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Elucidating Mechanisms of Base Excision Repair and Genetic Instability in *Saccharomyces cerevisiae*

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Abstract

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A large subset of DNA damage acquired by cells is repaired by the base excision repair (BER) pathway. Though defects in many BER genes have been associated with neurodegenerative diseases and cancer, the molecular basis for such associations is not well understood. Further, when cells cannot repair oxidative DNA lesions normally targeted by BER, large-scale genome destabilization can occur. The major goals of the studies presented here are to better understand BER mechanisms at the level of individual proteins and on the genome-wide level. We employed *Saccharomyces cerevisiae* because the biochemical steps of BER are highly conserved, and *S. cerevisiae* is a well developed model for DNA repair studies. AP endonucleases play a central role in the repair of DNA damage through the BER pathway, thus our studies focus on the major yeast AP endonuclease, Apn1, to better understand how BER protects cells against genomic instability, an important characteristic of cancer.

In an unbiased, forward genetic screen to identify mutations in *APN1* that impair cellular DNA repair capacity we identified and characterized variant Apn1 V156E, which was predicted to decrease catalytic function based on homology modeling. We found that, unlike wild type Apn1, the V156E is targeted for degradation by a proteasome-independent mechanism, leading to decreased steady-state levels. Inducing transcription of *APN1-V156E* using a regulatable promoter restored protein to levels comparable to wild type Apn1 and functionally restored DNA repair capacity. Thus, the V156 residue plays a critical role in maintaining Apn1 protein levels and normal levels of repair independent of catalytic function.

In genome-wide chromatin immunoprecipitation studies aimed at exploring the relationship between DNA damage repair and genomic instability using Apn1 as the target protein, we found that the level of oxidative stress dictates the distribution of Apn1 across the genome. Regardless of oxidative stress level, Apn1 binding sites are enriched for C and G nucleotides, suggesting that Apn1 targets particular regions in a base content-specific manner. These results have implications for understanding how the genomic distribution of DNA repair activities preserves genome integrity and for understanding how defects in the major human AP endonuclease may contribute to disease.

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CHAPTER 1

GENERAL INTRODUCTION

1. DNA Damage

Cellular DNA constantly incurs damage due to persistent encounters with genotoxic agents [1]. The base, sugar and phosphate components are all subject to attack (Fig.1), and the types of damages that result include base loss, base lesions that are either small or bulky, damage to the sugar-phosphate backbone including single-strand breaks and double-strand breaks, and inter- and intrastrand crosslinks. It is important to note that there is much overlap between the types of damage caused by the different classes of DNA damaging agents. For example, while ultraviolet radiation and ionizing radiation cause both base damage and strand breaks, they can also react with water molecules within the cell to produce reactive species that can then cause oxidative damage to the DNA [2]. In fact, many DNA damaging agents, regardless of the class, can also induce increased levels of intracellur reactive oxidants. For the present discussion, types of DNA damaging agents will be divided into different classes, and endogenous and exogenous sources as well as the types of damaged caused by the different agents will be discussed.

1.1. Oxidative DNA Damage

As an inevitable consequence of aerobic metabolism, reactive oxygen species (ROS) are continuously generated within cells and can damage proteins, lipids, carbohydrates and nucleic acids. For many years, the intracellular generation of ROS was recognized as merely a by-product of important cellular processes, such as the leakage of electrons from the mitochondrial electron transport chain and the subsequent reactions of electrons with oxygen in the cell to produce oxygen radicals [3]. ROS were mainly appreciated for their pathogenic effects. Upon the discovery that a free radical species called nitric oxide could be produced within the cell, and that it played important

physiological roles in vascular endothelial cells [4], there was a major paradigm shift in the field [5], and it is now appreciated that cells utilize ROS, in addition to reactive nitrogen species [6], as signaling molecules in a wide variety of cellular processes. Therefore, oxygen is a reactive molecule that is essential for life, but can become detrimental for the cell if not properly regulated.

As the name indicates, reactive oxygen species (ROS) are chemically reactive oxygen-containing molecules including radical species and non-radical species. ROS act as signaling molecules through their reactivity with target proteins, leading to post-translational modification to affect protein activity. Physiologically relevant ROS include the non-radical hydrogen peroxide (H_2O_2), superoxide ($^{\cdot}O_2^-$), and the hydroxyl radical ($^{\cdot}OH$). With respect to their role as signaling molecules H_2O_2 mainly modifies cysteine residues, $^{\cdot}O_2^-$ mainly modifies iron-sulfur clusters in proteins, and $^{\cdot}OH$ modifies proteins indiscriminately [7]. The mechanism by which ROS damage genomic DNA is thought to occur within the nucleus primarily by $^{\cdot}OH$ [8, 9] via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$) [10-12]. This is thought to operate in a continuous manner whereby $^{\cdot}O_2^-$ can reduce Fe^{3+} back to Fe^{2+}, and the reaction generating $^{\cdot}OH$ is repeated.

Aside from production within the mitochondria, ROS are also produced as metabolic by-products of the activities of many other cellular enzymes including cytochrome p450, which plays a major role in drug metabolism [13]. In contrast to the incidental production of ROS, the NADPH oxidase (Nox) enzymes within immune cells produce ROS and utilize them to attack invading pathogens [14]. The Nox enzymes are also a major source (but not the only source) of ROS used for signaling within various other cell types [15, 16]. Many environmental pollutants and chemical and physical

agents are potential sources of ROS, which include but are not limited to char-broiled foods, combustion products, long wavelength UV light, IR, and chemotherapeutic drugs [17].

ROS-induced modification of DNA occurs mainly through attack of the double bonds of the base rings and of the hydrogen atoms of the deoxyribose sugar moiety [17] (Fig. 1). A common, well-studied mutagenic lesion resulting from oxidative damage is 7,8-dihydro-8-oxo-guanine (8-oxoG) (Fig. 2A), which sometimes codes for dAMP incorporation and can be mutagenic following replication [18-23] and transcription [24]. The dNTP pool is an important source of 8-oxoG [25], and 8-oxodGMP can be misincorporated across from A by bacterial and human DNA polymerases [26]. Arguably one of the most mutagenic types of DNA damage is oxidation of cytosine [27], and in particular the resulting 5-hydroxycytosine and 5-hydroxyuracil (Fig. 2A) lesions have been shown to play a role in cytosine mutagenesis [28-31]. There are over 80 known oxidative base lesions that occur in cells [32], many of which cause little if any distortion to the DNA helix. A less well studied class of oxidative damage are bulky lesions [33], such as 8,5'-cyclo-2'-deoxyadenosine [34], resulting from the formation of covalent bonds between a purine base and sugar either within the same nucleotide or with an adjacent pyrimidine base. These types of bulky adducts can be mutagenic and can also block DNA replication and transcription [33]. ROS can also induce single-strand breaks (SSBs) following deoxyribose sugar oxidation, producing 3'-phosphoglycolate or 3'-phosphate ends, which are not substrates for DNA polymerase [35, 36].

1.1.2. Biological Consequences of Oxidative DNA Damage

The basal level of ROS necessary for proper cell function is kept in check by the activity of cellular anti-oxidant enzymes (e.g. peroxiredoxins [37]) and peptides (e.g. glutathione [38]) as well as dietary small molecules such as Vitamin E compounds[39] that scavenge and effectively neutralize ROS species [40]. A state of "oxidative stress" occurs when the balance is shifted in favor of ROS production to an extent that exceeds the ability of cellular defense mechanisms to buffer oxidative damage [41]. The cell may undergo oxidative stress following disruption of any of the aforementioned cellular processes that contribute to production of ROS. This is dangerous not only because of the vulnerability of macromolecules to oxidative damage, but also because of the potential for dysregulation of redox signaling.

Both oxidative and non-oxidative genotoxic agents can induce the production of intracellular ROS by directly damaging the DNA [42-44]. Intracellular ROS levels are also increased in response to spontaneous DNA damage that accumulates when DNA repair is defective. This increase represents a ROS-mediated DNA damage stress response [42-45], which is thought to be a signal transduction mechanism that activates genes involved in responding to the effects of DNA damage. Importantly, the ROS produced in response to different types of DNA damage can cause further damage to the DNA. A precise physiological role for this proposed stress response has not yet been elucidated.

The increased frequency of oxidative damage in the genomes of a variety of tumor types reveals a potential role of ROS-induced DNA damage in the development of cancer [46]. However, a mechanistic link to the etiology of cancer has not yet been

established because it is difficult to distinguish between the presence of damage occurring as a causative factor versus damage occurring as a consequence of disease development. Indirect evidence for a role of oxidative DNA damage in cancer development includes the finding that the common oxidative damage signature, GC → TA transversion mutations, has been identified in the *ras* oncogenes [47] and the p53 tumor suppressor gene within human tumors [48]. Another piece of indirect evidence for the role of oxidative DNA damage in human cancer is the identification of disease risk associated SNPs and mutations in repair proteins that are directly involved in the repair of oxidative DNA damage. Many of these SNPs and mutations are predicted to negatively impact protein function [49], and several have been shown to functionally decrease the repair function of the affected protein [46]. The reduced ability to repair oxidative DNA damage can result in acquisition of many cellular abnormalities including large scale genome rearrangements, as shown in *Saccharomyces cerevisiae* [43, 50], and such instability is a hallmark of cancer in complex organisms such as humans [51, 52].

1.2. Hydrolysis of DNA

An additional important source of spontaneous DNA damage results from another inevitable consequence of the cellular environment, the hydrolytic attack of nucleotide substituents. The loss of bases from the DNA backbone is thought to be the most common type of spontaneous damage, occurring at an estimated 20,000-50,000 bases/mammalian cell/day [1, 53]. Purines are lost 20 times more often than pyrimidines due to a weakened N-glycosidic attachment to the deoxyribose moiety [1]. The resulting lesion, termed an apurinic/apyrimidinic (AP) site (or abasic site) (Fig. 2D), lacks coding information and can block replication [1]. AP sites can also be bypassed during

replication and transcription, and phage, *E. coli* and human systems often show a preference for dAMP incorporation [19, 54-58]. The DNA bases containing an exocyclic amino group [1] (A, G and C) (Fig. 1) are subject to deamination under physiological conditions with cytosine being the most commonly targeted base [59]. Uracil in DNA can occur as a result of cytosine deamination, and may lead to C→T transition mutations following replication.

1.3. Alkylation Damage to DNA

Experimental evidence that protection against alkylation DNA damage is physiologically relevant came from the observation that DNA alkylation excision activity is present in *E. coli* [60]. The cloning of alkylation damage-specific DNA repair genes from yeast [61, 62], and genetic analysis of yeast cells lacking the repair enzymes encoded by these genes [63] demonstrated the importance of possessing repair pathways to protect eukaryotic cells against spontaneous alkylation DNA damage [64].

Exogenous sources of DNA alkylating agents include products of biomass burning [65] and chemotherapy [66]. The major intracellular source of alkylation damage to DNA is thought to be S-adenosylmethionine (SAM), which normally serves as a methyl group donor to cellular DNA methyltransferase enzymes [67]. SAM can aberrantly transfer methyl groups to nucleophilic centers on the DNA [68] (Fig. 1), producing N7-methylguanine, O6-methylguanine and N3-methyladenine base lesions [69] (Fig. 2B). Importantly, N7-methylguanine induces replicative misincorporation and N3-methyladenine causes replication blockage. Exposure to alkylating agents, namely MMS, has also been shown to induce an increase in intracellular ROS levels in yeast [42].

1.4. Radiation Damage to DNA

Ultraviolet (UV) radiation, which comes from solar light, can cause single-strand breaks [70] and the production of 8-oxoG due to increased levels of ROS following chronic UV exposure [71, 72]. Most prominently, UV radiation causes the formation of covalent bonds between adjacent pyrimidines to produce bulky adducts, such as cyclobutane pyrimidine dimers (CPDs) [73] and pyrimidine-pyrimidone (6-4) photoproducts (Fig. 2C) [74, 75]. UV light has also been shown to produce monoadducts such as a variety of pyrimidine photohydrates [76] as well as thymine glycols *in vitro* [77, 78].

Ionizing radiation (IR)-induced DNA damage comes principally from cosmic radiation, naturally occurring radionucleotides [17], and medical and dental X-rays [79]. IR induces direct damage to the DNA by ionizing the base and sugar substituents [2], leading to formation of strand breaks or base damages. IR also causes indirect damage, the major mode occurring through radiolysis of water to eventually generate ROS that go on to directly damage the DNA [2].

1.5. Chemotherapeutic Agents

The large majority of anti-cancer agents target the DNA as inducing DNA damage is an effective way to selectively kill cancer cells [66]. One example of an important anti-cancer agent is temozolomide (TMZ), which causes alkylation damage to cellular DNA [66]. Alkylation base damage is repaired mainly by a pathway known as base excision repair (BER) [17]. Small molecule inhibitors of several BER proteins are

being developed for use in the clinic in combination with agents such as TMZ to increase treatment efficacy. TMZ and alkylating agents such as MMS also have clastogenic effects [80, 81], meaning that they can induce double-strand breaks (presumably due to stalling of replication forks [82]), which must be repaired through pathways such as non-homologous end joining or homologous recombination. There are examples of anti-cancer drug classes dealt with by one or more of the major DNA repair pathways that are discussed below [83]. Several types of chemotherapeutic agents have been shown to cause intracellular increases in ROS, which causes damage types that are repaired primarily by BER.

1.6. DNA Damaging Agents Employed in the Present Studies

1.6.1. Hydrogen peroxide (H_2O_2)

H_2O_2 is a biologically relevant ROS that has been extensively used as a model DNA damaging agent to assess the cellular consequences of oxidative DNA damage. Attributes that make H_2O_2 a suitable experimental agent include that it is more stable than the other physiologically relevant ROS [84], and that it can readily enter cells with permeability similar to H_2O, in certain contexts [85].

1.6.2. Methyl methanesulfonate (MMS)

Methylating and ethylating chemicals have been used by investigators to elucidate mechanisms of DNA repair and biological consequences of DNA damage. Methyl methanesulfonate is a commonly used model monofunctional alkylating agent that

induces mainly N7-methylguanine and to a lesser extent N3-methyladenine [86], two of the lesions produced endogenously by SAM [68].

2. DNA Damage Management Systems

As discussed, much of the DNA damage incurred by cells comes from both spontaneous events and environmental agents. Organisms have evolved several pathways to protect against the mutagenic and cytotoxic effects of DNA damage, and these pathways are collectively referred to as damage management systems and can be divided into two major modes: DNA damage repair and DNA damage tolerance. While repair mechanisms replace the damaged DNA to maintain genetic stability, damage tolerance mechanisms do not repair the DNA damage per se, but allow replicative and transcriptional bypass of the DNA damage to promote cellular survival, often at the expense of increased genetic instability.

The major mode for the repair of oxidative, alkylation and hydrolytic attack of the DNA is the base excision repair (BER) pathway, which primarily repairs small, helix non-distorting lesions resulting from the aforementioned sources throughout the cell cycle. DNA lesions normally repaired via BER can also be substrates for other damage management pathways, especially when the amount of damage exceeds the capacity of BER to repair it [87].

2.1. DNA Repair Systems

The budding yeast, *Saccharomyces cerevisiae*, has been widely employed to elucidate cellular DNA damage repair pathways because the proteins and basic

biochemical steps of DNA repair pathways are conserved from yeast to human [88]. The major DNA repair pathways include base excision repair (BER) (Fig. 3 and Fig. 4), nucleotide excision repair (NER) (Fig. 4), and mismatch repair (MMR).

2.1.1. Base Excision Repair

The base excision repair (BER) pathway (Fig. 3) repairs DNA damages, such as single base lesions resulting from oxidation, alkylation and hydrolysis as well as single-strand breaks, which all cause minimal distortion to the DNA helix. The basic biochemical steps of BER can be described as proceeding stepwise. The first step is typically cleavage of the N-glycosidic bond by a damage-specific glycosylase to release a base lesion, leaving behind an AP site. AP sites are also generated via spontaneous hydrolysis of the N-glycosidic bond. AP endonucleases (AP endo) recognize AP sites and cleave the DNA backbone, producing 3' hydroxyl and 5'-deoxyribose phosphate end. AP sites may also be processed by the lyase activity of bifunctional glycosylase/lyase enzymes, which leaves behind a 3' α,β-saturated aldehyde group and a 5' phosphate. Once the backbone is cleaved, the ends must be processed to the appropriate 5'-phosphate and 3'hydroxyl groups in order for DNA repair synthesis by DNA polymerase and sealing of the gapped DNA by DNA ligase to complete the repair process. (See Table 1 for BER protein homologs from *E. coli*¸ yeasts and human).

2.1.1.1. Apurinic/ apurimidinic (AP) Endonucleases

The major AP endonuclease (AP endo) families are exemplified by and named for the prototypical proteins in *E. coli*, exonuclease III (exo III) and endonuclease IV (endo IV). Many organisms have both a major, constitutive AP endo and a minor, inducible AP

endo. In *E. coli* the major, constitutive AP endo is exonuclease III [89], and the minor one is endonuclease IV [90], which is inducible by oxidative stress [91, 92]. In *S. cerevisiae* the major AP endo is the endo IV homolog Apn1 [93, 94], and the minor AP endo is the exo III homolog Apn 2 [95]. In humans, both the major and minor AP endos, APE1 and APE2 [96], are exo III homologs. The presence of the minor AP endo is thought to serve as a back-up repair function [97, 98]. Another major associated repair function of AP endos is a 3'-phosphodiesterase activity, which is involved in the processing of the 3' groups resulting from AP lyase activity and of 3'-deoxyribose fragments resulting from oxidative and IR damage to the DNA [92, 99-103].

