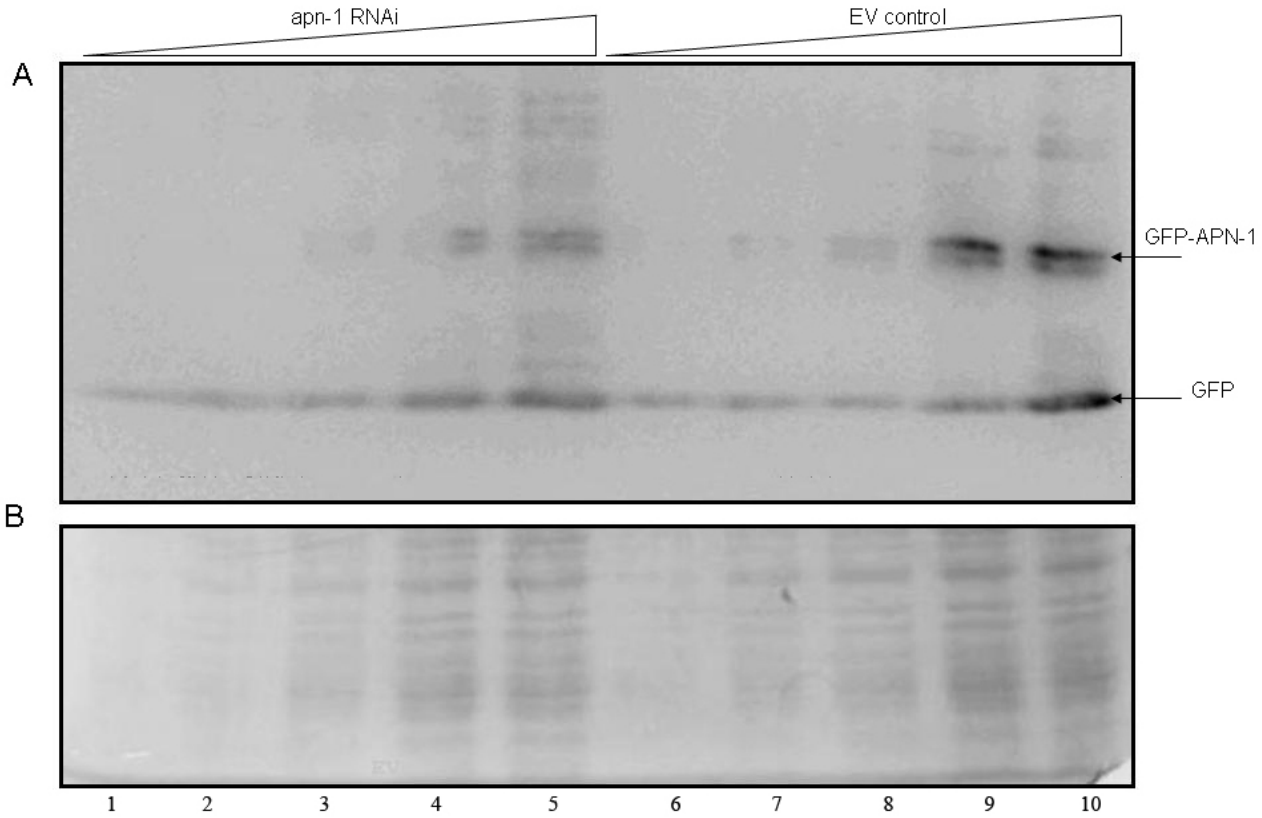


Figure 3. Western blot analysis showing the expression level of APN-1-GFP in the transgenic line fed control and apn-1 RNAi. Harvested worms were washed and total protein extracts prepared and analyzed by Western blot probed with anti-GFP antibody. A) western blot and B) Coomassie stain to monitor for protein loading

2. Overexpression of the long N-terminal domain of APN-1 is toxic

The original annotation of the *C. elegans* genome identified APN-1 as an 278 amino acid protein [176]. Recently, an additional in-frame start codon, upstream of the gene that would add additional N-terminal 118 amino acids to the protein, was identified. We have demonstrated through RT-PCR that these 118 amino acids are indeed transcribed but we haven't attempted to validate if they are translated as a part of the full length protein (data not shown). This unusually long

N-terminal region is not present in any of the other Endo IV family members from other species, except for close relatives to *C. elegans* such as *C. briggsae* (figure 4).

In yeast, the C-terminal sequence of Apn1 interacts with Pir1, which mediates translocation of Apn1 into the mitochondria [102]. Based on this, and since the long N-terminal region of APN-1 is found in other close relatives to *C. elegans*, we wished to test whether the additional sequence of APN1 might serve as a localization signal to target the protein to a specific subcellular compartment. In order to verify this hypothesis we attempted to construct a *C. elegans* transgenic line, over-expressing the N-terminal 118 amino acids tagged with GFP. We were hoping to follow the distribution of the N-terminal region of APN-1 and eventually attempt to identify factors that might be interacting with this portion of the protein. However, the injection of the plasmid expressing the N-terminal-GFP fusion was found to be toxic for the worms, and no transgenic lines were isolated even after the injection of very low plasmid concentrations (data not shown)(see discussion).

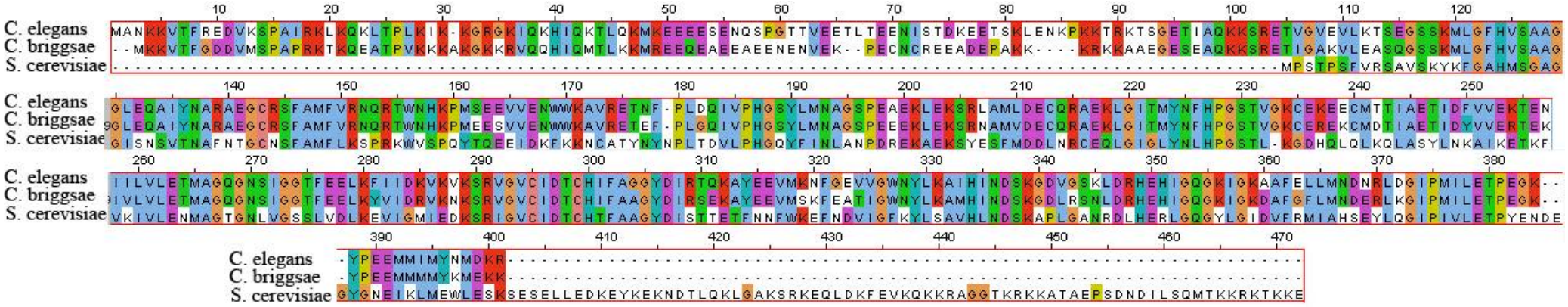


Figure 4. Alignment of the Endo IV family members of C. elegans, C. briggsae and S. cerevisiae. Protein sequences, obtained from the models' database were aligned using EMBL-EBI for sequence alignment.

3. Confirmation of knockdown by Q-RT PCR

The development of reverse genetics techniques presented ways for the easy assessment of gene function. In our study, we used RNAi knockdown to decrease the expression of the apn-1 gene and study the consequence of this decrease on different life aspects of the nematodes.

To determine whether our RNAi construct knocked down apn-1 message, we used real time and RT PCR to detect changes in mRNA levels in wild type animals, as it is thought that gene knockdown with RNAi in *C. elegans* acts through the degradation of the corresponding mRNA. Total RNA was extracted from the parental N2 worms obtained from either apn-1 knockdown or the RNAi control plates as described previously. The quality of the extracted RNA was verified by agarose gel electrophoresis (figure 5).