The exo III and endo IV families of AP endonucleases also possess 3'→ 5' exonuclease activity against oxidative DNA lesions and AP sites [103-108]. It has been suggested that this activity may be most relevant under oxidative stress conditions [105], and that it may be associated with misincorporation of 8-oxoG during replication [104]. However, the physiological relevance of the 3'-5' exonuclease activity associated with these families of AP endonucleases is not known.

E. coli, yeast and human AP endonucleases have also been shown, *in vitro* and *in vivo*, to nick DNA on the 5' side of a variety of oxidative base lesions [109-111]. This activity leaves behind a 3'-hydroxyl group and a 5'-phosphate group at the nicked termini, and no further processing is needed for DNA polymerase and ligase to restore the DNA to the original, undamaged state. This alternative pathway has been designated the nucleotide incision repair pathway and may contribute significantly to the repair of spontaneous damage within cells. Utilizing this sub-pathway over others may provide an

advantage to the cell as damages are repaired without the generation of the toxic and mutagenic AP site as a repair intermediate.

In addition to AP endo function, the human APE1 protein containing a non-DNA repair domain and also acts as a redox co-activator of a number of transcription factors [112], and has other less well-elucidated cellular functions [113, 114]. An important issue is that, while there are some mutations in either the redox domain or the nuclease domain that disrupt either the gene regulatory or DNA repair functions specifically [115], experimental manipulation of APE1 may present a challenge in terms of determining whether a phenotype is due to disruption of the non-DNA repair functions or the nuclease functions. One advantage of studying AP endonuclease biology in yeast, unlike human APE1, is that yeast Apn1 does not have any known additional non-DNA repair activities [113-115].

2.1.1.2. Endo IV Family of AP Endonucleases: Microbial Model Systems

The endo IV and Apn1 homologs share 41% amino acid identity. In addition, and *E. coli* endo IV and *S. cerevisiae* Apn1 can rescue the DNA repair deficiency phenotype of Apn1-defective yeast cells and endo IV-defective bacterial cells, respectively, via cross-species complementation assays [116, 117]. Thus, *E. coli* endo IV can potentially be used to model aspects of Apn1 biology. Significantly, *E. coli* endo IV structure-function relationships are well characterized because the high-resolution structure, mutational analyses and biochemical studies have defined the DNA binding and catalytic mechanism for this AP endonuclease [118-120].

Apn1 represents 97% of the major AP endonuclease activity in yeast cells under normal growth conditions [94], and was initially characterized as a DNA repair diesterase for 3'-deoxyribose fragments [102]. Deletion of the *APN1*gene results in cellular sensitivity to both oxidative and alkylating agents [121]. In addition, *apn1Δ* cells exhibit a mutator phenotype [121] in which the majority of changes are base substitutions resulting in AT→CG transversion mutations.

2.1.2. Nucleotide Excision Repair (NER)

The nucleotide excision repair (NER) pathway primarily repairs bulky DNA damage that causes distortion to the DNA helix. Such distortions are thought to provide a mode of damage recognition and binding for NER proteins. NER proceeds step-wise whereby a complex of enzymes unwinds the DNA encompassing the damage. Next, two enzyme complexes incise the DNA, one on the 3' side of the lesion and another on the 5' side of the lesion. The resulting oligonucleotide fragment is released from the DNA helix, which leaves a gap that is filled in by DNA polymerase followed by sealing of the nicked DNA strand by a DNA ligase.

Aside from the repair of bulky damage, NER shares substrate specificity with BER. For example, *E. coli* and human NER enzymes were shown to excise AP sites, small oxidative base lesions and methylated base lesions *in vitro* [122-125]. Further, genetic studies in yeast where BER and NER pathways were simultaneously deleted revealed a synergistic relationship regarding repair of spontaneous DNA damage [87].

2.1.3. Mismatch Repair (MMR)

The mismatch repair (MMR) pathway removes and replaces bases misincorporated during replication and also repairs other replication-associated aberrations, such as insertion loops or deletion loops resulting from polymerase slippage events during DNA synthesis [126]. MMR proceeds by the recognition and binding of the mismatch, discrimination between the nascent strand and the template strand to ensure that the misincorporated nucleotide is the one that is removed, and incision of the backbone by an endonuclease on 5' side and the 3' side of the target nucleotide. A helicase and an exonuclease work to remove the DNA patch containing the error, followed by DNA synthesis via DNA polymerase and sealing of the nicked backbone by a DNA ligase [126].

The MMR pathways can cooperate with BER to repair oxidative DNA damage. For example, a component of the MMR machinery (hMutSα) physically interacts with the BER glycosylase hMYH to remove dAMPs that are misincorporated across from 8-oxoG during DNA synthesis [127]. Yeast genetic studies where MMR and BER components were simultaneously deleted revealed a synergistic relationship toward repair of spontaneous DNA damage whereby the double pathway-deficient mutants accumulate GT to TA mutations [128], which is the signature for mutations induced by A:8-oxoG misincorporation events.

2.2. DNA Damage Tolerance Pathways

Under conditions when DNA damage is not repaired or bypassed during replication, cells can employ mechanisms that allow for cell survival via tolerance of the DNA lesions. Such pathways do not repair the DNA damage, but allow replicative and transcriptional bypass of the DNA damage, often at the expense of increased genetic instability. One such mechanism, homologous recombination (HR), is necessary during meiosis for chromosomes to segregate properly and to produce genetic variation [129, 130]. HR also plays an important role in repairing strand breaks as well as strand gaps resulting from restoration of replication forks blocked by DNA lesions. DNA damage tolerance via recombination can proceed by one of several homologous recombination sub-pathways [17]. Simultaneous deletion of both the BER and NER pathways results in a hyper-recombinogenic phenotype in yeast that is accompanied by the acquisition of large-scale chromosomal aberrations [43, 50, 87]. This reveals a role for recombination in the tolerance of spontaneous oxidative DNA damage, especially when the amount of damage exceeds the capacity of the cell to handle it via the excision repair (BER, NER) pathways.

Different types of lesions typically repaired by BER can be bypassed during replication by another important DNA damage tolerance mechanism, translesion synthesis (TLS) (Fig. 4). When the replication machinery encounters a blocking, non-coding or miscoding DNA lesion in the template strand, the cell can employ one of a handful of translesion DNA polymerases, which either add a nucleotide across from a DNA lesion or assist the replicative polymerase in extending past nucleotide incorporation opposite a DNA lesion [20-23, 54, 131-134] . TLS has been shown to

bypass AP sites, 8-oxoG, O6-methylguanine and uracil in DNA *in vitro* [18, 19, 54, 131-133]. Genetic studies in yeast have shown that TLS plays an important role in the tolerance of spontaneous DNA damage *in vivo* [43, 50, 87].

3. DNA Repair in Mitochondria

DNA damage management pathway components also protect the mitochondrial genome against damage in eukaryotic cells [135]. These pathways include BER, MMR, TLS and REC. The presence of repair proteins in the mitochondria was discovered via proteosomal analysis aimed at identifying nuclear repair protein homologs in the mitochondria in yeast [136]. Thus, many of the same proteins important for the repair of the nuclear genome also localize to the mitochondria for efficient DNA repair in this organelle.

4. DNA Repair in the Context of Chromatin Structure

Within cells, repair proteins must interact with DNA within the context of chromatin structure. The basic unit of chromatin, the nucleosome, consists of ~146 base pairs of DNA wrapped around a complex of proteins called histones. This arrangement allows for the compaction of DNA into higher order structures so that it can fit into the confines of the nucleus. Regulation of chromatin topology occurs through post-translational modifications on histones and ATP-dependent chromatin remodeling complexes and is necessary to provide DNA binding proteins access to their target loci in the DNA. Much of this knowledge is based on studies of NER and DSB repair.

In yeast cells, the repair of pyrimidine dimers by NER was found to occur more efficiently in DNA free nucleosomes [137]. Upon exposure of human cells to UV irradiation, histones are hyperacetylated [138] and nucleosomes undergo positional rearrangements [139]. Chromatin becomes more accessible following UV-induced histone acetylation [140]. And NER-associated DNA synthesis is more efficient in hypoacetylated nucleosomes [141]. Studies in yeast revealed a requirement for histone acetylation in the repair of DSBs via HR [142]. Chromatin remodeling complexes are recruited to double-strand breaks and participate in repair through HR [143, 144]. Phosphorylation of the variant histone H2AX (termed γ-H2AX), occurs at double-strand breaks in DNA [145] and is a commonly used marker for DNA damage. Chromatin remodeling factors are recruited to an induced DSB through interaction with γ-H2AX [146, 147]), and this recruitment to DSBs is dependent on physical association with the DNA damaged-induced γ-H2AX present at the DSB site. [146, 147].

The presence of nucleosomes on DNA has been shown to reduce the efficiency of BER toward uracil [148, 149] and 8-oxoG [150] repair *in vitro* as well as N-methyl purines in vivo [151]. The presence of an ATP-dependent chromatin remodeling complex can stimulate BER of the 8-oxoG lesions at the same level of repair as in naked DNA [150]. In addition, the 8-oxoG-specific BER glycosylase Ogg1 is recruited to open chromatin regions following induction of oxidative damage [152]. Information regarding BER in chromatin is accumulating and our studies will add to this growing body of work.

5. *Elucidating Mechanisms of DNA Repair and Genetic Instability in Saccharomyces cerevisiae*

Genetic instability is a major biological consequence of unrepaired DNA damage and is defined as reduced fidelity in the transmission of genetic information [153]. DNA repair is at the center of preventing genetic instability. The types of genetic instability resulting from unrepaired DNA damage include a range of alterations to the genetic information from point mutations and small insertion/deletion mutations to large scale chromosomal aberrations [154].

The studies reported in this dissertation explore the contribution of the base excision repair (BER) pathway in protecting the cell against genetic instability. Unlike the case with BER, the genetic basis for DNA repair dysfunction in disease is well-defined for nucleotide excision repair and mismatch repair [155, 156]. In addition, the biological consequences of DSBs have been very well-elucidated [157]. While defects in BER components have been linked to various diseases, there are only a few examples of specific genetic defects in BER components associated with disease development, including the glycosylase enzymes UNG in immunodeficiency [158] and MUTYH in familial colorectal cancer [159], as well as the BER-specific DNA polymerase β in various cancers [160]. Remarkably, there are SNPs resulting in missense substitutions associated with disease risk in virtually every BER component [49], but few functional relationships between polymorphism or mutations and disease have been experimentally validated [49]. A current model in the field is an extension of the somatic mutation theory of cancer [161] and states that SNPs in BER genes associated with disease development may cause subtle deficiencies in BER capacity that, combined with long-term exposure to

DNA damaging agents, such as endogenous ROS, lead to a mutator phenotype and eventually, genomic instability and cancer [162]. In addition to gaining a better understanding of BER defects in disease, assessing the role of individual proteins in DNA repair is a useful strategy because one can take various experimental approaches to learn about how the parts (repair proteins) contribute to cellular function (DNA repair capacity) as a whole. The studies described here will explore the contribution of the Apn1 repair protein to protecting yeast cells against the deleterious effects of unrepaired DNA damage.

Oxidative stress-induced genomic instability has been observed in a number of experimental systems [50, 163-168], but the molecular mechanisms underlying the conversion of DNA damage to chromosomal rearrangements are not well elucidated. A particular investigation in *Saccharomyces cerevisiae* revealed a genomic instability phenotype that resulted from the accumulation of chronic oxidative DNA damage in cells with genetic deficiencies in both the BER and NER pathways [50], attempted replication past said damage, and error-prone repair of the damage via recombination. The yeast model just described is an informative system , as genomic instability is a hallmark of cancer [51, 52].

6. Summary of Project Objectives

We employed *Saccharomyces cerevisiae* as a simple model eukaryotic system containing the basic biochemical components of BER that are conserved in humans. More specifically, we focused our studies on a central BER enzyme, the major yeast AP endonuclease Apn1, both as a model for AP endonuclease function in higher eukaryotes.

The major goal of objective 1 (Chapter 2) is to explore the effects of point mutations in *APN1* on DNA repair capacity through structure-function analysis. The information gained will provide insight into how mutations in human BER proteins may contribute to disease etiology, a largely unexplored question in the DNA repair field. The results will provide important information that could eventually lead to development of novel treatments for certain DNA damage-associated pathologies. The major goal of objective 2 (Chapter 3) is to generate a genome-wide map of Apn1 occupancy to represent the localization of BER across the yeast genome. This approach will aid in identifying cis-features, including base content, that make certain genomic regions unstable under oxidative stress and will help further elucidate how DNA damage is physically translated to large-scale chromosomal changes.

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Table 1 Base excision repair genes from bacteria, yeast and humans

Enzyme	*E. coli*	*S. cerevisiae*	*S. pombe*	Human	References
DNA glycosylase					
Uracil DNA glycosylase	*ung*	*UNG1*		*UDG1*	[169, 170]
3MeA DNA glycosylase	*alkA* *tag*	*MAG*	*mag1* *mag2*[b]	*AAGa*	[61, 171-174]
8-oxoG DNA glycosylase/ AP lyase	*fpg*	*OGG1**		*OGG1*[a]	[175-180]
MutY G:A mismatch glycosylase/ AP lyase	*mutY*			*MYH*	[181]
Thymine glycol DNA glycosylase/AP lyase	*nth*	*NTG1/OGG2* *NTG2*	*ntg1*	*NTG1*	[182-184]
TDG T:G mismatch DNA glycosylase				*TDG*	[185]
AP endonuclease					
Exonuclease III	*xth*	*ETH1/* *APN2*	*eth1*[b]	*APE /* *REF1* */ HAP1*	[95, 97, 186-188]
Exonuclease IV	*nfo*	*APN1*	*apn1*[b]		[94, 95]]
Additional factors					
Flap endonuclease		*RTH1 / RAD27*	*rad2*	*FEN1*	[189]
Proliferating cell nuclear antigen		*POL 30*	*pcn1*	*PCNA*	[190]
Replication Factor C		*RFC*	*rfc*	*RFC*	[191, 192]
XRCC1				*XRCC1*	[193]
DNA polymerase		*POL IV*		*Pol β*	[194-198]
		POL 3	*pol 3*	Pol δ	
		POL 2	*cdc20*	Pol ε	
DNA ligase	*ligA*	*CDC9*	*cdc17*	*LIG1*, *LIG3*	[199, 200]

[a]Gene product possess similar enzymatic activity to *E. coli* homology, but share no sequence similarity.

[b]Identified by database search, enzymatic activity has not been verified.

Figure 1.

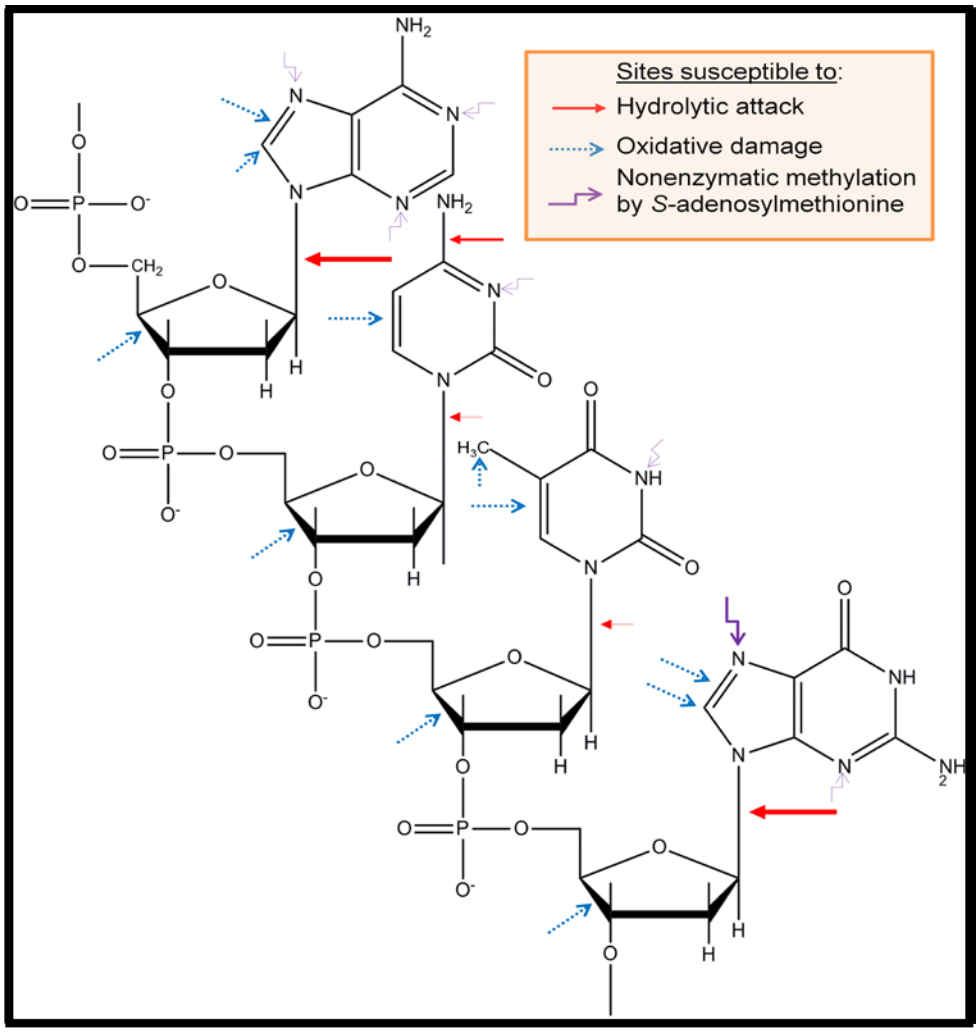

Figure 1.Target sites for intracellular DNA decay. A short segment of one strand of the DNA double helix is shown with the four common bases (from top: A-adenine, C-cytosine, T-thymine, G-guanine). Sites susceptible to hydrolytic attack are indicated by solid red arrows, oxidative damage by dashed blue arrows, and nonenzymatic methylation by *S*-adenosylmethionine as zig-zagged purple arrows. Major sites of damage are indicated by the large arrows. Hydrolytic and oxidative damage, but not

methylated residues would accumulate in fossil DNA. Adapted from Lindahl [1] with permission from the publisher.

Figure 2.

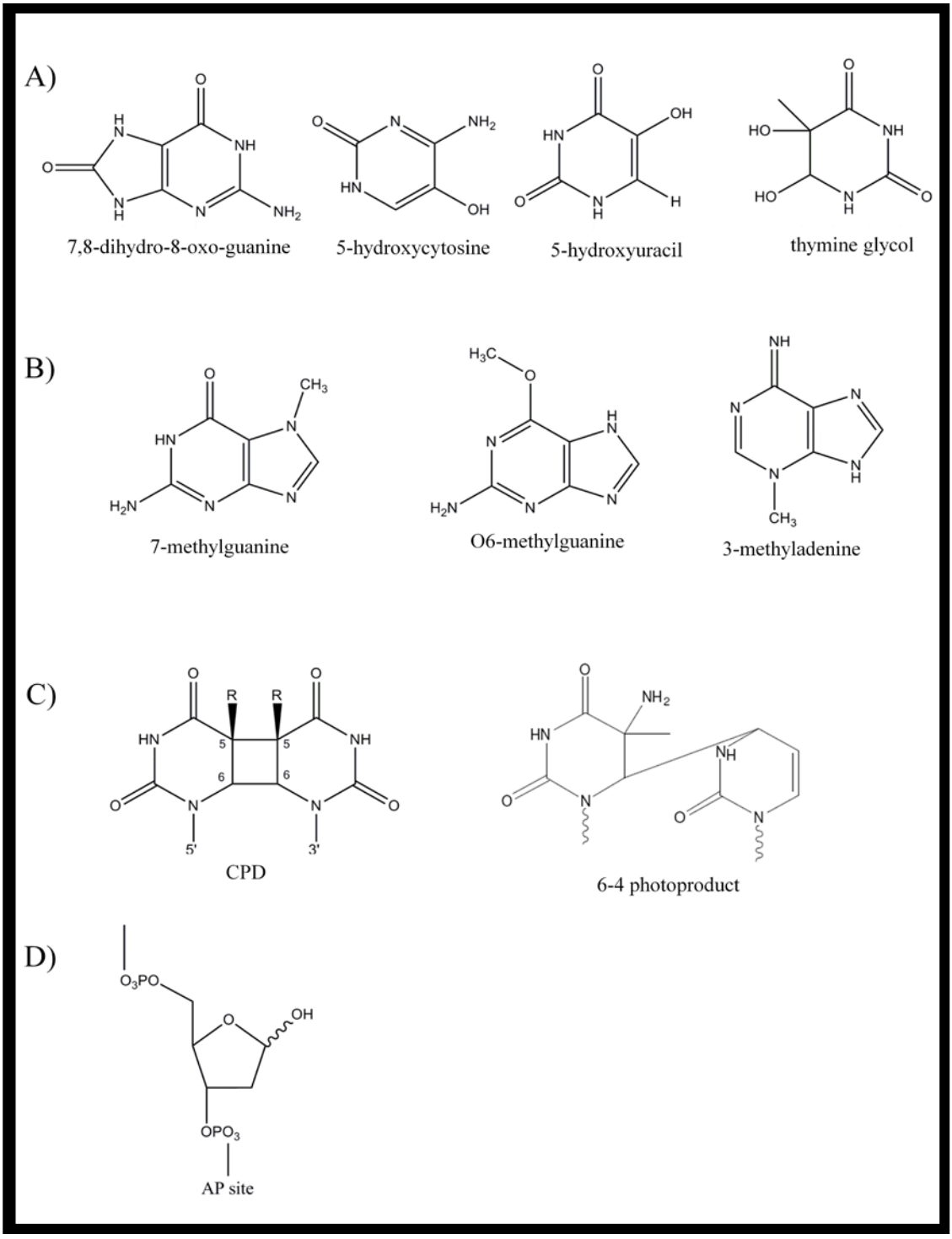
A)
7,8-dihydro-8-oxo-guanine
5-hydroxycytosine
5-hydroxyuracil
thymine glycol
B)
7-methylguanine
O6-methylguanine
3-methyladenine
C)
CPD
6-4 photoproduct
D)
AP site

Figure 2. Examples of base lesions caused by DNA damaging agents. (A) DNA base lesions caused by reactive oxygen species includes 7, 8-dihydro-8-oxo-guanine; 5-hydroxycytosine; 5-hydroxyuracil; thymine glycol. (B) DNA base lesions caused by alkylation include 7-methylguanine; O6-methylguanine; 3-methyladenine. (C) Bulky DNA lesions caused by UV irradiation include CPD and (6-4) photoproducts. (D) Apurinic/apyrimidinic (AP) site caused by spontaneous hydrolysis of the N-glycosidic bond or by processing of the N-glycosidic bond by an AP lyase.

Figure 3.

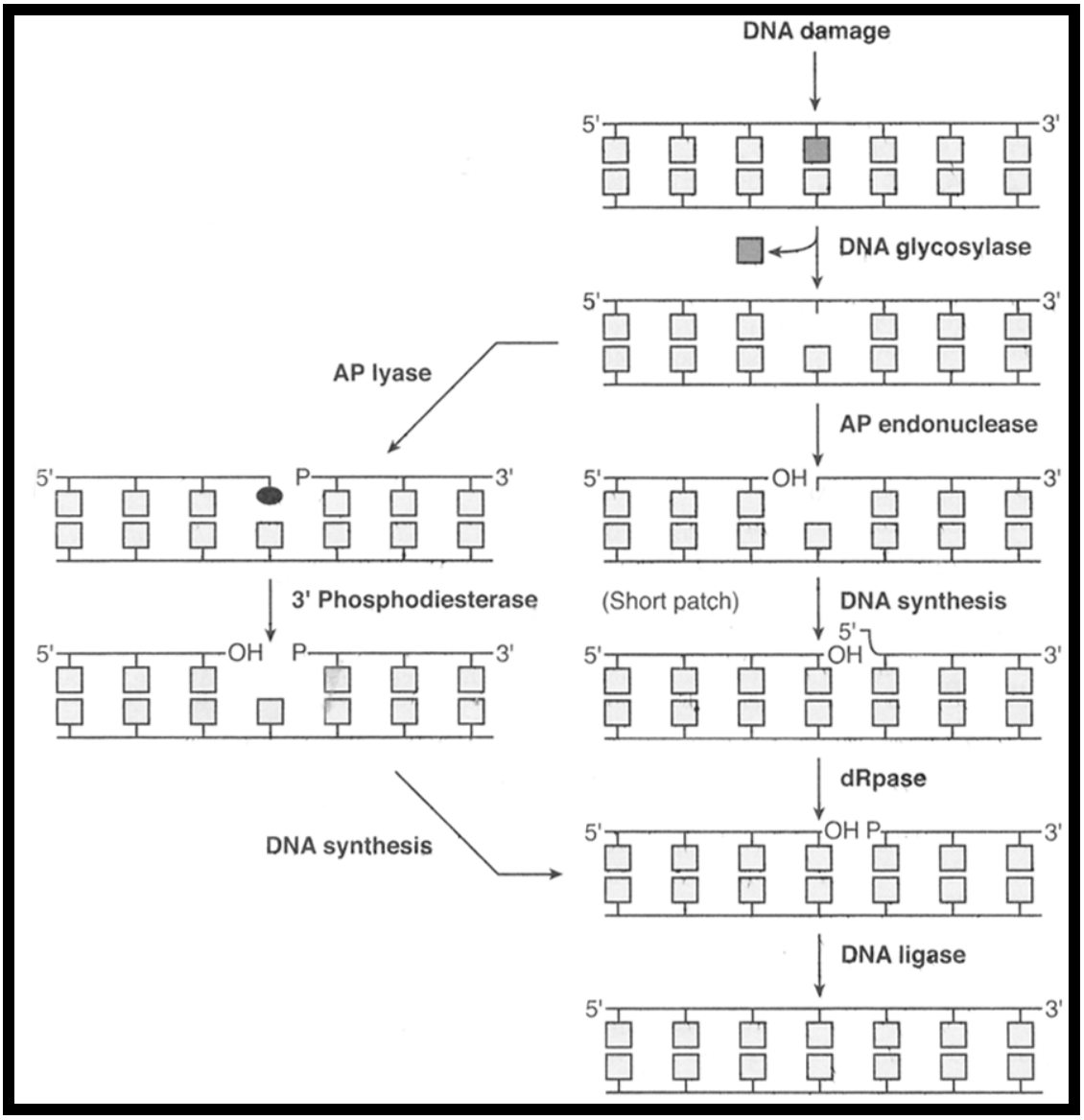

Figure 3. Schematic representation of the base excision repair pathway. BER is a multistep process that corrects endogenous damage to DNA caused by hydrolysis, ROS, and other metabolites. It is catalyzed by a lesion-specific DNA glycosylase, an AP site-specific endonuclease, another endonuclease or 2'-deoxyribose phosphodiesterase, DNA polymerase, and DNA ligase. Initially, the damaged base is excised from DNA by

cleavage of the N-glycosyl bond between the base and a deoxyribose sugar, and then the remaining abasic nucleotide (AP site) is excised and replaced by repair synthesis. Some DNA glycosylases have an associated lyase activity that cleaves the DNA backbone 3’ of the damaged nucleotide (left branch of pathway). The resulting 3’ modified nucleotide is removed by a phosphodiesterase before repair synthesis and ligation to complete the repair. Adapted from Friedberg [17] with permission from the publisher.

Figure 4.

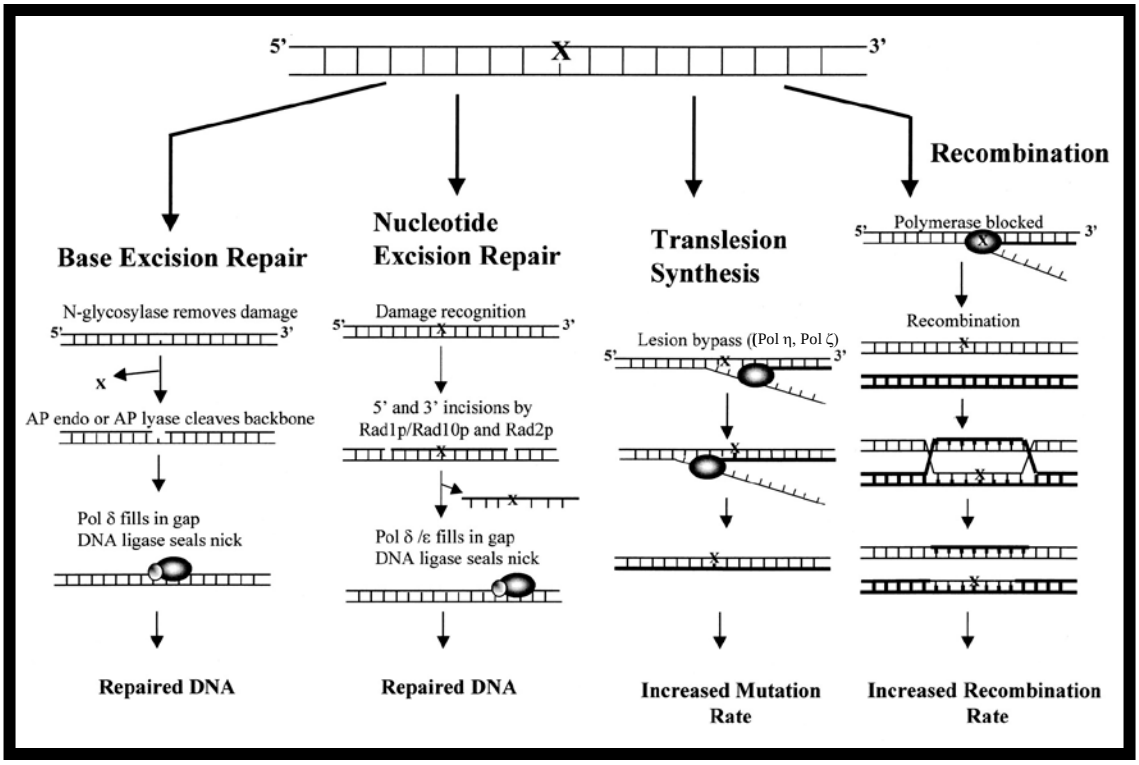

Figure 4. Processing of oxidative and spontaneous DNA damage in *Saccharomyces cerevisiae.* Processing of oxidative and spontaneous DNA damage in *S. cerevisiae.* "X" represents either a base damage which can be recognized and removed by BER or NER pathway, can be bypassed by TLS, or can block replication causing recombination to occur or an AP site which feeds into any of the four pathways illustrated. The amount of damage that is processed by each pathway varies depending on the repair background genotype. Adapted from Swanson [87] with permission from the publisher.

CHAPTER 2

SACCHAROMYCES CEREVISIAE APN1 MUTATION AFFECTING STABLE PROTEIN EXPRESSION MIMICS CATALYTIC ACTIVITY IMPAIRMENT: IMPLICATIONS FOR ASSESSING DNA REPAIR CAPACITY IN HUMANS

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Abstract

Apurinic/apyrimidinic (AP) endonucleases play a major role in the repair of AP sites, oxidative damage and alkylation damage in DNA. We employed *Saccharomyces cerevisiae* in an unbiased forward genetic screen to identify amino acid substitutions in the major yeast AP endonuclease, Apn1, that impair cellular DNA repair capacity by conferring sensitivity to the DNA alkylating agent methyl methanesulfonate. We report here the identification and characterization of the Apn1 V156E amino acid substitution mutant through biochemical and functional analysis. We found that steady-state levels of Apn1 V156E were substantially decreased compared to wild type protein, and that this decrease was due to more rapid degradation of mutant protein compared to wild type. Based on homology to *E. coli* endonuclease IV and computational modeling, we predicted that V156E impairs catalytic ability. However, overexpression of mutant protein restored DNA repair activity *in vitro* and *in vivo*. Thus, the V156E substitution decreases DNA repair capacity by an unanticipated mechanism via increased degradation of mutant protein, leading to substantially reduced cellular levels. Our study provides evidence that the V156 residue plays a critical role in Apn1 structural integrity, but is not involved in catalytic activity. These results have important implications for elucidating structure-function relationships for the endonuclease IV family of proteins, and for employing simple eukaryotic model systems to understand how structural defects in the major human AP endonuclease APE1 may contribute to disease etiology.

1. Introduction

The base excision repair (BER) pathway is essential for protection against the continuous oxidation, alkylation and hydrolysis of DNA due to normal cellular metabolism and environmental DNA damaging agents. Such genotoxic exposures result in the formation of base lesions, apurinic/apyrimidinic (AP) sites and DNA strand breaks. The production of AP sites, which are potentially cytotoxic [1, 2] and mutagenic [1, 3], occurs frequently as bases are spontaneously lost from the DNA backbone at an estimated rate of 10,000 per cell per day in mammals [4, 5]. AP sites are also generated as byproducts of the BER process through enzymatic removal of damaged bases by lesion-specific glycosylases that recognize and cleave base lesions from the DNA backbone [6, 7]. AP endonucleases recognize and nick the DNA backbone at AP sites followed by repair synthesis by DNA polymerase and sealing of the nicked DNA strand by ligase. AP endonucleases also function as 3' diesterases to process 3' blocked termini of single strand breaks produced as a result of oxidative damage [8]. Thus, AP endonucleases are central to the repair of a large subset of DNA damage within the cell via their ability to process major types of DNA damage that arise directly or that are produced as DNA repair intermediates.

Point mutations and SNPs in the human AP endonuclease APE1 that cause non-synonymous amino acid substitutions have been a focus of many epidemiological disease association studies [9, 10], and combined with computational modeling and experimental validation [11, 12], have provided clues regarding their roles in disease. For example, a previous study reporting *in vitro* biochemical characterization of APE1 variants identified from amyotrophic lateral sclerosis patients and several variants reported in the NCBI

database of SNPs, confirmed that some of the APE1 variants with predicted catalytic defects indeed cause decreased catalytic activity while other variants exhibited normal activity [10]. This suggests that if such “neutral” substitutions are associated with disease risk, the defects may influence aspects of APE1 biology that were not measured, such as steady-state expression level, *in vivo* repair capacity or interactions with other BER proteins. Studies in higher eukaryotes are complicated by factors such as inter-individual genetic variation and molecular differences in repair capacity from one cell type to another, which present major challenges to identifying the molecular basis of AP endonuclease dysfunction in disease [10].

In order to explore how structural defects that compromise a critical, central step in the BER pathway manifest themselves in eukaryotic cells, we have investigated Apn1, the major AP endonuclease in *Saccharomyces cerevisiae* [13]. Apn1 is a functional homolog of mammalian APE1 as cross species complementation studies have shown that Apn1 can functionally complement the absence of APE1 DNA repair activity in human and other mammalian cells [14-16]. A major advantage of utilizing yeast for our studies is that Apn1 has no known DNA repair-independent activities, unlike human APE1, which does have other activities [17] including functioning as a transcriptional co-activator of a number of genes . Studying yeast Apn1 allows for directly examining the cellular effects of AP endonuclease catalytic deficiency, whereas genetic manipulation of the human APE1 could also influence its non-DNA repair activities.

Apn1 is a member of the endonuclease IV (endo IV) family of AP endonucleases. Previous biochemical and structural studies of *E. coli* endo IV have defined the molecular mechanisms by which DNA binding and phosphodiester bond

incision are achieved for this family of enzymes [18-21]. This information provides a framework for exploring the functional consequences of particular changes in endonuclease structure.