While RT- PCR experiments clearly indicated decreased levels of apn-1 mRNA as verified by 1.5% agarose gel (data not shown), it failed to give a quantitative assessment of the knockdown. For this purpose, q-RT-PCR reactions were conducted. The collected data clearly indicates a reduction of 60% of the level of the apn-1 message in the knockdown sample as compared to the control (figure 6). It is important to note here that the level of apn-1 knockdown was lower than the level of the control unc-22, a muscle gene typically used in RNAi experiments [185].

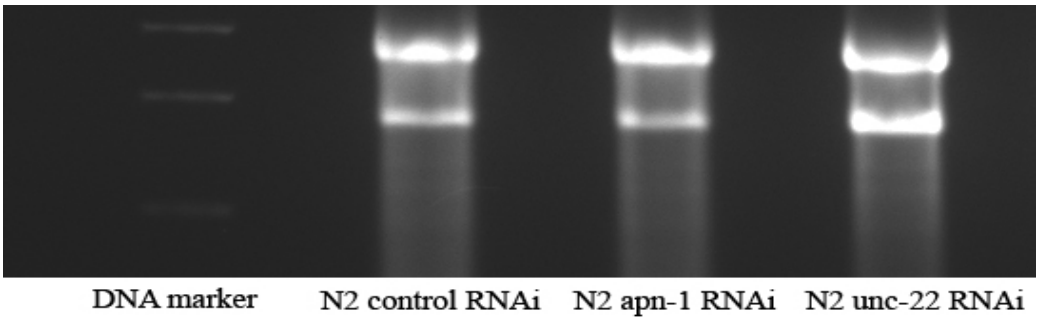


Figure 5. RNase free agarose gel revealing the quality of the extracted RNA samples. RNA extracts treated with DNase, were run on a 1% RNase free agarose gel and stained with ethidium bromide.

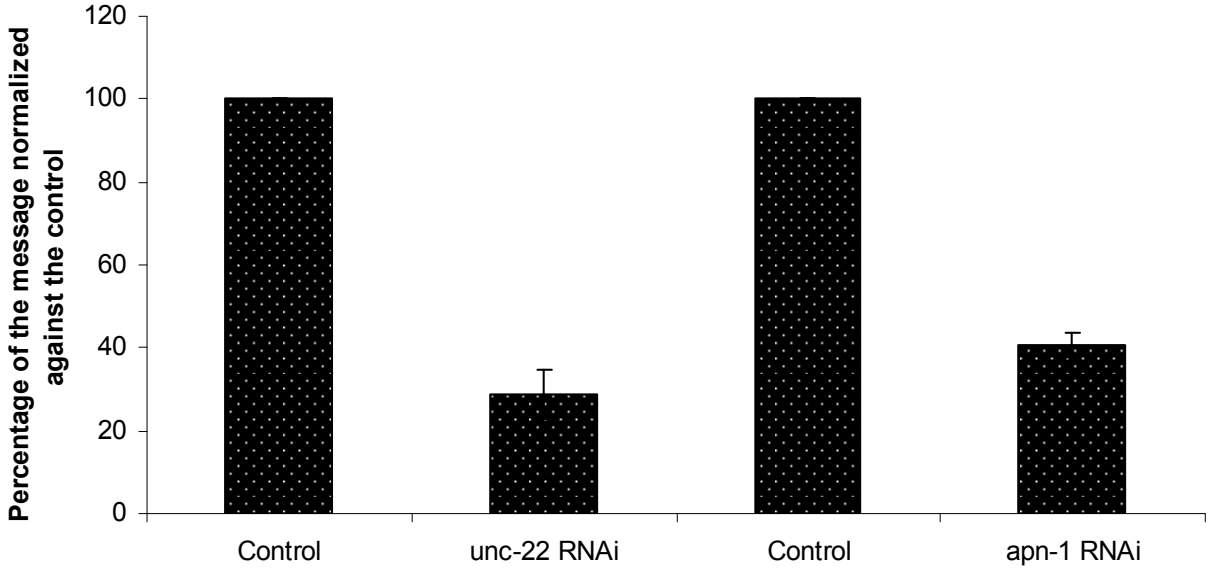


Figure 6. Percentage of the remaining unc-22 and apn-1 message following gene specific RNAi. unc-22 and apn-1 genes were knockdown separately through feeding (materials and methods). RNA was extracted and qRT-PCR experiments were conducted using standard protocols.

4. Confirmation of knockdown by Western Blot

The very low expression level of the APN1 protein in the parental *C. elegans* N2 strain and the absence of an appropriate anti-APN-1 antibody to detect it, led us to exploit the APN-1-GFP line to verify the efficiency of knockdown. For this purpose, increasing amounts of crude extracts from the transgenic line derived from the control and the apn-1 RNAi plates were run on a 10% SDS-polyacrylamide gel, transferred into a nitrocellulose membrane and probed with an anti-GFP monoclonal antibody. As shown in figure 6, the APN-1-GFP level clearly decreased following the RNAi treatment as compared to the control vector (figure 3 lanes 6-10 versus lanes 1-5).

5. apn-1 knockdown increases the frequency of spontaneous frameshift mutations

Since AP endonucleases have a direct role in maintaining the stability of the genome, we reasoned that knockdown of this gene could result in an increase in the mutation frequency. This is normally due to translesion synthesis that bypasses DNA lesion, by the possible incorporation of a wrong nucleotide, when DNA repair is compromised [45, 48]. To assess this, we used an established and sensitive β-galactosidase reporter assay that allows the detection of frameshift mutations resulting from small insertions and deletions in somatic cells of *C. elegans* [186]. The β-gal reporter is placed out of frame by a 17 A repeat inserted between the ATG and the gfp-LacZ open reading frame, and integrated in the *C. elegans* genome as a multicopy array to create the NL3400 strain [186]. A mutation that restores the reading frame of any single copy of the reporter can be visualized by β-galactosidase staining as a blue cell or a group of blue cells corresponding to a mutated cell that has since divided [186]. This assay has been used to identify several genes in *C. elegans* that affect genetic stability, such as the DNA mismatch repair gene *msh-6* [187].

Interestingly, increased blue coloring was observed in a population of apn-1 knockdown worms as compared to the control RNAi population, indicating an increased frequency of mutations (fig. 7). Furthermore, direct comparison of the apn-1 knockdown sample and the control sample under optical microscopy revealed ~ 5-fold increase in the mutation frequency following apn-1 knockdown. At least 85% of the knockdown worms contained mutations as compared to 18% in the control (figure 8). In addition, apn-1 knockdown worms often had multiple blue patches, indicating that multiple mutation events occurred in a single worm (figure 9 panel B).

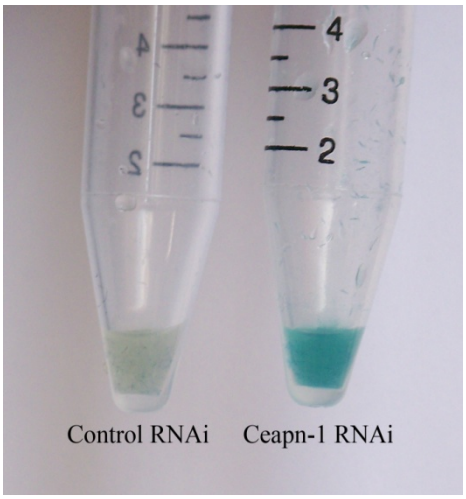


Figure 7. apn-1-RNAi worms exhibit elevated mutations as assessed by the β-galactosidase reporter gene. Control and apn-1 RNAi worms (4 days) were harvested and processed for β-activity (see materials and methods) and total worm populations were photographed by a digital camera.