To identify functionally relevant changes in Apn1 structure, we performed an unbiased random mutagenesis screen for mutants displaying sensitivity to the DNA alkylating agent methyl methanesulfonate. We report here the investigation of Apn1 structure-function relationship through characterization of a recessive mutation in the endogenous *APN1* locus that affects cellular repair capacity by an unanticipated mechanism. The V156E substitution leads to production of a full-length mutant protein with intact catalytic function even though predictions based on homology modeling suggested the possibility of catalytic domain dysfunction. Instead, we find that accelerated degradation of V156E leads to decreased cellular protein levels and defects in DNA repair. This unexpected mechanism of impaired DNA repair capacity suggests a role for V156 in the maintenance Apn1 structural integrity. Our findings have important implications for elucidating the functional consequences of SNPs mapping outside the known APE1 functional domains predicted to impact DNA repair, and illustrate the utility for employing simple model systems for such studies.

2. Materials and Methods

2.1. Yeast cell culture conditions and transformation

Standard yeast media and cell culture conditions were used as previously described [22, 23]. Methyl methanesulfonate (MMS) (Sigma) was added to YPD medium at 0.08% after autoclaving and cooling. The lithium acetate method for yeast cell transformation was employed as previously described [23]. The sequences of primers and oligonucleotides used in this study are available upon request.

2.2. Plasmid construction

Details for plasmids used in this study are listed in Table S1.

Plasmid pD428, a CEN plasmid with wild type *APN1* inserted at the multiple cloning site (MCS) of pRS316 [24], was constructed by gap-repair cloning [25]. Briefly, PCR products with homology upstream of the SacI restriction site at the 5' end and homology downstream of the Kpn1 restriction site at the 3' end (with respect to the pRS316 MCS) were amplified using genomic DNA from strain DSC320 (Table 1). The resulting *APN1*-SacI/Kpn1I fragment was co-transformed with linearized pRS316 (digested with SacI and KpnI). Plasmids were then recovered, propagated in *E.coli*, and verified via restriction analysis and sequencing.

Plasmids pD433, pD434 and pD435 containing mutant versions of *APN1* (*apn1-V156E*, *apn1-E207V*, *apn1-V156E, E207V,* respectively) were constructed via site-directed mutagenesis of plasmid pD428 using the QuickChange site-directed mutagenesis kit from Stratagene.

2.3. Genetic manipulations

All *S. cerevisiae* strains used in this study are listed in Table 1.The parental strain DSC320 is a haploid spore of the diploid strain hDNP16 [26], and strains DSC386, DSC393 and DSC378 are haploid spores of the diploid strain hDNP19 [26]. Strain DSC320 does not contain mutations in BER genes *APN1*, *NTG1* or *NTG2*. Strain DSC386 is *APN1*-deficient (*apn1Δ*). Strain DSC393 is *NTG1*- and *NTG2*-deficient (*ntg1Δ ntg2Δ*). Strain DSC378 is deficient in *APN1*, *NTG1* and *NTG2* (*apn1Δ ntg1Δ ntg2Δ*).

The *delitto perfetto* method [27] was used to target random PCR-generated mutations to the chromosomal *APN1* locus in a random mutagenesis screen (described in this section below), and to create site-directed mutations in the *APN1* locus (described in this section below). *I-SceI* CORE insertion strains were constructed using plasmid pGSKU as a PCR template to amplify an *apn1*::*KlURA3*-KanMX4-I-SceI fragment for transformation. The resulting *apn1::I-SceI* CORE insertion strains are DSC501 and DSC502 (Table 1).

For the random mutagenesis screen, plasmid pD428 was used as a template to generate a library of mutant *apn1* PCR fragments via amplification of the *APN1* coding sequence with a relaxed-fidelity PCR protocol described previously [28]. The library of mutant *apn1*fragments was introduced into the chromosomal *APN1* locus of CORE insertion strains DSC501 or DSC502 via *delitto perfetto*. Mutants were selected based on sensitivity to MMS by replica plating of transformants to medium containing 0.08% MMS. Only G418-sensitive and Ura$^-$ clones were considered for further analysis. Once mutant clones were purified, sensitivity to MMS was re-checked via serial dilution assay

(described in section 2.4). Strain YDM10 was selected as an MMS-sensitive transformant in the genetic screen as described below.

Strains DSC517, DSC518, DSC519 and DSC520 were constructed in order to introduce the mutant alleles *apn1-V156E, E207V* and *apn1-V156E* and *apn1-E207V*, respectively, into non-mutagenized backgrounds. This was done by performing site-directed mutagenesis via *delitto perfetto* of CORE insertion strains DSC501 and DSC502. CORE deletion was performed by transforming the CORE insertion strains with PCR-generated fragments using as templates plasmids pD433, pD434 and PD435 that carry the different mutant versions of *APN1* (section 2.2 and Table S1).

Wild type and mutant strains expressing C-terminal Tandem Affinity Purification (TAP)- tagged Apn1 variants were constructed as follows: an *APN1*-TAP PCR fragment was generated via PCR amplification using the template plasmid pBS1479 [29]. The PCR product was used to transform strains DSC0320, DSC517, DSC519 and DSC520 to create DSC503, DSC521, DSC522, and DSC523 C-terminal TAP-tagged strains, respectively.

Wild type and mutant strains containing the *GAL1* promoter integrated directly upstream of the chromosomal *APN1* coding sequence were constructed as follows: a P_{GAL1}-*APN1* DNA fragment was amplified by PCR using pBS1761 [29] as a template. Strains DSC320, DSC522 and DSC523 were transformed with the resulting PCR fragment to create DSC504, DSC525, DSC526, respectively. Integration of the *GAL1* promoter at the *APN1* locus was confirmed by PCR. Wild type and mutant *APN1* strains expressing N-terminal TAP tagged versions of Apn1 under the control of the *GAL1* promoter at the chromosomal *APN1* locus were constructed as follows: a P_{GAL1}-TAP-

APN1 DNA fragment was amplified by PCR using pBS1761 as a template. Strains DSC320 and DSC519 were transformed with the resulting PCR fragment to create DSC436 and DSC545, respectively. Integration of the P_{GAL1}-TAP construct at the *APN1* locus was confirmed by PCR.

2.4. Analysis of MMS sensitivity

To test sensitivity of yeast cells to MMS, approximately equal numbers of cells were picked from streaks on YPD plates, and 5-fold serial dilutions of cells were plated onto media containing only YPD or YPD with 0.08% MMS. For P_{GAL1}-*APN1* overexpression experiments, cells were plated onto YPD as described above and media containing only YP-galactose or YP-galactose with 0.08% MMS. Plates were incubated for 2 days at 30^{o}C and then analyzed for sensitivity.

2.5. Homology modeling

The amino acid residue sequences of Apn1 and endo IV orthologs from bacterial, fungal, and metazoan organisms were downloaded from the UniProt database [30]. A sequence alignment was then performed with the ClusterX program using the BLOSUM protein weight matrix [31]. One of the closest homologs of Apn1 (41% amino acid identity) with an available protein crystal structure is *E.coli* endo IV. The structure of endoIV (PDB ID: 1qum) was used as the main template to build a homology model of the central region of Apn1. Because the C-terminus of endo IV is 67 amino acid residues shorter than Apn1, it cannot be used as a template to model the C-terminus of Apn1. A BLAST search was performed for the C-terminal part of Apn1 and sequence fragments from three proteins: foldase protein PrsA (PDB ID: 2JZV), fructose-1, 6-bisphosphatase

(PDB ID: 2JJK), and hexokinase-1 (PDB ID: 1BG3). Although Apn1, PrsA, F16BP and hexokinase are not functionally related, these other proteins share significant sequence similarity with the C-terminal domain of Apn1, and can thus be used as acceptable templates for homology modeling. The sequence fragments from these three proteins were highly identical with the sequence fragments of the C-terminal domain of Apn1. The sequences of these three proteins were aligned with the Apn1 sequence, and their structures were used as templates. The homology model of wild type Apn1 was generated with the Modeller v9.7 program [32]. The default values were used for all parameters, except the following: the number of models to generate = 25, library_schedule = 10000, library_schedule = autosched.slow, md_level = refine.very_slow. Ten additional models were generated for each of the loop regions with the md_level parameter set to refine.very_slow. The most accurate model was selected based on the calculated values of DOPE, GA341, and normalized DOPE scoring functions. The Apn1 model was further refined with the Protein Preparation Wizard implemented in the Schrödinger Suite [33-35], and subjected to 1000 iterations of Polak-Riber Conjugate Gradient minimization in the MMFFs force field with the Schrödinger MacroModel program [33-35].

Homology models of Apn1 protein variants were derived from the refined wild type Apn1 model with the Maestro program of the Schrödinger Suite [36], and optimized by energy minimization as described above. Models of Apn1 variants were subjected to Monte Carlo Conformational Search analysis performed for the mutated residue and all residues within 5Å. The residues within an additional 2Å were used as a constrained shell. The Mixed torsional/Low-mode sampling method was utilized, the maximum number of steps was set to 500, and 100 steps per rotatable bond were used. The

calculations were performed with the MacroModel program of the Schrödinger Suite [37].

2.6. Protein analysis

Yeast cell lysates were prepared as follows: A saturated cell culture was inoculated into 50 mL liquid YPD (or 2% YP-Galactose for P_{GAL1} induction) media and growing the cultures to OD_{600} 0.8-1.0. Cells were harvested, washed with sterile water and then frozen at -80^{o}C. Lysis buffer (PBS-Tween plus Roche Complete Mini Protease Inhibitor Cocktail Tablets) was added to the cell pellet and cell breakage was accomplished by adding glass beads to equal the volume of the cell pellet, and then by vortexing for 30 seconds followed by 30 second incubation on ice. This procedure was repeated 20 times. Samples were pelleted and the resulting supernatant was recovered. Protein concentration determinations were made via the Bradford protein assay (BioRad).

AP endonuclease activity was measured by assessing the ability of cellular lysates to cleave an oligonucleotide containing the stable abasic analog, tetrahydrofuran (THF) as well as a 3'-3' phosphodiester linkage at the 3' end, which allowed for labeling of both ends with ^{32}P, as previously described [38-40]. This assay is specific for Apn1 activity as THF is not processed by AP lyases. Oligonucleotides are degraded *in vivo* mainly by cellular 3'-5' exonucleases. The two 5' ends block such degradation. In addition, the 3'-3' phosphodiester linkage is extremely resistant to 5' exonuclease activity. A 0.1 μg aliquot of cell lysate from each strain expressing an Apn1 variant was incubated with oligonucleotide for 15 minutes. Reactions were then loaded onto denaturing-urea polyacrilamide gels to separate the cleavage products. 0.5 μg and 1.0 μg aliquots of cell

lysates from the strains overexpressing Apn1 variants from the integrated P_{GAL1} were incubated with oligo. For thermal stability assessments, 0.5μg aliquots of cell lysates from each Apn1 variant expressing strains was pre-incubated at different temperatures as previously described [41, 42]. Lysates were then incubated with oligonucleotide, and reactions were resolved as described above Dried gels were analyzed for band intensity using a TYPHOON™ phosphoImager and ImageQuant software. The % incision activity results are calculated by taking into account and correcting for labeling efficiency, which is determined by measuring the relative amounts of the 3' and 5' products following cleavage with the purified endonuclease IV enzyme.

For SDS-PAGE and Western blot analysis, aliquots of lysates were boiled with 6X SDS-PAGE loading buffer and samples were run on precast NU-PAGE 10%Bis-Tris minigels (Novex). Western blot analysis was performed with primary anti-TAP (1:5000) antibody (ThermoScientific) or with primary anti-Apn1 (1:200) antibody (Santa Cruz Biotechnology Inc.) Secondary HRP-conjugated anti-rabbit (1:5000) (Promega) and anti-goat (1:5000) (Santa Cruz) were employed for TAP and Apn1 detection, respectively. Blots were stripped and reprobed with anti-Pgk1 primary antibody (1:5000) (Molecular Probes) and anti-mouse secondary antibody (1:5000) (Promega) to determine the relative levels of protein loaded. Chemiluminescence was used to detect immunoreactive proteins, and protein abundance was quantified based on band intensities using ImageQuant software.

Cycloheximide chase experiments were performed as previously described [43] with the following modifications. After aliquots of cells were collected at appropriate time points following addition of cycloheximide to the growth medium, cultures were

spun down and cell pellets were lysed by boiling in Laemmli urea sample buffer (63mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 0.01% Bromophenol blue, 5M Urea, 2% 2-Mercaptoethanol). Samples were then pelleted, and the resulting supernatants were subjected to SDS-PAGE and Western blotting with the anti-TAP antibody as described above. For each strain genotype, half-lives were estimated for each of four individual protein decay curves by fitting a straight line to the data by regression analysis to a semi-log plot of the % protein remaining with the time of incubation plotted along the x-axis using Microsoft Excel.

MG132 experiments were performed as previously described [44]. Lysates were prepared and analyzed in the manner described above for cycloheximide chase experiments.

2.7 Reverse transcriptase-PCR analysis

Cells were cultured in YPD medium and harvested as described in section 2.6. Total RNA was extracted from yeast cell pellets with the MasterPure yeast RNA purification kit from Epicentre. Reverse transcriptase (RT)-PCR was performed with the Quantitect RT-PCR kit from Qiagen. PCR optimization was performed by first determining the exponential range of the PCR reaction by analyzing PCR products at different cycle numbers by agarose gel electrophoresis. Subsequently, varying concentrations of PCR template (RT-PCR reaction) were utilized for validation. No-RT and no-RNA controls for the RT-PCR reaction were amplified by PCR and subjected to agarose gel electrophoresis alongside experimental samples. Diagnostic PCR was performed with primers internal to the *APN1* coding sequence. PCR amplification with

primers internal to the coding sequence of housekeeping gene *SRB4* was used as an internal standard.

3. Results

3.1. An Unbiased Genetic Screen Identifies MMS-Sensitive apn1 Mutants

In order to define structural components important for *in vivo* Apn1 function, we performed a small-scale unbiased, forward genetic screen for *APN1* mutations that affect cellular DNA repair capacity. Our previous studies revealed that in the absence of Apn1 its substrates can be efficiently repaired *in vivo* by two oxidative damage-specific *N*-glycosylase-associated lyases, Ntg1 and Ntg2 [26, 45-49]. For this reason the screen was carried out in a wild type background as well as a background that was both *NTG1*- and *NTG2*-deficient (*ntg1Δ ntg2Δ*). We sought to identify strains that displayed increased sensitivity to the DNA damaging agent methyl methanesulfonate (MMS), an alkylating chemical that induces cytotoxic and mutagenic DNA base damages [49-51]. For the initial screen we targeted a library of mutagenized fragments to the chromosomal *APN1* locus via the *delitto perfetto* cloning technique ([27] and section *2.3*). All transformants were replica plated onto media containing 0.08% MMS to identify sensitive mutant clones. Following confirmation of MMS sensitivity, the *APN1* locus and upstream promoter region of 13 clones were sequenced to identify mutations (Table S2). For our analysis, we selected only clones for which resulting base changes would encode no more than two non-synonymous missense mutations (Table S2).

For further analysis, we re-introduced mutations into the chromosomal *APN1* locus in “clean” non-mutagenized backgrounds to ensure the MMS sensitivity phenotype was due only to mutations in *APN1*. We performed site-directed mutagenesis of plasmid pD0428, which contains the *APN1* coding and promoter sequences, to generate plasmids carrying specific mutant alleles. These plasmids were then used as PCR templates to

construct strains expressing the mutant alleles from the endogenous *APN1* locus in non-mutagenized wild type and *ntg1Δ ntg2Δ* backgrounds. Newly constructed strains were tested for sensitivity to 0.08% MMS.

Sequencing of the MMS-sensitive mutants revealed that strain YDM10 contained two single nucleotide substitutions $T_{467}\rightarrow A$ and $A_{620}\rightarrow T$ in *APN1* coding region, which result in two amino acid substitutions V156E and E207V, respectively. This mutant was of particular interest because V156 is a conserved residue (Fig. 1, indicated by asterisk) for which a mutation at the corresponding residue in the *E.coli* endo IV mutant V143E has been previously shown to exhibit catalytic deficiency and decreased functional repair capacity that is independent of cellular protein levels [52]. This provided the opportunities to elucidate structure-function relationships for endonuclease IV family endonucleases and to explore how this deficiency manifests itself in eukaryotic cells. The E207V substitution is located at a non-conserved residue (Fig. 1A, indicated by star) with no readily predictable structural or functional defects based on its position within the primary amino acid sequence.

3.2. Functional Analysis of apn1-V156E, E207V Mutant

The original mutant strain identified in the screen (YDM10) containing the V156E and E207V substitutions in the *ntg1Δ ntg2Δ* background, was as sensitive to MMS as the strain containing an *APN1* deletion in the *ntg1Δ ntg2Δ* background (Fig. 2A, compare rows 3 and 4). To determine if there was contribution of second site mutations to the MMS sensitivity phenotype, we re-assessed MMS sensitivity after introduction of the $T_{467}\rightarrow A$ and $A_{620}\rightarrow T$ mutations in non-mutagenized backgrounds. In a wild type

(*NTG1, NTG2*) background, *apn1-V156E, E207V* (DSC517) exhibited sensitivity comparable to the *apn1Δ* strain (DSC386) (Fig. 2B, compare rows 2 and 3; Fig. 2C compare rows 2 and 5), indicating that it is a null mutant. In the *ntg1Δntg2Δ* background, which was not subjected to random mutagenesis, *apn1-V156E, E207V* (strain DSC518) exhibited MMS sensitivity similar to *apn1Δ ntg1Δ ntg2Δ* (strain DSC393) (Fig. 2B, compare rows 4 and 5), supporting the conclusion that *apn1-V156E, E207V* is a null mutation.