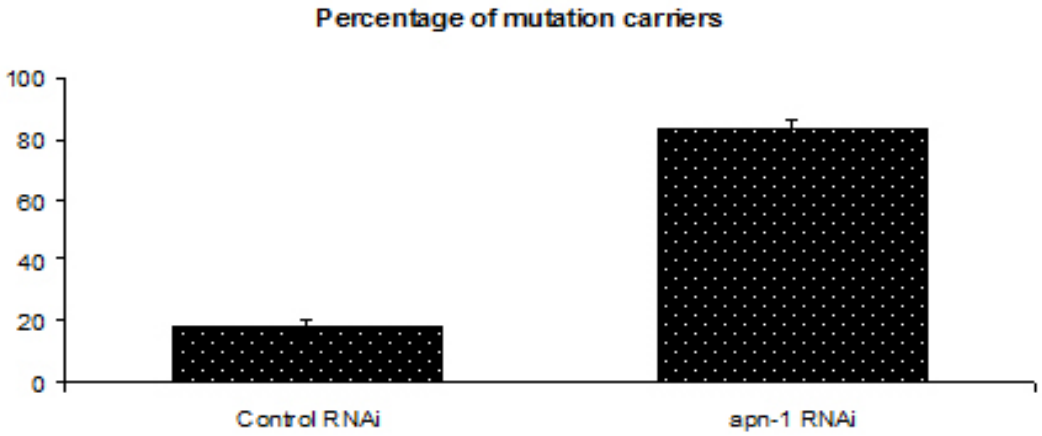


Figure 8. Percentage of worms with mutations in the 17A inserted adjacent to the ATG of the β-galactosidase gene. Control and apn-1 RNAi worms (4 days) were harvested and processed for β-gal activity before being visualized and counted under standard light microscopy.

Since aged worms were stained in both populations, the above results represent the staining of the young L1 and L2 worms (figure 9). Moreover, what was remarkably different between the two samples was the fact that eggs were almost exclusively stained when apn-1 gene was knockdown (figure 10 B marked by arrows). This data suggest that APN-1 is implicated in maintaining genomic integrity and might be particularly needed during early development.

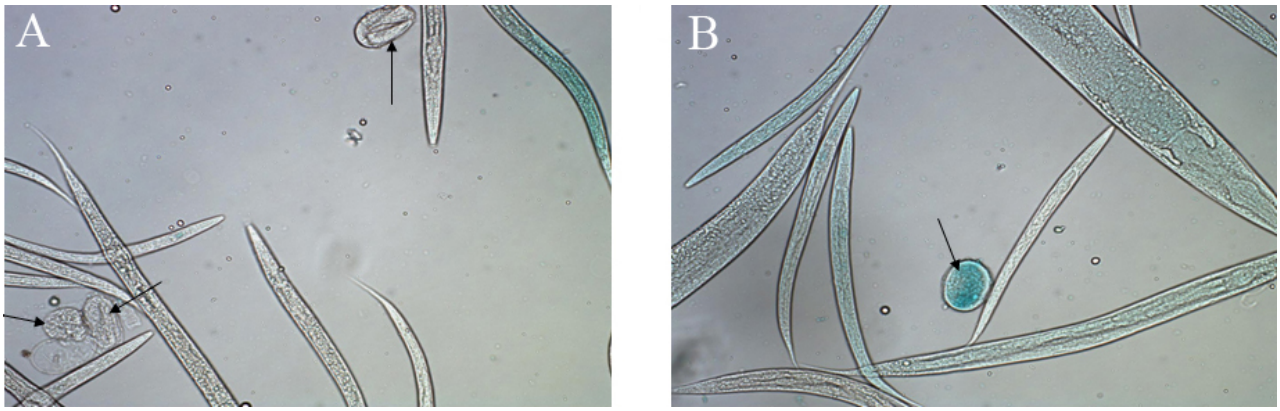


Figure 9. apn-1 RNAi worms exhibit elevated mutations as assessed by the β-gal reporter. Control and apn-1 RNAi worms (4days) were harvested and processed for β-gal activity (materials and methods). A) control RNAi and B) apn-1 RNAi worms photographed under a light microscope. The eggs are marked with black arrows.

Surprisingly the β-gal staining was mainly located in the gut and the central nervous system of the worm (fig. 10) suggesting that the cells of these tissues are more susceptible to mutations then other tissues when the DNA repair gene apn-1 is knocked down. This observation suggests a possible tissue specific distribution of the APN-1 protein in the worm or that APN-1 is more needed to preserve the DNA integrity specifically in these tissues.

Figure 10. The X-Gal staining is mainly localized to the gut and central nervous system of the worms.

6. Knockdown of apn-1 causes the accumulation of unhatched eggs

The results obtained from the mutation frequency assay pointed towards loss of genomic stability due to the decreased expression of an important DNA repair gene. We therefore anticipate that the elevated mutation frequency would present some consequences on the living organism. We observed unhatched eggs accumulating in the plates with the knockdown as compared to the control animals (fig. 11 A vs B). This observation coincides with exclusive β-galactosidase staining of eggs in knockdown plates as compared to the control.

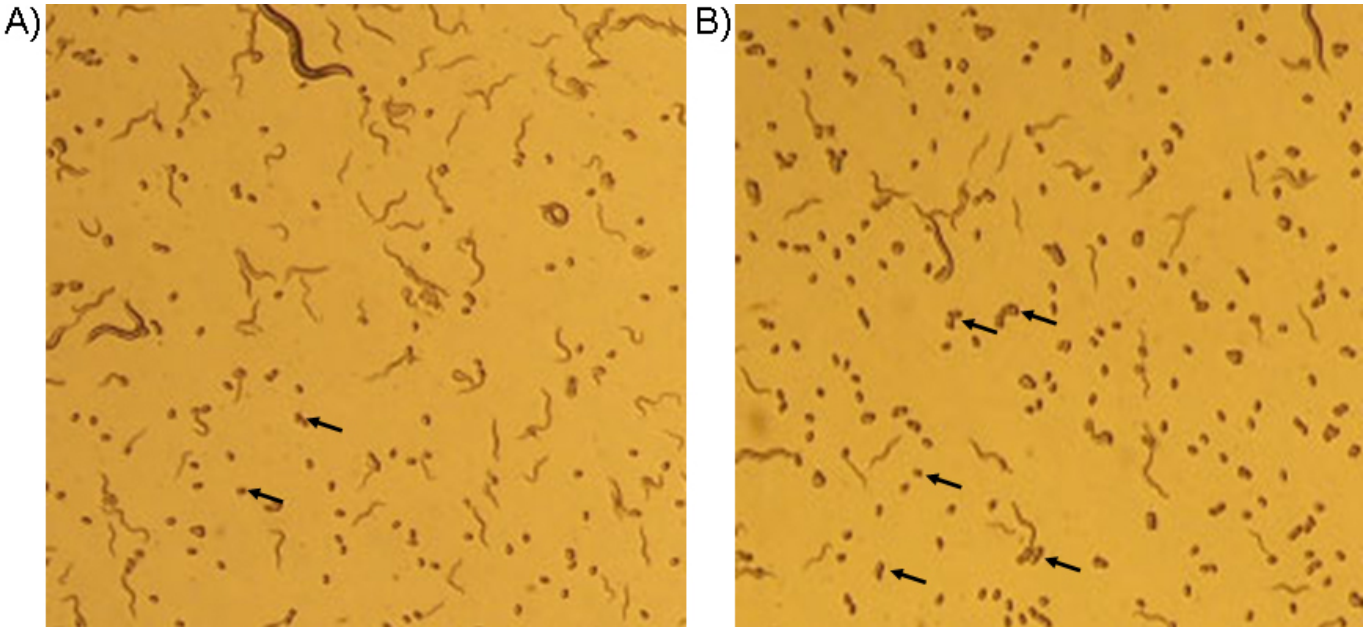


Figure 11. The knockdown of the apn-1 gene causes the accumulation of unhatched eggs. Control and apn-1 RNAi N2 worms were photographed (4-5 days after incubation at 16ºC) using a digital camera fixed on the binocular lens (microscope type). Unhatched eggs are marked with an arrow.

7. RNAi-apn-1 knockdown delays the development of the parent N2 strain and sensitizes worms to different DNA damaging agents

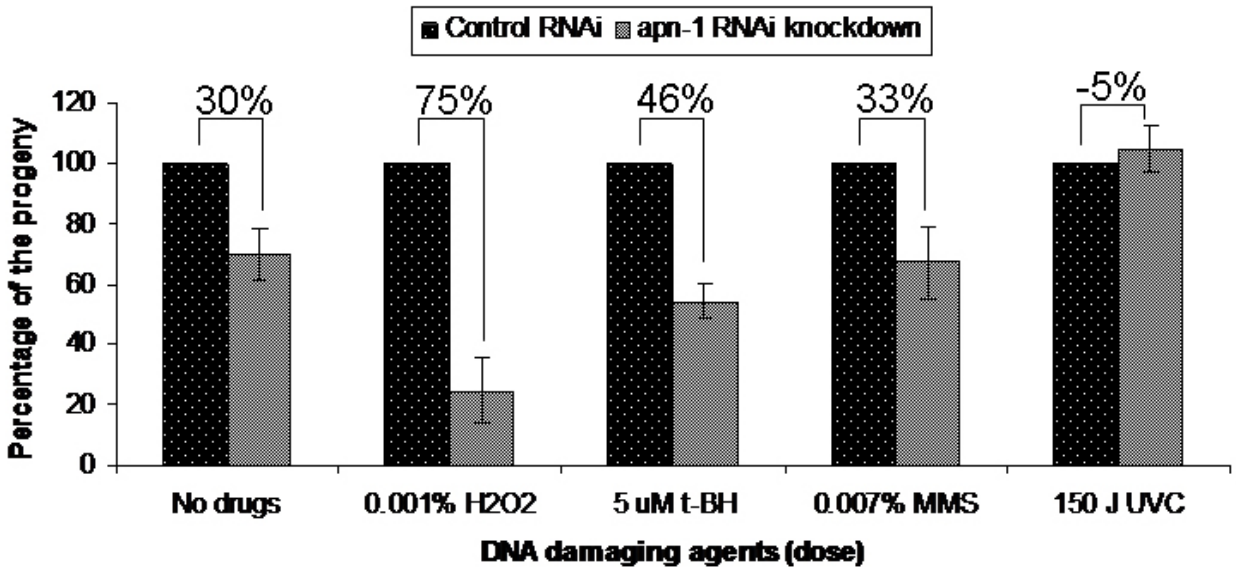
In order to further analyze the effect of the apn-1 knockdown on egg viability, a protocol was developed in our laboratory to measure the percentage of hatched eggs in the presence or absence of DNA damaging agents. Briefly, apn-1 knockdown and control worms were synchronized and three L4-adult worms were transferred to plates containing DNA damaging agents. After 12-15 hours, the adults were removed and the eggs were counted. The plates were then re-incubated before being scored at two different time points, namely 24 and 36 h. For UVC treatment, the L4 worms were irradiated directly on the plates followed by the same steps as above.

When no DNA damaging agents are used, apn-1 knockdown animals showed 30 $\pm$ 8% reduction of the population at 24 hours when compared to the control animals fed either the empty L4440 vector (Figure 12 panel A) or expressing a negative control RNAi that is RNAi-6G5 (data not shown). Interestingly, at 36 hours, the observed difference in the progeny size between the apn-1 knockdown and the control decreased from 30 to 10 $\pm$ 3% (figure 12 panel B). Further, incubation did not improve the progeny size, suggesting that RNAi-apn-1 knockdown diminishes the survival of a fraction of the progeny (data not shown).

When treating with DNA damaging agents, apn-1 knockdown animals were found to be sensitive to hydrogen peroxide (H_2O_2), tert-butylhydroperoxide (t-BH) and methyl methane sufonate (MMS), showing 75 $\pm$ 11, 46 $\pm$ 6 and 33 $\pm$ 12% decrease in the progeny size at 24 h, respectively, normalized to the control RNAi for each DNA damaging agent tested (figure 12 panel A). On the other hand, at 24h, the UVC treatment did not show any decrease in the progeny size between the control and the apn-1 animals (figure 12 panel A).

Similarly to what was observed in the absence of genotoxic stress, the observed gap in the progeny size between the control and the apn-1 knockdown was partially recovered after an additional 12 hours incubation of the plates when treated with H_2O_2 and t-BH. At 36 h, the observed gap in progeny size between control and apn-1 RNAi animals, following H_2O_2 and t-BH treatment reached 30 $\pm$ 8 and 25 $\pm$ 7 respectively (figure 12 panel B). Further incubations did not improve the progeny size (data not shown). Interestingly, the MMS-treated RNAi-apn-1 knockdown did not show any recovery showing 31 $\pm$ 11 % decreased in the progeny size (figure 12 panel B).

We interpret these data to indicate that RNAi-apn-1 knockdown is causing (i) a delay in the progeny size, which is substantially elevated by H_2O_2 treatment and to a lesser extent by tert-BH, but not by MMS, and (ii) death in a fraction of the population which is enhanced by H_2O_2, T-BH and MMS treatment, but not by UVC (see discussion). Thus, it would appear that APN-1 plays a more prominent role in protecting the animals from the genotoxic effects of certain types of oxidized DNA lesions, as opposed to alkylation DNA damage and pyrimidine dimers. In fact, it is somewhat surprising that the RNAi-apn-1 knockdown animals were no more sensitive to MMS than the untreated control during the first 24 h of exposure.