To determine whether the MMS-sensitivity phenotype could be attributed to either one or both mutations in *APN1*, we constructed strains expressing either *apn1-V156E* or *apn1-E207V* in the non-mutagenized *NTG1, NTG2* background and assessed their MMS sensitivity. The *apn1-V156E* mutant (DSC519) was more sensitive to 0.08% MMS than wild type, but less sensitive than *apn1Δ* (DSC386) (Fig. 2C, compare rows 1, 2 and 3). Further, the *apn1- V156E* mutant (DSC519) appeared to be less sensitive to MMS than the double mutant (DSC517) (Fig. 2C, compare rows 3 and 5), demonstrating an interaction between the two amino acids substitutions whereby the *apn1*-E207V mutation, which does not cause an apparent MMS sensitivity phenotype by itself, exacerbates the repair defect of the *apn1-V156E* mutation.

3.3. Homology Modeling Provides Insight into Structural Consequences of Amino Acid Substitutions

As there is no published high resolution structure for Apn1, our initial approach for elucidating the molecular basis of the mutant phenotypes was to employ computational molecular modeling to predict the 3D structure of Apn1 utilizing

information from alignment between amino acid sequences of endo IV orthologs in bacteria, fungi and *C. elegans* and from the high resolution structure of *E. coli* endo IV (PDB ID: 1qum). Endo IV and Apn1 share 41 percent total amino acid identity. Furthermore, they share 100% amino acid identity with respect to the nine conserved metal binding amino acid residues that comprise the endo IV active site (Fig. 1A, bolded, underlined text), which together are important for DNA incision activity [18, 21, 52].

Residue V156 is located in the hydrophobic core of the protein. The substitution of V156 by glutamic acid (V156E) is predicted to disrupt hydrophobic interactions of C190. Alanine and tryptophan substitutions in the corresponding C190 residue of E. coli endo IV (C177) were shown to substantially affect enzyme activity [45]. In addition, V156E would form additional polar interactions, ultimately affecting hydrophobic interactions of E158 (Fig. 3A). E158 is one of the conserved residues involved in Zn^{2+} ion binding (Fig. 3A and 3B), and its mutation to glycine has been shown to substantially reduce enzyme activity in yeast [53]. Thus, we predict that the substitution of V156 for glutamic acid leads to decreased repair activity by a disruption of the catalytic site and that this structural change is responsible for the MMS sensitivity phenotype (Fig. 3A).

Based on the homology modeling predictions, residue E207 would be located on the surface of the protein at the beginning of the predicted Apn1 alpha-helix 6 (Fig. 3B), away from both the DNA binding surface and the catalytic center. The side chain of E207 would be involved in a network of intra-molecular interactions. Thus, the substitution of surface residue E207 for valine is predicted to disrupt a network of interactions that might be important for other functions, such as protein-protein interactions, but may not be substantial enough to affect functional repair capacity as measured in our assay.

3.4. AP Site Incision Activity is Compromised in apn1-V156E and apn1-V156E, E207V Mutant Cells

Based on predictions inferred from previously reported amino acid substitution mutants and homology modeling, *apn1-V156E* is expected to influence the AP site incision activity of Apn1. We measured the AP site incision activity in cellular lysates using an oligo containing the stable AP site analog, tetrahydrofuran (THF) (Fig. 4A) [38, 39]. Lysates from *apn1-E207V* mutant cells displayed incision activity comparable to wild type (Fig. 4B, compare lanes 3 and 7, and 4C), indicating that, as expected, the predicted structural changes do not impact enzyme activity. Lysates from *apn1-V156E* mutant strains exhibited a 70% reduction in incision activity compared to wild type (Fig. 4B, lanes 3 and 6), which supports the prediction that this substitution negatively affects the active site. There was no detectable incision activity in *apn1- V156E, E207V* mutant lysates (Fig. 4B, lanes 2 and 5), which is consistent with the hypothesis that the E207V substitution can further enhance the defect caused by V156E.

3.5. Cellular Levels of Apn1 V156E and Apn1 V156E, E207V Mutant Proteins are Reduced Compared to Wild Type Apn1 Levels

An alternative explanation for the decreased incision activity in cell lysates from strains expressing Apn1 variants could be variable cellular protein concentrations. Thus, we measured the steady-state levels of Apn1 protein in lysates from wild type and mutant cells. The endogenous expression of Apn1 in cell lysates is difficult to detect using antibodies against Apn1 [54-56]. We therefore integrated the tandem affinity purification (TAP) tag directly downstream of the chromosomal Apn1 locus, and then determined

endogenous Apn1 protein expression levels in cell lysates using an antibody to the TAP moiety [22, 57, 58]. It is important to note that addition of the TAP tag alone did not negatively influence functional repair capacity, as assessed by cell growth in the presence of MMS (Fig. S1).

Cellular levels of Apn1 E207V were similar to levels of the wild type protein. Apn1 V156E was detected at ~15% of wild type levels, and there was no detectable protein expression in lysates from the Apn1 V156E, E207V double mutant (Fig. 5A and 5B). Interestingly, the expression level of the mutant proteins closely correlates with the amount of incision activity within cell lysates (compare Fig. 4C and 5B). These results suggest that Apn1 V156E and Apn1 E207V are both functionally equivalent to wild type Apn1, and that reduced repair capacity in cells containing the Apn1 V156E variant may be due to decreased steady-state protein levels.

The observed decrease in cellular levels of Apn1 V156E mutant protein could be the result of a decrease at the level of either the mRNA transcript or the protein. To investigate whether there was a defect in steady-state expression of mutant *APN1* mRNA transcripts, we performed reverse transcriptase-PCR analysis. *APN1* mRNA transcript levels were similar in mutant and in wild type cells (Figure 5C), suggesting that the decrease in levels of mutant Apn1 (V156E) proteins is not caused by defects in *APN1* mRNA metabolism. Taken together, the data suggest that the *apn1-V156E* mutation plays a prominent role in the molecular phenotype of the double mutant. Therefore, we focused our attention on understanding the molecular basis of Apn1 V156E dysfunction.

3.6. Apn1-V156E Is Thermostable but Degraded More Rapidly than Wild Type Apn1

The decrease in steady-state expression level of Apn1 protein in *apn1-V156E* mutants could be due to a decrease in protein production and/or an increase in protein degradation. The former possibility would suggest that mutant Apn1 V156E polypeptide can be produced but may be translated inefficiently. This is unlikely because there are no mutations in the regulatory region upstream of the *APN1* locus in this mutant (data not shown). Moreover, the $T_{467} \rightarrow A$ base substitution in the *apn1-V156E* mutant results in usage of the preferred glutamic acid codon in yeast (GAA versus GAG) [59], ruling out a codon bias mechanism. A more plausible explanation for the decreased mutant protein level is that the V156E substitution leads to degradation by causing the protein to be unstable. To test this prediction, we assessed the thermostability of Apn1 variants within cellular lysates. Following pre-incubation at increasing temperatures, lysates from wild type and Apn1 mutant cells displayed similar resistance to heat treatment with respect to AP incision activity, with the loss of activity for each lysate occurring at 72^{o} C (Fig. 6A). This suggests that Apn1 V156E, when in its functional conformation, is not less stable than wild type Apn1.

Missense substitution mutations can also impair the proper folding of a protein into its mature form, often leading to degradation. We predicted that the V156E substitution causes less of the functional protein to accumulate due to accelerated elimination from the cell. To determine whether mutant Apn1 V156E protein is degraded more quickly than wild type Apn1, we determined the half-life for the protein variants by employing the translational inhibitor cycloheximide and measuring protein levels via SDS-PAGE and Western blot analysis on extracts from cells expressing C-terminal TAP-

tag versions of Apn1. Following addition of cycloheximide to the culture medium, the amount of Apn1 V156E protein decreased faster than wild type protein (Fig. 6B and 6C). While the half-life of wild type Apn1 was estimated to be ~90 minutes, the half-life of V156E was reduced by ~3-fold to an estimated ~30 minutes (Fig. 6D). To determine whether proteasome function was involved in regulating the turnover of Apn1 V156E we employed the proteasome inhibitor MG132 in combination with cycloheximide and performed a chase analysis experiment. Inhibition of proteasome function led to accumulation of the wild type but not the variant protein in cells (Fig. 6E). Our results suggest that, while wild type protein turnover may be regulated by the proteasome, cells use an alternative pathway to eliminate the mutant Apn1 V156E protein. Taken together, the data support the prediction that the mutant protein is degraded more rapidly than wild type, leading to the observed decrease in steady-state levels of Apn1 V156E.

3.7. Overexpression of apn1-V156E Restores Cellular DNA Repair Activity

Since the AP site incision activity of Apn1 variants correlates with the steady-state protein level in cell lysates, we hypothesized that increased cellular Apn1V156E levels would result in increased AP site incision activity. To overexpress *APN1*, we integrated the galactose-inducible *GAL1* promoter upstream of the chromosomal *APN1* open reading frame and induced expression in galactose-containing growth medium. We examined the galactose-induced expression levels of the Apn1 variants in cell lysates via SDS-PAGE and Western blot analysis using the anti-Apn1 antibody. (Fig. 7A). Galactose-induced overexpression of wild type Apn1 and both variants lead to production of comparable amounts of protein, although Apn1 V156E accumulated to lower levels.

We next decided to measure the difference in endogenous and galactose-induced levels of Apn1. To estimate the relative overexpression levels of Apn1 variants versus endogenous wild type Apn1 expression levels, we first integrated a P_{GAL1}-TAP construct directly upstream of the chromosomal Apn1 locus. We then compared galactose-induced overexpression levels of TAP-Apn1 and TAP-Apn1-V156E (C-terminal tags) to that of Apn1-TAP expressed from the endogenous *APN1* promoter at the chromosomal locus using the anti-TAP antibody. Compared to normal Apn1 protein levels, galactose-induced *APN1* overexpression resulted in an ~30-fold increase in protein levels (Fig. 7B and 7C). The difference in protein levels in wild type without galactose induction and P_{GAL1} − *apn1-V156E* strain subjected to galactose-induced overexpression was barely distinguishable (Fig. 7B, first and third lanes, and 7C).

To test the prediction that the Apn1 V156E retains catalytic function, we measured AP incision activity in lysates from cells overexpressing the mutant alleles. In lysates containing increased levels of Apn1 V156E following galactose-induced expression, incision activity was increased compared to the level of activity in lysates from cells expressing *apn1*-V156E from the endogenous *APN1* promoter (Fig. 7D, lanes 4 and 7). In addition, the incision activity in lysates harboring overexpressed Apn1 V156E was comparable to incision activity in lysates from cells expressing Apn1 from the endogenous promoter (Fig. 7D, lanes 4 and 6). Notably, the *E. coli* endo IV variant V143E previously reported by Jilani, *et al.* did not display catalytic activity upon overexpression in cell lysates [52]. These results indicate that while the Apn1 V156E substitution leads to decreased accumulation of native protein within the cell, this protein variant retains catalytic activity comparable to wild type Apn1.

To determine whether overexpression of *apn1-V156E* could restore *in vivo* DNA repair capacity, we assessed growth of cells expressing Apn1 V156E under the control of the *GAL1* promoter in the presence of galactose and MMS. The MMS sensitivity of cells overexpressing Apn1 V156E appeared to be similar to the MMS sensitivity of the cells expressing endogenous levels of wild type Apn1. Galactose-induced overexpression of *apn1-V156E* leads to accumulation of mutant protein at levels similar to endogenous wild type levels under non-induction conditions. (Fig. 7E, compare rows 1 and 6). Galactose-induced overexpression of wild type *APN1* and the functionally equivalent *apn1-E207V* led to inability to grow in the presence of MMS (Fig. 7E, rows 5 and 7). This is presumably due to accumulation of toxic BER intermediates upon increased repair activity [58]. Nonetheless, accumulation of Apn1 V156E at increased concentrations functionally restored cellular DNA repair capacity.

4. Discussion

The absence of major AP endonuclease function in cells results in inability to repair a large subset of spontaneous and genotoxic agent-induced DNA damage [60]. In humans, genetic defects in APE1 are associated with diseases including cancer, but the precise molecular mechanisms related to pathology are largely unknown [9-11, 61]. A major challenge to elucidating such mechanisms is the complexity of studies in human cells including inter-individual genetic variability, cell-type specific gene expression and the multiple DNA repair-independent cellular functions of APE1.

We studied genetic defects in Apn1, the major AP endonuclease in *Saccharomyces cerevisiae,* since the basic biochemical mechanisms of BER are highly conserved from yeast to humans. We initiated our studies in a functionally relevant context by directly screening random mutations at the chromosomal *APN1* locus to discover aspects of structure that are important for *in vivo* repair activity. We identified amino acid variant Apn1 V156E, which renders cells sensitive to growth in the presence of MMS. The V156E amino acid substitution is homologous to the previously characterized endo IV V143E that also confers MMS sensitivity [52]. Thus, this study represents the independent identification of an amino acid substitution at an evolutionally conserved residue outside the active site, but within the hydrophobic core, that disrupts Apn1 biological function. The endo IV V143E mutant displayed defective repair capacity independent of its steady-state expression level [52]. Therefore, along with the structural information from *E. coli* endo IV, we predicted that the Apn1 V156E substitutions would cause catalytic deficiency via disruption of the important active site residue, E158 [53]. Unlike the *E. coli* V143E substitution, Apn1 V156E retained catalytic ability and

exhibited normal levels of repair when expressed at increased steady-state levels. Surprisingly, the repair defect of this amino acid change could not be attributed to impaired catalytic function, but was instead due to decreased cellular protein levels compared to wild type Apn1. Although the specific mechanism that targets Apn1 V156E for elimination from the cell are unknown, the substitution of the hydrophobic valine residue for the hydrophilic glutamic acid residue within the hydrophobic core suggests that the V156E substitution may cause protein instability and/or a defect in protein folding. It will be important to elucidate the precise molecular defects resulting from this particular amino acid substitution in future studies.

Our study revealed different molecular phenotypes for the mutant yeast Apn1 versus the homologous mutant *E. coli* endo IV , indicating that endo IV V143 and Apn1 V156 may play somewhat different roles in endonuclease structure. The predictive potential of our analysis was limited by the fact that no high-resolution structure of Apn1 has been reported to date. Nonetheless, apparent differences between Apn1 and endo IV, however subtle, can be exploited to further elucidate structure-function relationships for this family of AP endonucleases.

A common readout currently used in the field to measure protein dysfunction caused by missense mutations in APE1 is the catalytic activity of purified enzymes, which has only been validated for a small number of variants [10, 11]. While it has been proposed that alterations in repair enzymes likely to affect repair capacity will occur in the catalytic domain of the protein [9], our study and various *in silico* analyses of APE1 missense variants [11, 12, 62] provide evidence that this may not always be the case. Disruption of Apn1 active site residues E158 and D192 has been shown to severely affect

catalytic capability and cellular repair capacity while leaving expression level intact [53]. Our results reveal that the opposite can be true: disruption of a residue outside the active site of Apn1 can also affect repair capacity by a mechanism that does not affect enzyme activity, but instead appears to disrupt folding and/or stability. Studies utilizing the high-resolution structure of APE1 to computationally predict the impact of amino acid substitutions found in the human population have identified many changes not expected to directly affect DNA binding, catalytic activity or protein-protein interactions [11, 12]. Some of these changes are expected to impact protein stability, ligand binding, or hydrophobicity [11, 12]. We predict that such perturbations in APE1 biology could have as significant an effect on repair capacity as missense substitutions in known functional domains. Whether or not these mutants affect repair capacity *in vivo* is yet to be determined, but based on our unexpected findings they should be prioritized for experimental validation of function. Such mutant proteins, especially those that may be associated with pathological conditions, may be candidates for pharmacological chaperone therapy, which seeks to specifically target unstable and/or misfolded mutant proteins to promote stabilization of the functional structure [63, 64].

The central role Apn1 plays in the repair of DNA damage is underscored by the fact that yeast cells completely lacking the major AP endonuclease display a mutator phenotype and are extraordinarily sensitive to oxidative and alkylation DNA damaging agents [65]. The results reported here illustrate that change in the cellular protein level of Apn1 and Apn1 variants above or below a critical concentration can substantially influence repair capacity. Such imbalances in other BER proteins are known to be mutagenic as exemplified by studies in yeast where overexpression of *MAG1* increases

spontaneous mutation rates by several hundred-fold [66]. In mice heterozygous for the gene encoding the APE1 mouse homolog *Apex*, steady-state expression levels are reduced by 50%, which is accompanied by increased frequency of spontaneous tumor development [67]. Our finding that reduced Apn1 V156E mutant protein levels lead to MMS sensitivity, supports a potential role for similar APE1 defects in cancer development according to a previously proposed model whereby spontaneous damage combined with BER defects over time lead to accumulation of mutations, genomic instability and eventually cellular transformation [68]. Accelerated protein degradation is an important mechanism for loss of function caused by missense mutations outside known functional regions of a protein [69]. Our studies have important implications for the use of simple model genetic organisms such as yeast to elucidate the functional role of missense SNPs predicted to influence DNA repair capacity and that are associated with risk for human disease development.