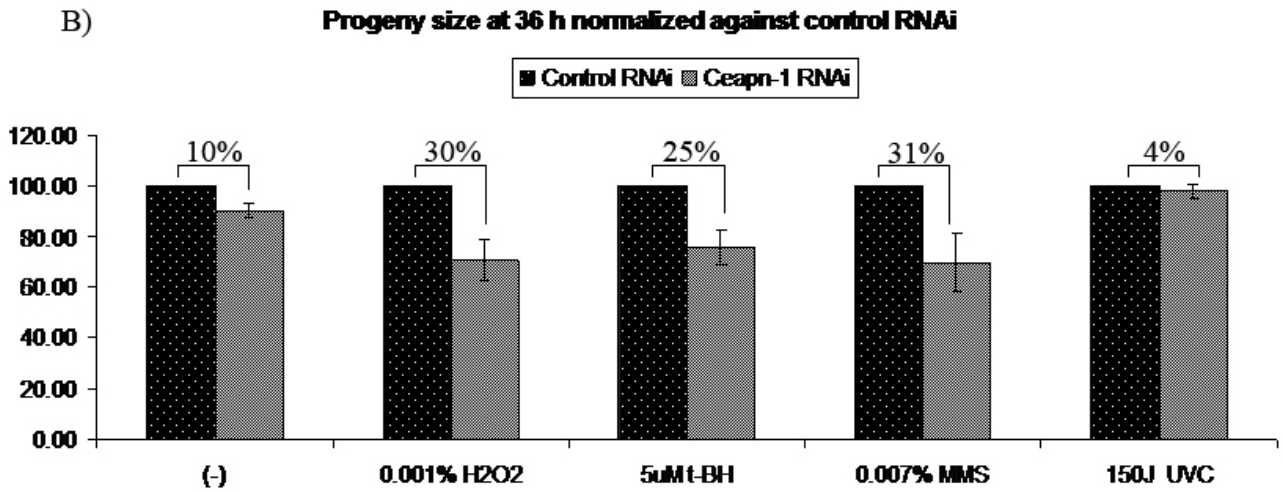


Figure 12. Spontaneous and drug-induced levels of mutations affect progeny size in apn-1 RNAi animals. Briefly, control and apn-1 RNAi treated adult worms (2-3) were transferred in quadruplicates onto plates without and with the indicated damaging agents. 12 to 15 hours after incubation, the adults were removed and the embryos were counted. The plates were reincubated before being scored at 2 time points: 24 and 36 hours. A) percentage of hatched eggs at 24 hours, B) percentage of hatched eggs at 36 hours. Percentage of hatching of the apn-1 RNAi in A) and B) were normalized against control RNAi.

8. RNAi-apn-1 knockdown delays cell cycle progression

We next examined the cell cycle progression of individual single cell embryos derived from the control and apn-1 knockdown animals using time-lapse microscopy. For this experiment, and for each treatment, we checked a total of 8 single cell embryos for the length of time for cell division from 2 to 4 cells to occur. The wild type single cell embryo, termed P0, divides asymmetrically into a larger anterior AB cell and a smaller posterior P1 cell. The AB cell then divides into an the anterior daughter ABa cell and the posterior daughter cell Abp, while ~ 2 min later, the P1 cell divides into P2 and EMS cells. The observed gap between the P1 and the AB divisions is partially due to the presence of checkpoint signaling in the P1 cell while it is believed to be silenced in the AB cell. Following apn-1

knockdown, we observed that the posterior P1 cell delayed by 38.8 ± 0.6 sec when compared to the control RNAi, whereas the anterior AB cell did not show any significant delay (figure 13 and data not shown). One possible explanation for the P1 delay could be the results of the accumulation of unrepaired spontaneous lesions in the apn-1 knockdown. Even though preliminary, treatment with t-BH led to an increased delay in P1 division. This delay reached 105 ± 48.3 sec between the control and the knockdown embryos, while once again no significant delay was detected in the AB cell division (figure 13 and data not shown). The dose of UVC used led to a delay in the cell cycle progression in the control as well as the apn-1 RNAi samples. However there was no apparent delay in either P1 or AB cell division between the control to apn-1 knockdown, indicating that the observed delay is only due to damages that are repaired by apn-1 (figure 13).

The collection of data suggests that the mutations observed as a result of apn-1 knockdown and the hatching delay caused upon exposure to the DNA damaging agents might be due to an arrest in cell cycle progression. We predict that apn-1 knockdown would promote the action of alternative mechanisms such as error prone translesion pathway to bypass the damage before allowing completion of the cell division.

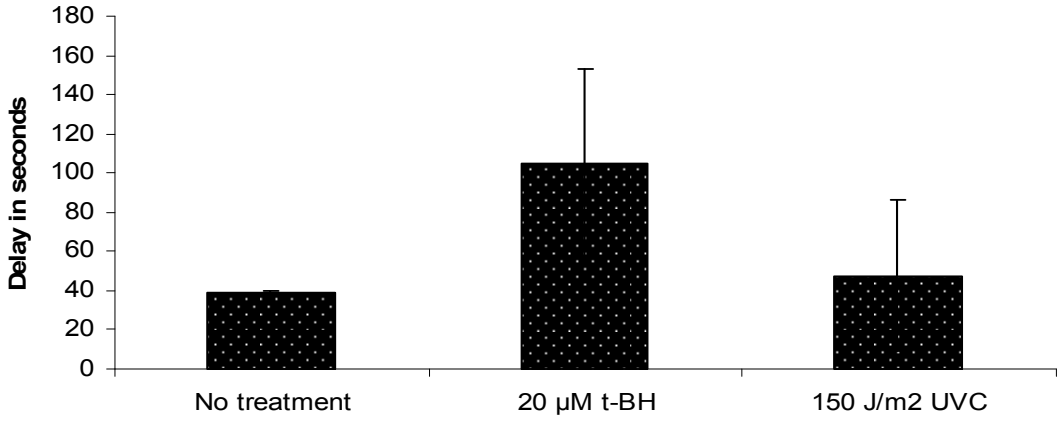


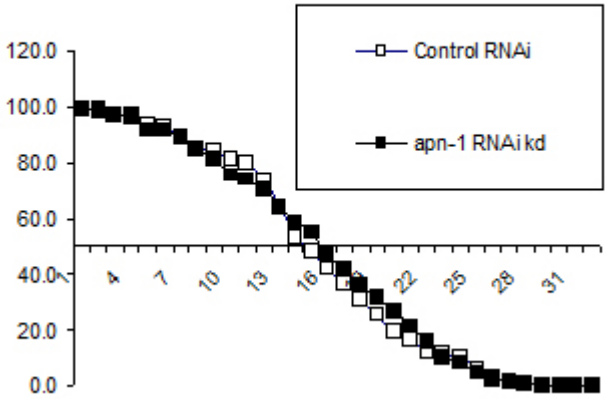
Figure 13. Cell cycle delay of embryonic P1 cell following apn-1 knockdown. Worms were dissected to extract the embryos which were monitored throughout the first 3 cell divisions using time-laps microscopy. The time interval between the 2 and 4 cell embryo was recorded.

9. Knockdown of apn-1 decreases lifespan in the presence of agents that cause AP sites

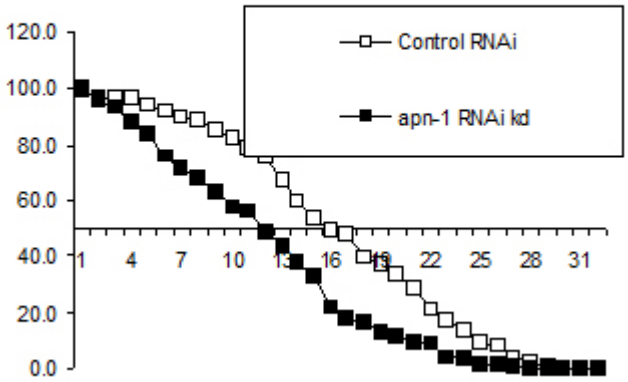
The fact that the knockdown of apn-1 decreased the percentage of hatched eggs raised the question of whether it could have an effect on life span of the worm. To answer this question, a longevity assay was conducted on worm cultures in the presence and absence of multiple DNA damaging agents as described in the materials and methods. The agents chosen for this study were t-BH (final concentration 20 μM), MMS (final concentration 0.07%), 4NQO (final concentration 1 μg/ml) and UVC (final dose 150J/m^2).

Interestingly, the knockdown of the DNA repair gene apn-1 alone was not found to have any effect on the overall longevity of the worms as 50% of the population died off in ~ 16 days in both control and apn-1 RNAi populations (fig 14 A). However, the addition of DNA damaging agents that are known to create AP sites, such as t-BH and MMS were found to slightly decrease the overall longevity of the apn-1 knockdown worms, with 50% of the population dying in 11 and 14 days respectively, while the vector control fed animals showed a 50% decrease in the population within 16 and 17 days respectively (figure 14 panels B and C). By contrast, the longevity of the worms was not found to be altered by the knockdown of apn-1, when the worms were challenged with agents that do not create DNA damages that are repaired by the AP endonuclease enzymes, such as 4-NQO and UVC, with 50% of the population dying at 15 and 11 days respectively Figure 14 D and E). These results further confirmed the importance of APN-1 in counteracting the deleterious effect of DNA damages and pointed once again to its primary importance during early embryogenesis as no decrease in longevity was observed upon apn-1 knockdown alone.

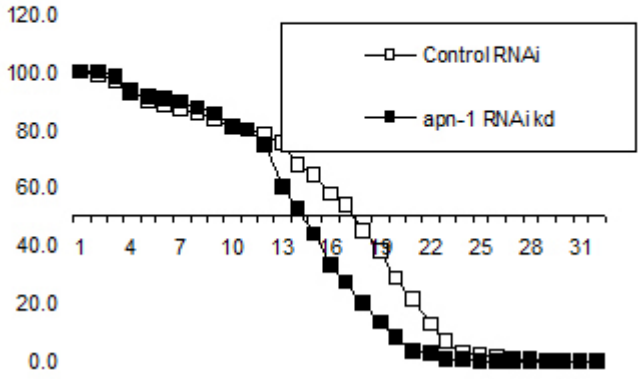
A) Longevity under no drug treatment



B) Longevity under 20 uM t-BH treatment



C) Longevity under 0.01 % MMS treatment



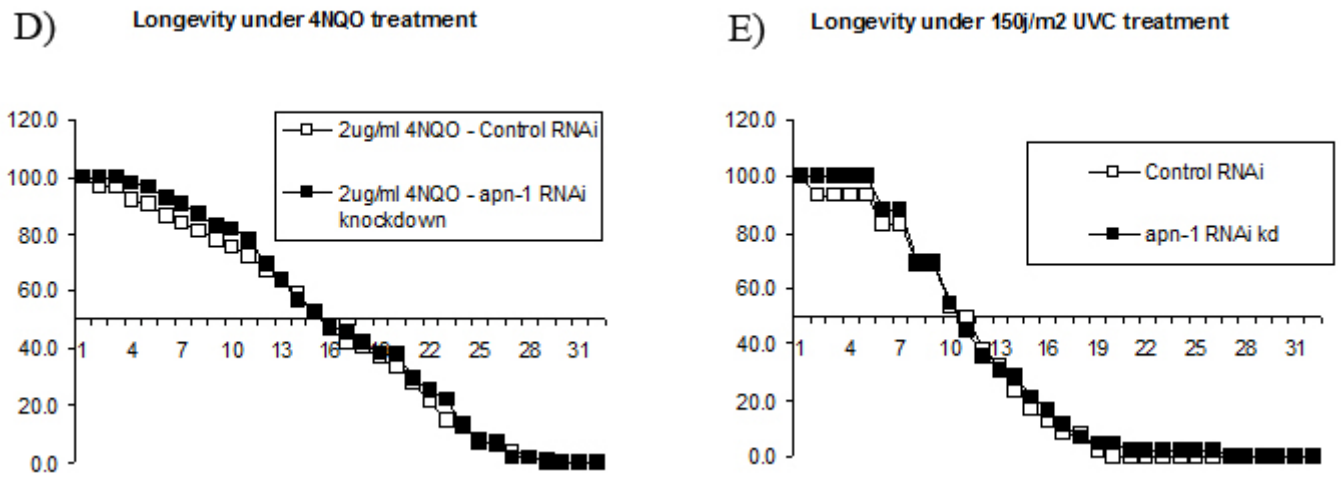


Figure 14. apn-1 RNAi worms display shorten life-span when exposed to t-BH and MMS, and not to 4-NQO or UVC. Briefly, control and apn-1 RNAi (12 worms each) were transferred in quadruplicates onto plates without and with the indicated DNA damaging agents (t-BH, MMS, and 4-NQO) and containing a line of the respective feeding bacteria. For UVC, worms were irradiated on the feeding plate with a single dose. Worms were transferred onto fresh drug plates on day 3 and day 7. The data is representative of three independent analyses.

Chapter 4 – Discussion

To date, the first and only multicellular organism where members of the two AP endonuclease families, Endo IV and Exo III, were identified is *C. elegans*. Despite the high structural homology that APN-1 and EXO-3 present when compared to their *E. coli* and *S. cerevisiae* homologues, all previous studies aiming at elucidating their role, were done in the context of ectopic expression in yeast, and not in *C. elegans* itself.

In the present study, we have investigated the *in vivo* function of one of these 2 genes, i.e. apn-1, using gene specific RNAi as a tool and the natural host as the biological model. Here, we report strong evidence implicating APN-1 in the repair of DNA damage *in vivo* as it was found essential in preserving the genomic integrity of *C. elegans*. This is supported by the observations that knockdown of apn-1 expression (i) substantially elevated the level of spontaneous mutations as determined by using a well-defined GFP::lacZ reporter [186], and (ii) caused sensitivity to a subset of genotoxic agents that are known to produce DNA lesions that require processing by AP endonucleases and 3'-diesterases [41]. This is entirely consistent with our previous study showing that expression of APN-1 in yeast produced a protein with both AP endonuclease and 3'-diesterase activities [178]. While we showed by quantitative PCR that apn-1 RNAi decreased the gene expression, and expected a corresponding decrease in APN-1 enzymatic activities, these activities were difficult to detect in total extracts derived from parental *C. elegans*. In fact, the AP endonuclease activity of APN-1 can only be detected if instead the total extract was derived from transgenic animals carrying additional copies of the apn-1 gene, suggesting that the APN-1 protein is weakly expressed, but likely bears high specificity towards DNA lesions.

1. Genome-wide screen for the identification of genes involved in the maintenance of DNA integrity failed to identify apn-1

Although we were able to clearly show increased mutagenesis in *C. elegans* following apn-1 knockdown, a previous genome wide screen for genes implicated in protecting the *C. elegans* genome against mutations, failed to identify apn-1 as an important contributor to genomic stability, with both studies using β-galactosidase assay to assess mutation frequency [187]. Thus, it seems that for an unknown reason, some genes have eluded the screen and were not picked up. One possibility that could explain the absence of apn-1 in the screen is the difference in the feeding conditions that were used in the two cases with the knockdown in our study performed at 16ºC and the genome-wide screen at 20ºC [187]. This might have led to an ineffective knockdown of apn-1 in the genome wide screen, as it has been previously reported that the knockdown effectiveness of a limited number of genes is somehow affected by temperature, whereas the knockdown of the rest of the genome is not [180]. Accordingly, the consequence of temperature on the effectiveness of apn-1 knockdown needs to be addressed in future experiments. Another possibility that could explain the obtained contradicting results is the protocol used for the β-galactosidase assay. Taking into consideration that the out of frame β-galactosidase genes are under the control of the heat chock hsp16 promoter in the NL3400 strain, we heat shocked culture plates for 2 hours at 33 ºC prior to collecting the worms in order to increase the expression of the β-galactosidase genes that were placed back into frame due to mutations. This induction step, however, was not done in the genome-wide screen, thus, suggesting that apn-1 might be a false negative in the latter study [187].