5. References

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Table 1 Genotypes of strains used in this study

Strain	Relevant genotype or description	Reference/Source
hDNP16	*MAT***a**/*MATα rad1*::*kanMX/RAD1 ntg1*::*hphMX4/NTG1 ntg2*::*BSD/NTG2 apn1*::*TRP1/APN1 his7-1/his7-1 lys2Δ5′*::*LEU-lys2Δ3′/lys2Δ5′*::*LEU-lys2Δ3′ ade5-1/ade5-1 trp1-289/trp1-289 ura3-52/ura3-52*	[26]
hDNP19	*MAT***a**/*MATα rad1*::*kanMX/RAD1 ntg1*::*hphMX4/NTG1 ntg2*::*BSD/NTG2 apn1*::*TRP1/APN1 DSF1*::*URA3/DSF1 his7-1/his7-1 lys2Δ5′*::*LEU-lys2Δ3′/lys2Δ5′*::*LEU-lys2Δ3′ ade5-1/ade5-1 trp1-289/trp1-289 ura3-52/ura3-52*	[26]
DSC320[1]	*MATa [lys2::Alu-DIR-LEU2-lys2D5'] ade5-1 his7-2 leu2-3 112 trp1-289 ura3-52*	this study
DSC386[2]	*MATa [lys2::Alu-DIR-LEU2-lys2D5'] ade5-1 his7-2 leu2-3 112 trp1-289 ura3-52 ntg1::hyg ntg2::bsd*	this study
DSC393[2]	*MATa his7-1 lys2Δ5′*::*LEU-lys2Δ3′ ade5-1 trp1-289 ura3-52 apn1::TRP1 ntg1::hyg ntg2::bsd*	this study
DSC378[2]	*MATa his7-1 lys2Δ5′*::*LEU-lys2Δ3′ ade5-1 trp1-289 ura3-52 apn1::TRP1*	this study
DSC501	DSC0320 *apn1::KlURA3 kanMX4 GAL1*-I-*SceI*	this study
DSC502	DSC0386 *apn1::KlURA3 kanMX4 ntg1::hyg ntg2::bsd GAL1*-I-*SceI*	this study
YDM10[3]	*MATa [lys2::Alu-DIR-LEU2-lys2D5'] ade5-1 his7-2 leu2-3 112 trp1-289 ura3-52 apn1 V156E, E207V*	this study
DSC517[4]	DSC320 *apn1-V156E, E207V*	this study
DSC518[4]	DSC320 *apn1-V156E, E207V ntg1::hyg ntg2::bsd*	this study
DSC519[4]	DSC320 *apn1-V156E*	this study
DSC520[4]	DSC320 *apn1-E207V*	this study
DSC503	DSC320 *APN1-TRP1-TAP*	this study
DSC521	DSC320 *apn1-V156E, E207V TRP1 -TAP*	this study
DSC522	DSC320 *apn1-V156E TRP1-TAP*	this study
DSC523	DSC320 *apn1-E207V- TRP-TAP*	this study
DSC436	DSC320 *TRP1-*P_{GAL1}*-TAP-APN1*	this study
DSC519	DSC519 *TRP1-*P_{GAL1}-TAP-*apn1-V156E*	this study
DSC504	DSC320 *TRP1-*P_{GAL1}*-APN1*	this study
DSC525	DSC320 *TRP1-*P_{GAL1}*-apn1-V156E*	this study
DSC526	DSC320 *TRP1-*P_{GAL1}*-apn1-E207V*	this study

1 This strain is a haploid spore of hDNP16.

2 These strains are haploid spores of hDNP19.

3 This strain was selected as MMS-sensitive after transformation of DSC502 with mutagenized *APN1* PCR fragments.
4 In these strains, the mutations selected for in the *APN1* locus of YDM10 were re-introduced in order to confirm the MMS-sensitivity phenotype.

Figure1

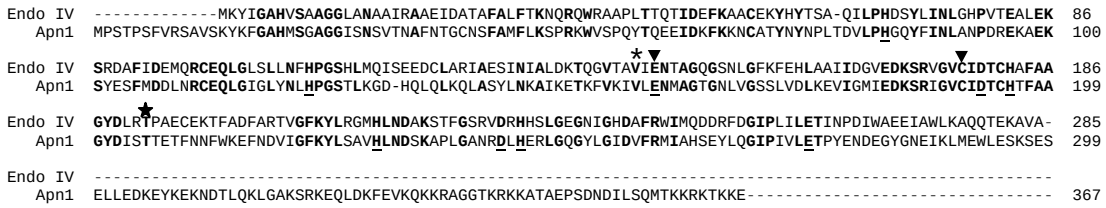


Figure 1: Amino acid alignment of *E. coli* endo IV and *S. cerevisiae* Apn1. Conserved residues indicated with bold font. Zn^{2+}-binding active site residues indicated in bold font and underlined. Residues V156 and E207 indicated by an asterisk and a star, respectively. Residues predicted to be affected by the V156E substitution are indicated with arrow heads.

Figure 2.

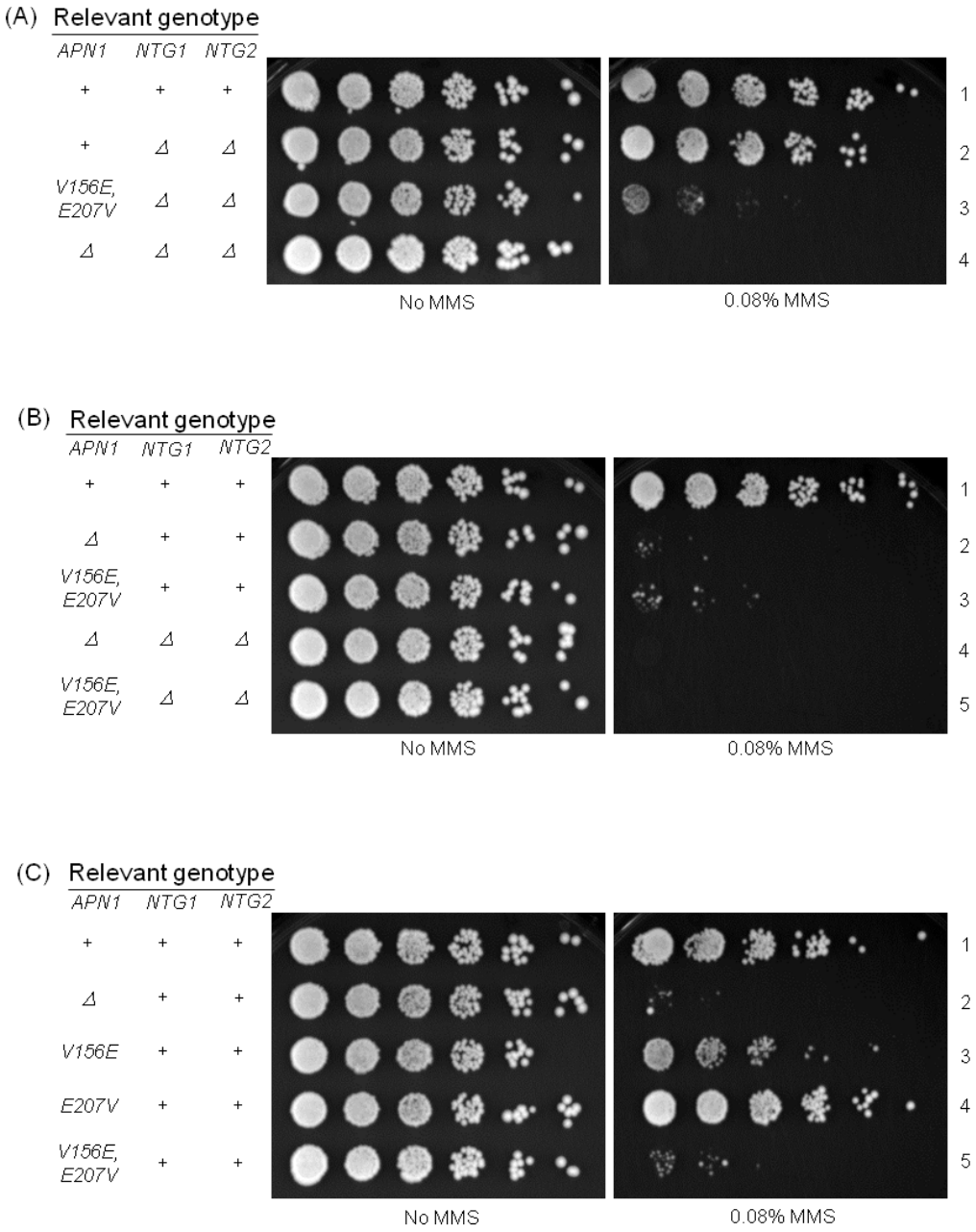


Figure 2: MMS sensitivity of *apn1* mutant strains. 5-fold serial dilutions of cells were spotted onto YPD plates containing 0.08% MMS. The genotypes with respect to *APN1*, *NTG1* and *NTG2* are designated +: wild type, *Δ*: deletion, or by amino acid substitution. **(A)** MMS sensitivity of the original mutant strain YDM10. Row 1: *APN1* (DSC320),

Row 2: *ntg1Δ ntg2Δ* (DSC386), Row 3: *apn1-V156E, E207V ntg1Δ ntg2Δ* (YDM10), Row 4: *apn1Δ ntg1Δ ntg2Δ* (DSC393). **(B)** MMS sensitivity of *apn1-V156E, E207V* in non-mutagenized backgrounds. Row 1: *APN1* (DSC320); Row 2: *apn1Δ* (DSC393); Row 3: *apn1-V156E, E207V* (DSC517), Row 4: *apn1Δ ntg1Δ ntg2Δ* (DSC378); Row 5: *apn1-V156E, E207V ntg1Δ ntg2Δ* (DSC518). **(C)** MMS sensitivity of single mutants *apn1-V156E* and *apn1-E207V,* and double mutant *apn1-V156E, E207V* in non-mutagenized background. Row 1: *APN1* (DSC320); Row 2: *apn1Δ* (DSC326); Row 3: *apn1-V156E* (DSC519); Row 4: *apn1-E207V* (DSC520); Row 5: *apn1-V156E, E207V* (DSC517).

Figure 3.

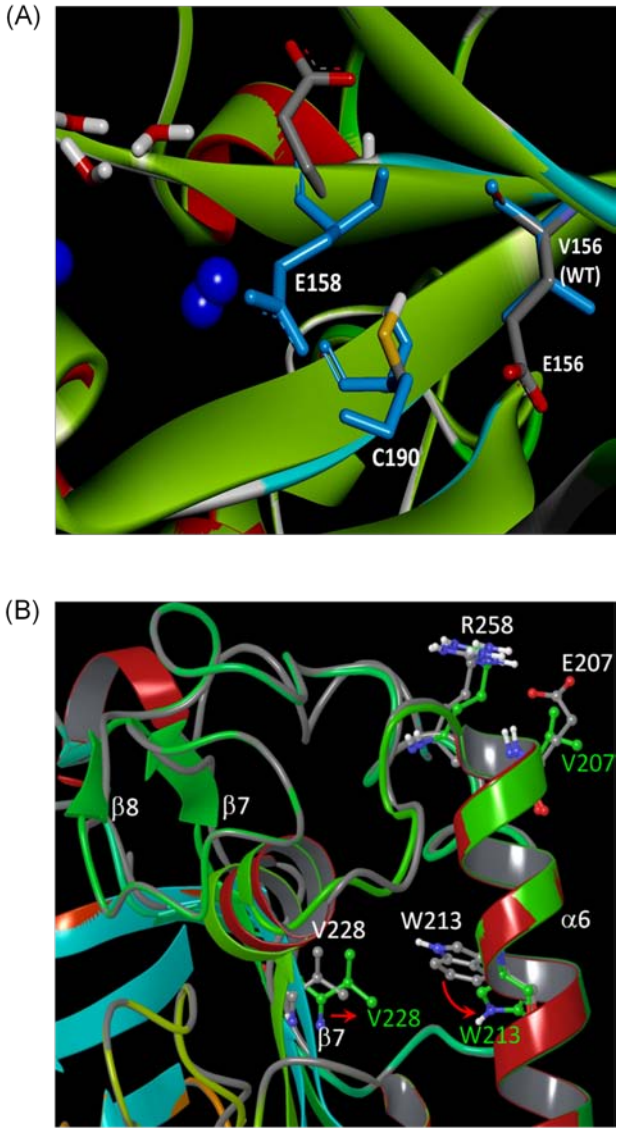
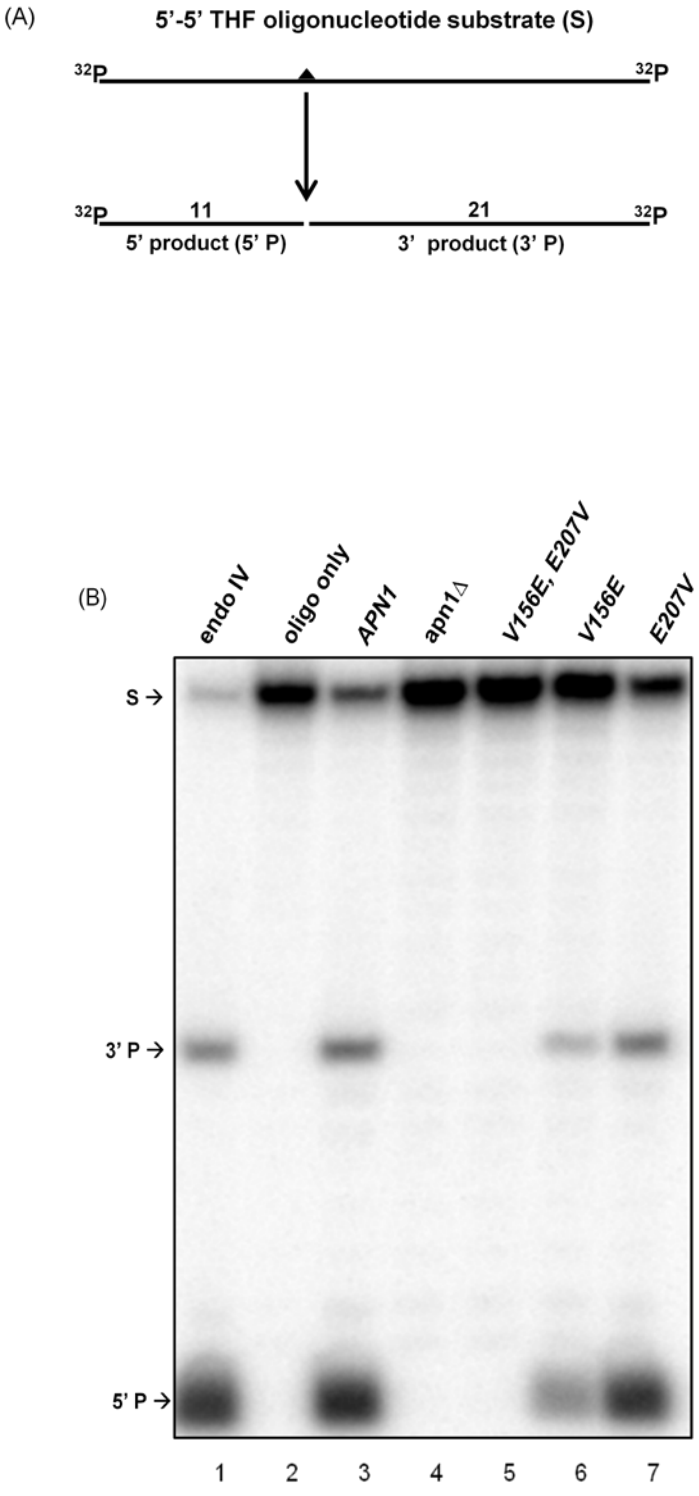


Figure 3: Homology modeling of Apn1. (A) Superimposed structures of the predicted models of wild type and Apn1 V156E, E207V zoomed in on the V156 region. Green: wild type backbone, Blue: wild type side chains, Red: mutant α-helices, Grey: mutant loops, Teal: mutant β-sheets, Blue spheres: Zn^{2+} ions. **(B)** Superimposed structures of the predicted models of wild type and Apn1 V156E, E207V zoomed in on the E207 region.

Grey ribbons: wild type backbone, Grey side chains: wild type residues, Green ribbons: wild type backbone, Green side chains: residues in the mutant, Red: wild type α-helices, Teal: wild type β-sheets.

Figure 4.