2. APN-1 function seems essential for *C. elegans* embryogenesis

In our study, we have showed that knockdown of apn-1 alone does not decrease the longevity of the studied nematodes, whereas it was found to decrease the viability of the embryos as shown in the egg hatching assay suggesting that APN-1 function is more required during the embryonic development of the nematode than it is during later developmental stages of the worm. In fact, it was shown in a previous study, using microarray technology, that apn-1 expression is highly enriched in *C. elegans* germline, suggesting a basic role of APN-1 during meiosis [188]. Taking into consideration that a high proportion of the genes that are enriched in the germline present an important role during embryogenesis [189], we propose that apn-1 is most probably important for the proper progression of embryonic development. This might suggest a similar enrichment in the expression level of apn-1 during embryogenesis as compared to later stages of the nematode's development. If this is the case, one would expect high levels of mutations in the embryos and young juvenile as compared to more aged worms, which is the case as shown in the β-galactosidase assay. Furthermore, it would be logical to assume that APN-1 is more needed during the embryonic development of the worm as it constitutes the phase with the most cellular divisions.

The relatively low numbers of blue stained young adult when compared to embryos and juveniles might be due to a lethality caused by the accumulated mutations from early embryogenesis till adulthood. Moreover, those that show normal survival and growth, might be either refractive for knockdown for a reason that is not very well understood, or did not accumulated enough mutations that lead to cell death. In either way, this data strongly suggest a more prominent role for APN-1 during the early development of *C. elegans*, and thus could suggest a similar expression of the APN-1 homologue in humans if ever one do exist.

3. Possible redundancy between APN-1 and EXO-3

Although we were able to identify some phenotypes associated with the elevated mutation frequency resulting from apn-1 knockdown, the level of the observed sensitivity was rather slim, suggesting that the spontaneous or drug induced damages were still repaired by some mean.

On one hand, we have revealed by qRT-PCR that the knockdown of apn-1 through feeding, while using the optimal conditions as described previously [180], resulted in a decrease in the apn-1 message by 60%. Considering that a corresponding decrease in the APN-1 level occurs following the knockdown, a 40% residual activity will still be present in the cells, and might account for the partial repair of the inflicted DNA damage, resulting in a weak phenotype, similarly to what was previously reported with yeast [101].

On the other hand, the presence of a redundant protein that can substitute for the absence of APN-1 could also explain the observed weak phenotype. Indeed, similarly to *E. coli* and *S. cerevisiae*, the *C. elegans* genome was found to contain a second AP endonuclease, EXO-3, homologue of *E. coli* exonuclease III. In fact, it has been well documented previously that, although the families have certain substrate preference, they can complement the absence of one another [178]. This was exemplified by the fact that yeast apn1Δ cells are not very sensitive to AP site-creating DNA damaging agents unless apn2 is also deleted [70]. Furthermore, multiple studies have revealed the predominance of one family over the other depending on the studied model system [41]. Whereas exonuclease III constitutes the major AP endonuclease enzyme in *E. coli*, the Endo IV family member Apn1 was found to be major AP endonuclease in *S. cerevisiae* [41]. Thus, *C. elegans* EXO-3 likely provides a second mechanism for repairing AP sites.

The inability of EXO-3 to fully complement the absence of APN-1 can be mainly explained as a difference in the substrate recognition specificity between the two enzymes rather than being related to the predominance of APN-1

over EXO-3. This is supported by the identification of EXO-3 as an important enzyme for the maintenance of genomic stability in the genome wide screen described earlier [187]. Furthermore, nothing is known so far regarding the contribution of each of the two families of AP endonuclease enzymes in *C. elegans*.

4. APN-1 is essential for the repair of H2O2 induced damage

In *E. coli* or yeast, the deficiency of endo IV or Apn1, respectively, showed no striking sensitivity to the chemical oxidant H_2O_2 [104]. Interestingly, in *C. elegans* the knockdown of apn-1 caused significant sensitivity to this agent. We reasoned that since *C. elegans* lacks the DNA glycosylase OGG1 that excises, for example, the H_2O_2-induced oxidized base damage 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dGuo), then removal of such lesion could depend upon the nucleotide incision repair activity of APN-1 (Yang et al., in preparation), and/or the 3' to 5'-exonuclease activity of APN-1 might be required to prevent the misincorporation of 8-oxo-dGuo from the oxidized dNTP pool into the DNA as we have previously shown for yeast Apn1 [98]. In addition, *C. elegans* genome also lacks another key enzyme Tpp1/PNKP of the BER pathway, which in yeast (Tpp1) or human (PNKP) functions to remove 3'-phosphate left at DNA single strand breaks following exposure to H_2O_2 [190]. Thus, removal of H_2O_2-induced 3'-phosphate would rely on the 3'-diesterase activity of APN-1.

5. Overexpression of apn-1 is toxic

To study the localization of APN-1 in the worm, we attempted to construct a transgenic *C. elegans* line that overexpresses APN-1-GFP fusion

protein. This is usually done through the injection of a plasmid expressing the fusion protein along with another expressing a mutated rol-6 gene that is used as a transformation maker. Since the used plasmids possess similar DNA backbones, they will create extrachromosomal arrays after injection through homologous recombination [191]. The isolated transformed lines showing the transformation maker will almost always contain the co-injected sequence in the formed array [149]. These arrays will express the two proteins at a level that is dependent on the concentration at which the plasmids were initially injected [149]. The transmission of the array from parent to progeny indicates successful line formation. In most cases the plasmid expressing the desired protein is injected at a concentration of 100 ng/µl whereas the transformation marker is injected at a concentration of 50 ng/µl [149]. Surprisingly, injection of the APN-1-GFP plasmid at any concentration higher than 1 ng/µl failed to produce stable lines. This observation might be due to the fact that Apn1 overexpression is either toxic to the worm or interferes with the array formation after injection.

In the first case, the massive conversion of primary damages, such as AP sites, that are highly mutagenic, but not very lethal, into a more toxic and/or mutagenic DNA repair intermediate, such as single strand breaks, might be at the origin of the observed toxicity. Similar switch to more toxic intermediates have also been previously reported in yeast where deletion of mag1 rescued the MMS sensitivity of an apn1 apn2 null strain by preventing 3-Methyladenine conversion to AP sites [192]. Furthermore, *C. elegans* APN-1 was found to take part in the NIR pathway (manuscript in preparation), thus allowing the direct conversion of oxidized bases into the highly toxic single strand break that will block DNA replication and may lead to apoptosis.

In the second case, the 3'→5' exonuclease activity of APN-1 might be removing the 3'-overhangs necessary for the invasion phase of homologous recombination, thus preventing the array formation. This is true only if APN-1-GFP plasmid start being expressed right after the injection.

Interestingly, the APN-1-GFP overexpression line that we were able to isolate after the injection of the APN-1-GFP plasmid at a concentration of 1ng/µl, showed a high incidence of males and a short life span. A high incidence of males may indicate genomic instability, as it normally results from meiotic non-disjunction of the sexual X chromosomes. When this occurs the resulting gamete that is void of the X chromosome gives rise to a male after fecundation. Thus, the high incidence of male observed might be explained by the fact that the high concentration of the APN-1 protein is creating many DNA lesions, leading to chromosomal fusion, re-arrangement as well as segregation defects, fusing in some cases the X chromosomes and leading to the production of males. If that is the case, the increased DNA lesions might also explain the sickness and the short life span observed in this line.

The surprising observation that overexpression of the N-terminal 118 amino-acids of APN-1 in *C. elegans* is toxic suggests that the toxicity observed when the plasmid expressing the full length APN-1-GFP fusion protein was injected at relatively high concentrations might also be due to the N-terminal portion and not the actual nuclease activity of the protein. Since this N-terminal part of the protein might be involved in the localization of the protein, its overexpression might lead to its accumulation in massive quantities in the nucleus and/or mitochondria, thus blocking the normal functioning of these two cellular compartments and leading to lethality. On the other hand, the over-expressed N-terminal portion of the protein might be causing a dominant negative effect by blocking, for example, the appropriate binding of the endogenous protein to the damage site on the DNA.

To try and solve this dilemma, we are presently preparing a transgenic line over-expressing the APN-1-GFP protein lacking the N-terminal sequence. The successful isolation of a transgenic line using high concentration of injected plasmid might confirm that the previously observed toxicity is due to the N-terminal sequence. However, the simple isolation of a transgenic line will not necessarily mean that the N-terminal portion is the only toxic part. In fact, another

possibility suggests that if the N-terminal region is a localization signal, then the protein might accumulate in the cytoplasm instead of being in the nucleus and/or mitochondria to cause the toxicity. Thus, additional experiments including immunostaining and GFP visualization will be needed to verify that the active part of the protein is still accessing the DNA.

6. The *C. elegans* RNAi hypersensitive rrf-3 strain behave similarly to the parental N2 strain

It has been previously shown that gene knockdown through RNAi is not effective for the genes expressed in the central nervous system [193]. As discussed earlier, we have found that the APN-1 protein is mainly localized in the gut of the worm. However, we have also noticed that the protein might be expressed in the central nervous system where it is refractory for knockdown through RNAi. For this purpose, we have assayed the *C. elegans* RNAi hypersensitive rrf-3 strain for sensitivity to multiple DNA damaging agents mainly through the egg hatching assay in the hope of conforming or rejecting the previously established observation. The main advantage of this strain is that it provides an effective knockdown in the central nervous system resulting in an increased sensitivity to certain DNA damaging agents if the APN-1 is also expressed in this tissue [193]. Surprisingly, the results obtained with the rrf-3 strain were closely comparable to those obtained with the parent N2 strain suggesting that the APN-1 protein is most probably not or weakly expressed in the central nervous system. Moreover, the observed difference between the N2 and the rrf-3 strains is due to a probable more effective knockdown when using the hypersensitive strain. However, the discussed results are rather preliminary and thus require further investigation to be confirmed.

7. Future plans

Despite the successful establishment of a biological role for APN-1 throughout the course of this study, more detailed aspects of the involvement of this enzyme in DNA repair still needs to be investigated.

7.1. Mutational spectrum resulting from apn-1 knockdown

The results obtained in the beta-galactosidase assay clearly indicated an increase in the mutation frequency but failed to provide an insight on the mutational spectrum that is occurring in the cells as a result of the apn-1 knockdown. In fact, the *C. elegans* NL3400 strain highly expresses an out of frame beta-galactosidase gene that can be put back into frame through unrepaired damages to produce a functional protein. The only mutations that allow this transition are frame shifts that occur through base addition or deletion. Base substitution on the other hand does not change the coding frame and thus cannot attribute to the observed increase in the mutation frequency.

To better understand the types of mutations that are normally prevented by the action of the apn-1 gene *in vivo,* it would be essential to get a better understanding of the mutation spectrum that is occurring in the genome. For this purpose we have chosen a well described *C. elegans* strain, the SP457 that possess an unc-93 (e1500) III mutation [187, 194]. Worms possessing an e1500 mutation show an abnormal muscle structure/function rendering the worms almost paralyzed and partially egg laying defective [194]. In fact it has been shown that the product of the e1500 allele is toxic and that this toxicity is abolished following mutations that generates null unc-93 gene [194]. Thus, any unrepaired mutation occurring in the unc-93 gene has the potential to allow the reversion of the SP457

worms to the wild type phenotype [187]. Revertants will then be selected and grown. The DNA will then be extracted from the population of revertants and the unc-93 gene will be sequenced to reveal the type of occurred mutations.

7.2. Study of the contribution of the two families of AP endonuclease/3'-diesterase in *C. elegans*

One of the missing chapters of the presented work is the detailed contribution of each of the two AP endonuclease enzymes present in the nematode. To investigate this, we are planning to knockdown exo-3 and perform a similar set of experiments as with apn-1 knockdown to investigate the implication of the EXO-3 enzyme in the BER pathway and in repairing specific types of lesions caused by specific DNA damaging agents. We expect to get a similar if not a higher sensitivity when exo-3 is knockdown as compared to apn-1, thus explaining the resistance observed in the present study. However, we also anticipate finding a certain preference of each of the two enzymes to repair specific types of lesions, such as seen when these enzymes were used in cross-species complementation assays [178].

To further understand the contribution of each of the two enzymes to DNA repair, we are planning to perform a double knockdown of both apn-1 and exo-3 genes and then study the resulting effect on the animals. This double knockdown is expected to produce a much higher sensitivity if not lethality if the knockdown is very effective, as the worms will be left with little defense against DNA damage.

An alternative and better way to achieve this goal is by creating knockout animals for each of the two genes. This will prevent complicated interpretation of possibly weak phenotypes that are due to the residual activity that is left following the knockdown. Then the knockout worms will be knocked down for the second enzymes and a similar set of experiments will be conducted to study the resulting

phenotype. However, it might be relatively expensive and hard to obtain knockouts for these genes as the knockout might be lethal, or the resulting animals might be extremely sick.

7.3. In depth Cell cycle arrest studies following apn-1 knockdown

The cell cycle progression delay that we observed in the P1 cell following apn-1 knockdown was significant and explained the observed delay in the egg hatching experiments. However, the exact mechanisms behind this delay are still unclear. Previous studies pointed out that replication stress leads to a delay in the S phase of the first cell cycles of a *C. elegans* embryo [195]. This delay was found to depend on the DNA replication checkpoint genes, notably atl-1 and chk-1, encoding the homologues of mammalian ATR and Chk1 kinases, respectively [195].

In order to further confirm our data, we propose to perform double knockdown experiments in which we decrease the expression of both apn-1 and one of the two checkpoint proteins. This will allow us to investigate whether the observed delay is indeed caused by checkpoint arrest triggered by the inflicted DNA damage that is increased due to apn-1 absence.

8. Conclusion

Members of the endo IV family, including *C. elegans* APN-1, possess multiple DNA repair activities that encompass AP endonuclease, 3'-diesterase, nucleotide incision and 3' to 5'-exonuclease allowing this class of enzymes to act on various DNA lesions [72]. As such, it seems logical that *C. elegans* has evolved to retain this versatile enzyme, particularly since it lacks a full

complement of the BER enzymes, as we noticed while searching the database (e.g., OGG1 and Tpp1/PNKP), as compare to either yeast or mammalian cells. Therefore, under normal aerobic condition, the knockdown of APN-1 is expected to cause the accumulation of a variety of oxidative DNA lesions that include AP sites, base damage, and single strand breaks with blocked 3'-termini. These lesions if not repaired efficiently are expected to cause cell cycle delay, increase mutations, as well as giving rise to more toxic lesions such as DNA double strand breaks that could lead to cell death. On the basis of our findings, we anticipate that deleting the apn-1 gene would result in mutant animal displaying dramatic phenotypes and may even be lethal.

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