(A)
5'-5' THF oligonucleotide substrate (S)
32P
32P
32P
11
21
32P
5' product (5' P)
3' product (3' P)
(B)
endo IV
oligo only
APN1
apn1Δ
V156E, E207V
V156E
E207V
S →
3' P →
5' P →
1
2
3
4
5
6
7

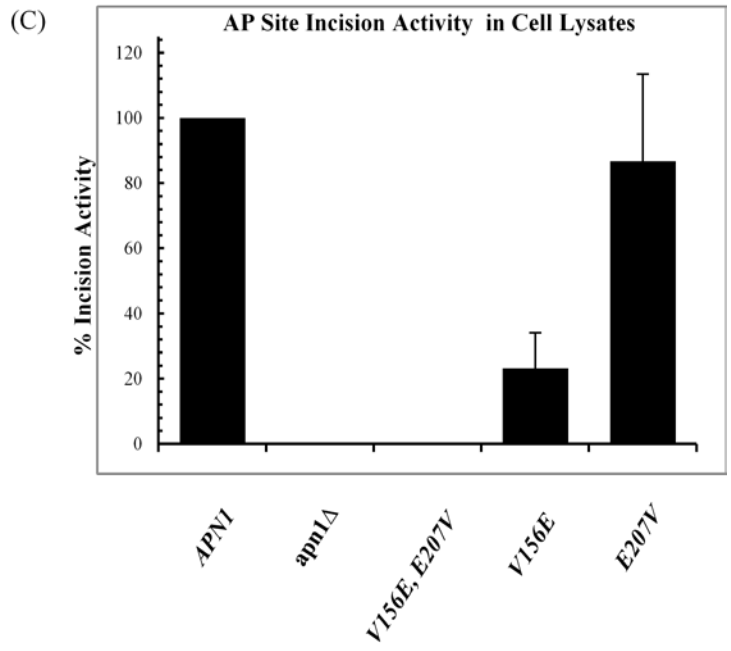
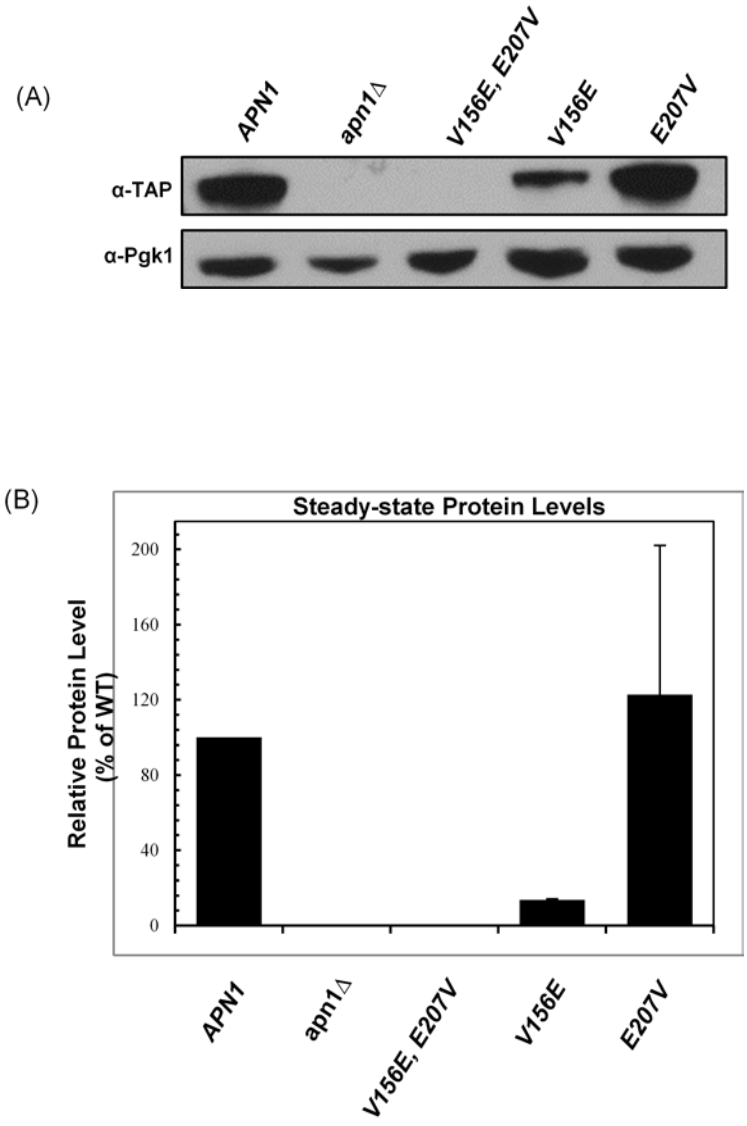


Figure 4: Measurement of AP site incision activity in cell lysates containing Apn1 variant proteins. (A) Schematic diagram of oligonucleotide substrate used in AP endonuclease incision assay. The oligonucleotide substrate contains a tetrahydrofuran (THF) site and is labeled at both ends with ^{32}P. Expected cleavage product sizes are denoted. **(B)** Representative image of oligo incision assay. AP endonuclease activity was determined by assessing the extent of cleavage following incubation of oligo with 1.0 μg aliqots of lysates from cells of the indicated *APN1* genotypes. No lysate was added to the "oligo only" reaction. Incubation of purified recombinant endo IV with oligo served as a positive control. Incision products were resolved on a polyacrilamide urea-denaturing gel and visualized via phosphorimager. Arrows indicate the positions of the oligonucleotide substrate (S), the 3' cleavage product (3' P), and the 5' cleavage product (5' P). **(C)** Quantification of AP incision activity from three independent experiments. Error bars represent standard deviation.

Figure 5.


(A)
APN1
apn1Δ
V156E, E207V
V156E
E207V
α-TAP
α-Pgk1
(B)
Steady-state Protein Levels
Relative Protein Level (% of WT)
200
160
120
80
40
0
APN1
apn1Δ
V156E, E207V
V156E
E207V

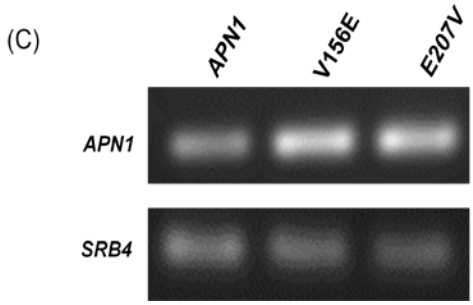
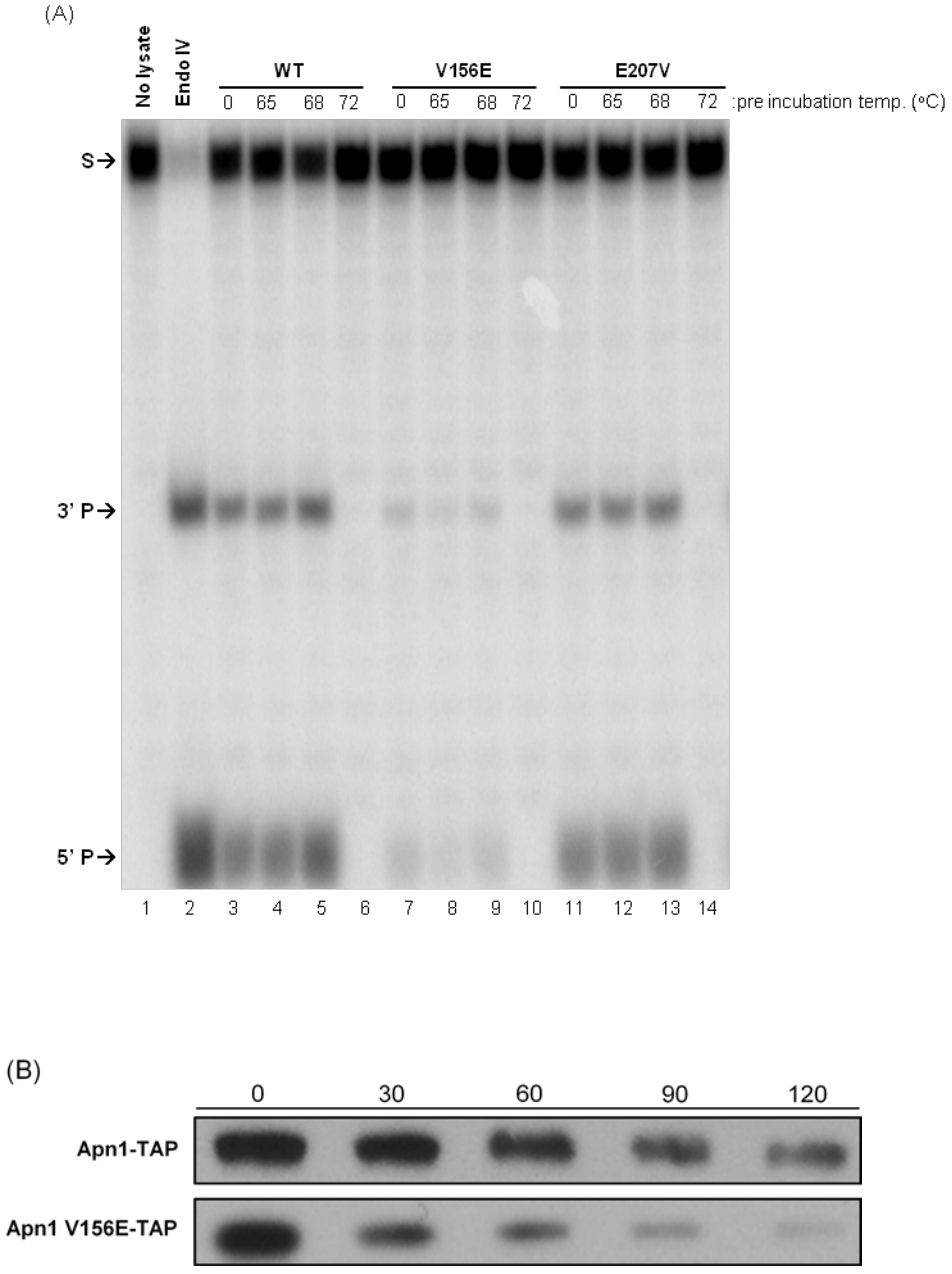


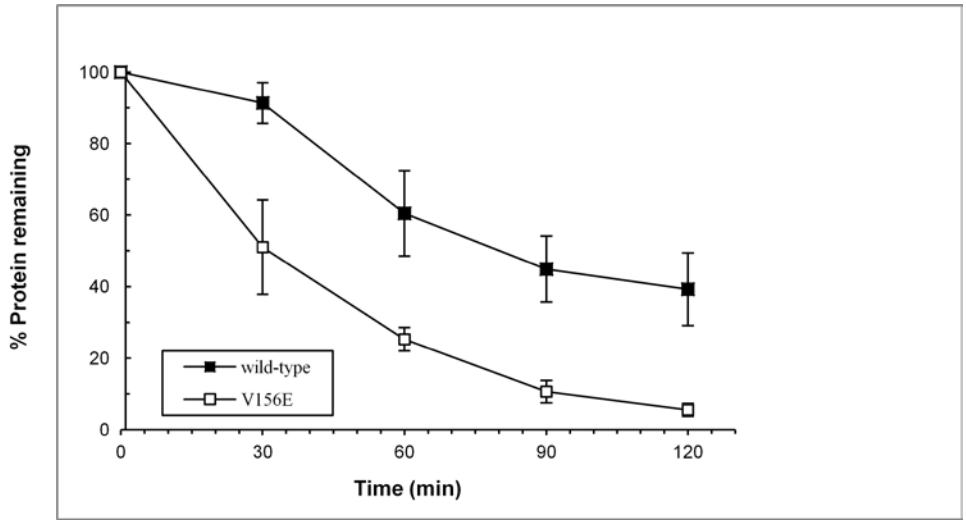
Figure 5: Quantification of endogenous Apn1 protein and *APN1* mRNA levels.

(A) Detection of endogenous protein levels in cells with TAP-tagged Apn1 variants. 50 μg of lysates from cells containing C-terminal TAP fusions of the wild type and variant Apn1 proteins were resolved by SDS-PAGE and subjected to Western blot analysis by probing with anti-TAP antibody. Blots were reprobed with anti-Pgk1 antibody to determine the relative levels of protein loaded. **(B)** Quantification of protein levels from three independent experiments. Error bars represent standard deviation. **(C)** Quantification of *APN1* mRNA levels. Total RNA from wild type and mutant strains was subjected to reverse transcriptase (RT)-PCR. RT-PCR products were subjected to PCR analysis and resolved on a 1.2% agarose gel stained with ethidium bromide for visualization. Gel image is representative of at least three independent experiments.

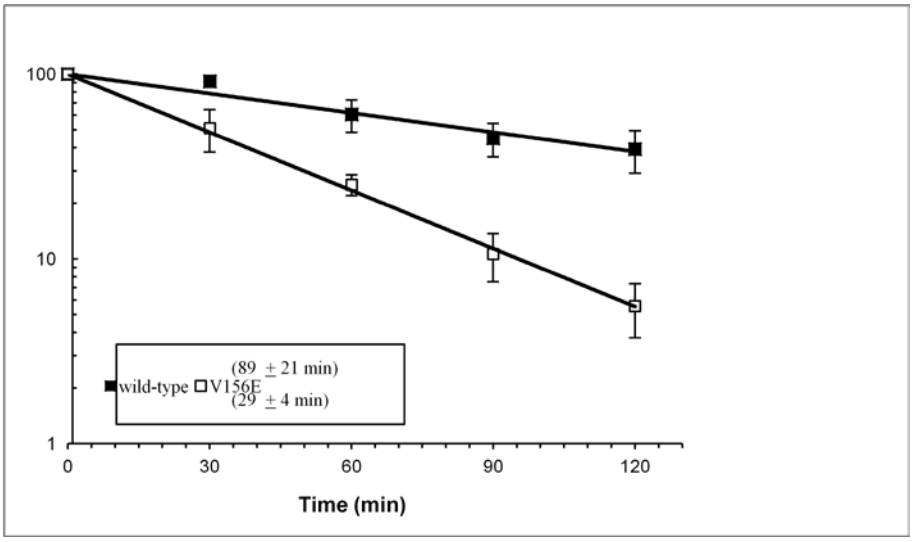
Figure 6.


(A)
No lysate
Endo IV
WT
V156E
E207V
0 65 68 72 0 65 68 72 0 65 68 72 :pre incubation temp. (°C)
S→
3' P→
5' P→
1 2 3 4 5 6 7 8 9 10 11 12 13 14
(B)
0 30 60 90 120
Apn1-TAP
Apn1 V156E-TAP

(C)


100
80
60
40
20
0
% Protein remaining
wild-type
V156E
0
30
60
90
120
Time (min)

(D)


100
10
1
% Protein remaining
wild-type
V156E
(89 ± 21 min)
(29 ± 4 min)
0
30
60
90
120
Time (min)

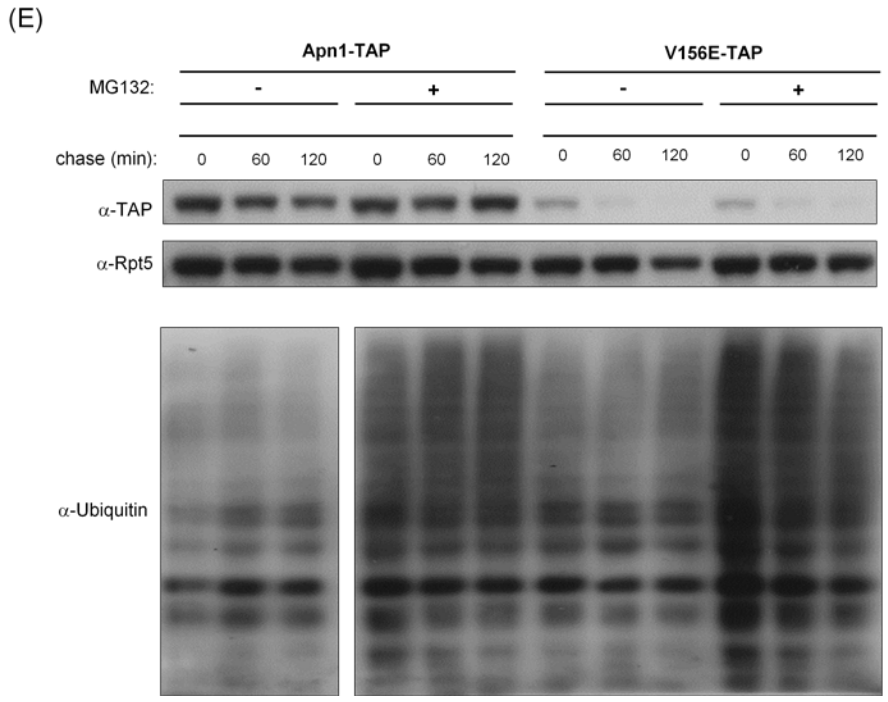
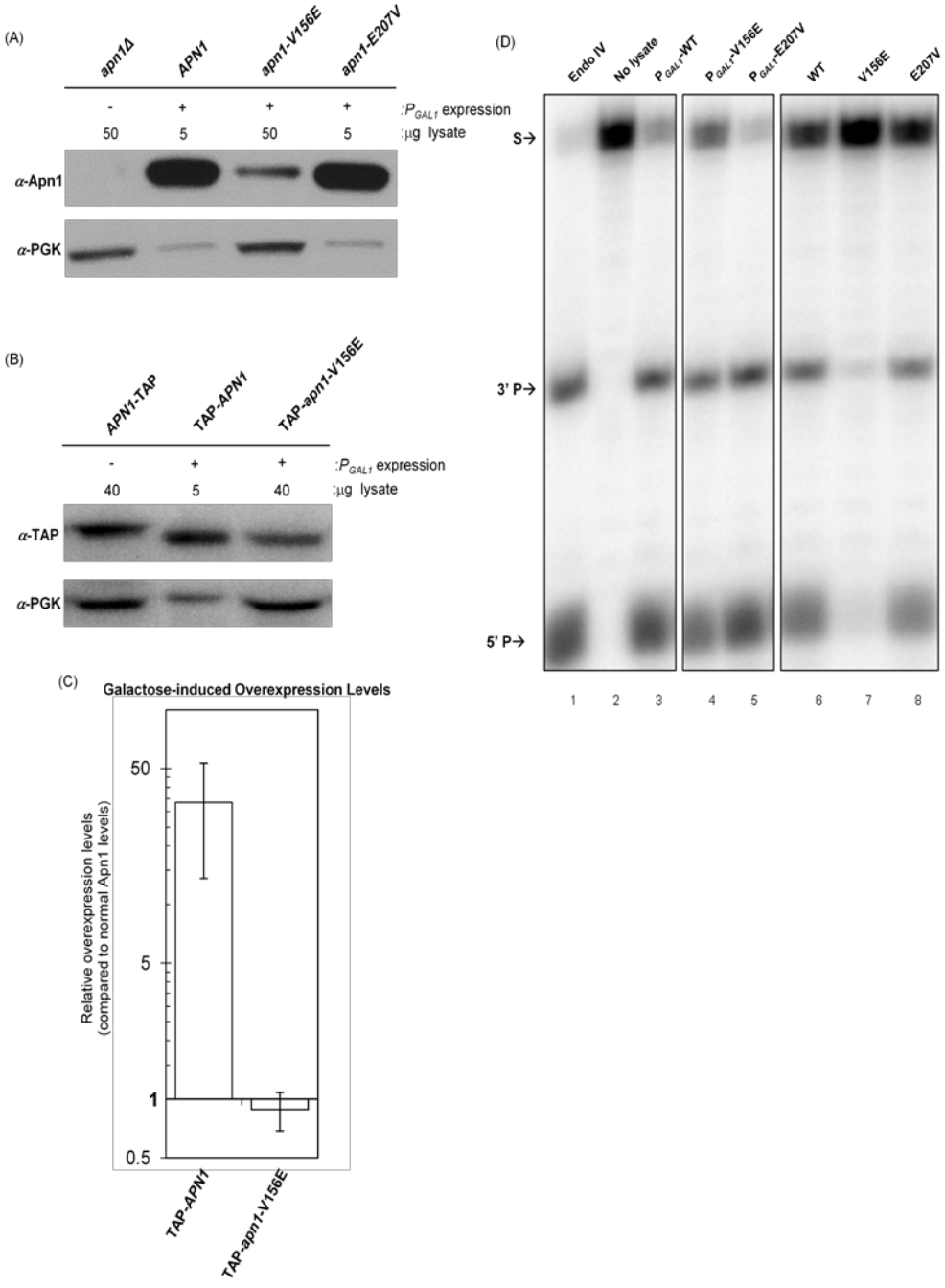


Figure 6. Thermostability and degradation of Apn1 variant proteins. (A) Thermal stability incision activity assay. Aliquots of 0.5 μg of cellular lysates from the indicated strains were pre-incubated at 0^{o} C, 65^{o} C, 68^{o} C or 72^{o} C for thirty minutes. Lysates were then incubated with a THF-containing oligonucleotide substrate labeled at both ends with ^{32}P. Reaction products were resolved on a polyacrylamide urea-denaturing gel and visualized via phosphorimager. Arrows indicate the positions of the oligonucleotide substrate (S) and the 3' and 5' cleavage products (3'P and 5'P, respectively). **(B)** Cycloheximide chase assay. Cells with the TAP tag fused to the C-terminus of Apn1 (genotypes indicated) were gown in YPD medium to log-phase, harvested, and adjusted to 2.5 OD_{600}. Cycloheximide was added to the growth medium and aliquots were removed from yeast cell cultures at the indicated timepoints. Cell lysates were resolved by SDS-PAGE and subjected to Western blot analysis by blotting with anti-TAP

antibody. **(C)** Quantification of cycloheximide chase analysis results. Graph represents the average of at least four independent experiments. Error bars depict standard deviation. **(D)** Protein half-life of wild type and Apn1 V156E variants. For each strain genotype, half-lives were estimated for each of four individual protein decay curves and then averaged as described in "materials and methods". Graph represents the decay curves based on average protein levels measured in four independent experiments for each data point for each strain. Intersection of dotted horizontal line and decay curves indicates half-life of the protein (50 on log scale). **(E)** Effects of proteasome inhibition on degradation of Apn1 variant proteins. Cells with the TAP tag fused to the C-terminus of Apn1 (genotypes indicated) were grown to log-phase in SD-complete media with proline as the nitrogen source and supplemented with 0.003% SDS. MG132 was added to the growth medium and after 30 minutes of growth at 30^{o}C. Cycloheximide was then added to the cell culture and aliquots were removed at the indicated timepoints.Cell lysates were resolved by SDS-PAGE and subjected to Western blot analysis by blotting with anti-TAP antibody. Blots were reporobed with anti-Rpt5 antibody (Abcam) to visualize the relative levels of protein loaded. Blots were also probed with anti-ubiquitin P4D1 antibody (Upstate) to ensure MG132 could enter the cells and affect proteasome activity as indicated by increased levels of poly-ubiquitinated proteins.

Figure 7


(A)
apn1Δ
APN1
apn1-V156E
apn1-E207V
- + + + :P_{GAL1} expression
50 5 50 5 :µg lysate
α-Apn1
α-PGK
(B)
APN1-TAP
TAP-APN1
TAP-apn1-V156E
- + + :P_{GAL1} expression
40 5 40 :µg lysate
α-TAP
α-PGK
(C)
Galactose-induced Overexpression Levels
Relative overexpression levels (compared to normal Apn1 levels)
50
5
1
0.5
TAP-APN1
TAP-apn1-V156E
(D)
Endo IV
No lysate
P_{GAL1}-WT
P_{GAL1}-V156E
P_{GAL1}-E207V
WT
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E207V
S→
3' P→
5' P→
1 2 3 4 5 6 7 8

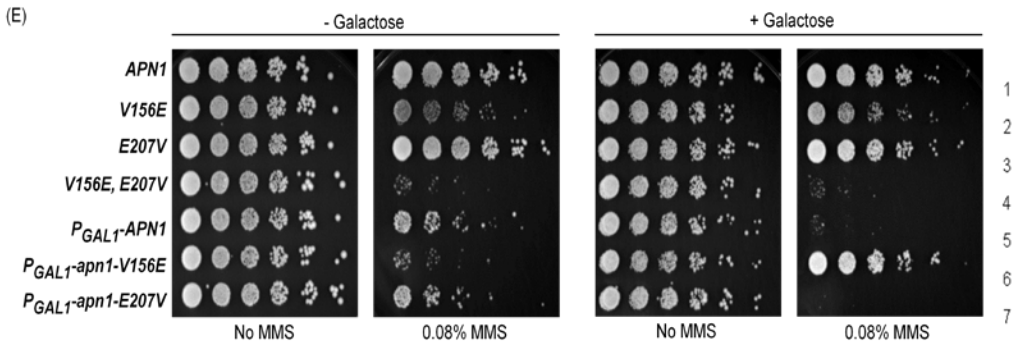

Figure 7: Apn1 V156E overexpression functionally restores cellular DNA repair activity. (A) Galactose-induced expression levels of Apn1 variant proteins. 5.0 μg aliquots of lysates from P_{GAL1}-*APN1* and P_{GAL1}-*apn1-E207V* cells and 50 μg aliquots of lysates from , *apn1Δ GAL-apn1-V156E* cells, , grown in 2% galactose to induce *APN1* overexpression (except for the *apn1Δ* strain, which was used for negative control), were resolved by SDS-PAGE and subjected to Western blot by probing with the anti-Apn1 antibody (top panel). Different amounts of lysates were used for comparison. Blots were reprobed with an antibody for PGK to determine the relative levels of protein loaded (bottom panel). **(B)** Galactose-induced expression levels of TAP-tagged Apn1 variant proteins. A 5.0 μg aliquot of lysate from P_{GAL1}-TAP-*APN1* and 40 μg aliquots of lysates from *APN1*-TAP and P_{GAL1}-TAP-apn1-V156E cells, grown in 2% galactose, were resolved by SDS-PAGE and subjected to Western blot by probing with the anti-TAP antibody (top panel). Blots were reprobed for PGK to determine the relative levels of protein loaded (bottom panel) **(C)** Quantification of protein levels relative to the levels of wild type protein expressed from the native promoter for three independent experiments. Error bars represent standard deviation. **(D)** AP incision activity in cells with galactose-induced overexpression of Apn1 variants. A THF site-containing oligonucleotide

substrate labeled at both ends with ^{32}P was incubated with 0.5 μg of lysates from cells of the indicated strains grown in the presence of 2% galactose to induce *APN1* overexpression (lanes 3,4 and 5) or in YPD (lanes 6, 7 and 8) without induction. Note that the strains in lanes 6, 7, and 8 contained endogenous levels of Apn1 and Apn1 variants. Reaction products were resolved on a polyacrilamide urea-denaturing gel and visualized via phosphorimager. Arrows indicate the positions of the oligonucleotide substrate (S) and the 3' and 5' cleavage products (3'P and 5'P, respectively). Separate panels represent reactions resolved in non-adjacent lanes on the same gel. **(E)** MMS sensitivity of cells overexpressing Apn1 variants. 5-fold serial dilutions of cells were grown on YPD (denoted as "-Galactose") or 2% galactose (denoted as "+ Galactose") to induce *APN1* overexpression with or without 0.08% MMS. MMS sensitivity was assessed after incubation for two days at 30 ^{o}C. Strain genotypes with respect to *APN1* allele are indicated to the left of each row.

Table S1. Plasmids used in this study

Plasmid	Description	Reference
pRS316	CEN *URA3* Amp^r	[23]
pBS1479	C-terminal TAP; *TRP1* Amp^r	[28]
pBS1761	N-terminal *GAL1*-TAP; *TRP1* Amp^r	[28]
pD0428	pRS316-*APN1*; CEN *URA3* Amp^r	this study
pRS467	pRS316-*apn1-V156E*; CEN *URA3* Apm^r	this study
pRS620	pRS316-*apn1-E207V*, CEN *URA3* Apm^r	this study
pRS467620	pRS316-*apn1-V156E,E207V* CEN *URA3* Apm^r	this study

Table S2: Mutations Identified in *APN1* Random Mutagenesis Clones

Mutant #	Nucleotide change	ORF position	Amino acid change	Amino acid position
1	A->G	621	synonymous	207
	G->A	781	Ala --> Thr	261
	A->G	906	synonymous	302
	A->T	1028	Lys-->Met	343
	A->G	1103	Stop--> Stop	367
2	A->G	725	Asp-->Gly	242
3	A->G	621	synonymous	207
4	T->C	576	synonymous	192
	A->T	1018	Thr--> Ser	340
	A->C	1018	Thr--> Pro	340
5	T->C	681	synonymous	227
	A->G	725	Asp-->Gly	242
	A->G	858	synonymous	286
6	T->A	467	Val--> Glu	156
	A->T	620	Glu--> Val	207
7	T->C	806	Ile-->Thr	269
	A->G	836	Asn--> Ser	279
	T->C	954	synonymous	318
8	T->C	103	Phe--> Leu	35
	A->G	294	synonymous	98
	T->A	317	Met--> Lys	106
	T->C	350	Ile-->Thr	117
	A->G	575	Asp-->Gly	192
	T->G	728	Leu--> Trp	243
	A->G	746	Gln--> Arg	249
	A->G	785	His--> Arg	262
	A->G	927	synonymous	309
9	A->T	584	His--> Leu	195
	A->G	616	Thr--> Ala	206

Non-point mutations

Mutant #	Mutation type
	No mutations: At least 2X coverage
11	Deletions starting at ref. position 169 up to end
12	Big N-term deletion
13	Large N-terminal deletion: first ~592 nt

Figure S1

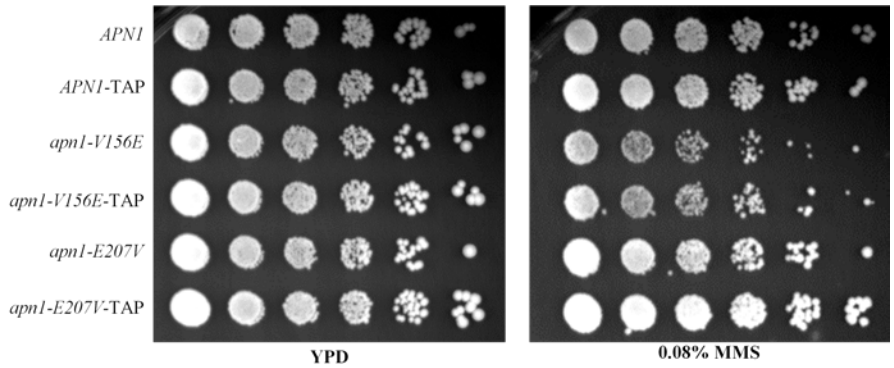

Figure S1 MMS sensitivities of strains containing C-terminal TAP-tagged versions of *APN1* wild-type and mutants. Approximately equal numbers of cells from strain isolates (as indicated) expressing the indicated TAP-tagged version of *APN1*from the chromosomal locus were diluted into sterile H_20. 5-fold serial dilutions were made and cells were spotted onto plates containing YPD (left panel) or YPD with 0.08% MMS (right panel).

CHAPTER 3

APN1 LOCALIZES TO SITES OF PRIORITIZED REPAIR OF OXIDATIVE DNA DAMAGE IN *SACCHAROMYCES CEREVISIAE*

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Abstract

Cells are continuously exposed to reactive oxygen species resulting in the production of toxic and mutagenic DNA damage. Previous studies revealed that disruption of base excision repair (BER), the major oxidative DNA damage repair pathway, alone or simultaneously with NER in *Saccharomyces cerevisiae* leads not only to increased mutation accumulation but also causes the production of large-scale genomic instability, a hallmark of cancer [1, 2]. In addition, a particular region of chromosome II is susceptible to such oxidative stress-induced chromosomal rearrangements, suggesting the existence of damage/repair hotspots. In the present study, we set out to investigate the relationship between oxidative DNA damage and genomic instability utilizing chromatin immunoprecipitation combined with DNA microarray technology to profile DNA damage sites along yeast chromosomes under different oxidative stress conditions. Because AP endonucleases are central to the BER pathway, we employed strains expressing a TAP-tagged version of the major yeast AP endonuclease, Apn1, from a galactose-inducible promoter, as a reporter to determine genomic regions bound by BER enzymes. Our initial findings indicate that oxidative stress induces changes in patterns of BER distribution across the genome; that Apn1 binding sequences are enriched for C and G nucleotides; and that Apn1 binding sites overlap with chromosome II oxidative stress-induced breakpoints of rearrangements. The results from our yeast studies can provide insight into how regional deployment of oxidative DNA damage management systems along chromosomes protects against genome destabilization in human systems.

1. Introduction

Reactive oxygen species (ROS)-induced damage to the DNA is one of the most commonly occurring types of spontaneous DNA damage, producing base modifications and strand breaks within the genome [3-6] at an estimated 10,000 lesions per mammalian cell each day [7]. The inability to repair such damage may lead to deleterious outcomes including genetic instability, cell death, and the development of a number of human pathologies [8]. Importantly, it is thought that oxidative DNA damage plays a significant role in the etiology of certain types of human cancers as it is present in a variety of human tumor types [9].

To maintain genetic integrity amid continuous oxidative insult, cells use the base excision repair (BER) pathway, which is responsible for repairing non-bulky DNA damage including oxidative lesions, alkylation-induced lesions, uracil in DNA and apurinic/apyrimidinic (AP) sites [10]. BER typically proceeds through the recognition of DNA damage by a lesion-specific glycosylase that removes the damaged base, leaving an AP site. The production of AP sites also occurs spontaneously due to hydrolytic attack on the DNA. Repair continues with cleavage of AP sites by an AP endonuclease. This is followed by processing of the nicked DNA ends, repair synthesis by DNA polymerase and sealing of the DNA backbone by a DNA ligase [10]. The majority of damage repaired through BER proceeds through AP sites as the common repair intermediate. Thus, AP endonucleases play a primary role in the repair of DNA damage through the BER pathway.

Although the nucleotide excision repair (NER) pathway is primarily responsible for the repair of bulky, helix-distorting lesions such as those resulting from UV exposure,

NER excision nuclease complexes, from both *E. coli* and humans can cleave several BER substrates *in vitro* [11-14]. Further, genetic studies in *Saccharomyces cerevisiae* revealed that when genes for the major proteins involved in both BER of oxidative damage (Apn1, Ntg1, Ntg2) and NER (Rad1) were simultaneously deleted (BER^-/NER^-), cells accumulate a substantial load of oxidative DNA damage as compared to single pathway mutants [15]. The handling of this damage in BER^-/NER^- strains by recombination and translesion synthesis, resulted in synergistic increases in spontaneous mutation and recombination rates, respectively, compared to the single pathway mutants [15-18]. A significant increase in the number of large-scale chromosomal rearrangements arise as a result of tolerance of the persistent oxidative DNA damage in these repair deficient strains, leading to development of genomic instability [18]. The genomic instability in BER^-/NER^- strains was characterized by mapping breakpoint hotspots within the genome. Most frequently, rearrangements occurred within a 10kb region on chromosome II.

The genome is like a topographical map where features such as chromatin state and base content are the hills and valleys of the DNA landscape. A detailed understanding of the genomic context of repair can be gained by studying DNA repair on a genome-wide level within the *in vivo* chromatin environment. We predict that uneven distribution of DNA damage and the underlying genomic features influence the locations of DNA repair and the oxidative stress-induced chromosomal rearrangements.

In *in vitro* reconstitution assays, the presence of nucleosomes is refractory to repair of oxidative DNA damage by BER, and the function of chromatin remodeling complexes has been shown to stimulate BER of oxidative damage to levels comparable to repair in naked DNA [19]. In a recent study, the human oxidative damage-specific

glycosylase, Ogg1, was shown to be actively recruited to open chromatin regions in order to repair DNA damage [20]. Thus, the repair of oxidative DNA damage requires open access to the DNA within chromatin.

In the present study, we employed the model eukaryote *Saccharomyces cerevisiae* and utilized the major yeast AP endonuclease, Apn1, as a marker for genomic localization of the BER machinery. We aimed to define the genomic sites occupied by Apn1 as a novel feature of the genomic map describing oxidative DNA repair in order to answer some important but largely unexplored questions. To explore how oxidative DNA damage translates to large-scale chromosomal changes, we sought to determine if and how cis-features of the genome influence localization of repair pathway components and how the location of repair machinery influences genome destabilization.

Genome-wide ChIP analyses have provided a wealth of information regarding the protein-DNA interactions of important DNA binding proteins, such as transcription factors and histones, but no previous studies have attempted to characterize excision repair enzyme occupancy using such methods. This is presumably due to the transient, dynamic and unpredictable manner in which repair enzymes interact with the DNA. Our experimental approach combined chromatin immunoprecipitation with DNA microarray analysis (ChIP-chip) to generate genome-wide maps of Apn1 binding sites under different levels of oxidative stress. We then assessed the underlying genomic landscape of these regions. Our findings suggest that Apn1 mostly binds GC-rich sequences and that the binding of Apn1 to either intra-or intergenic sequences is dependent upon the cellular oxidative stress level. Further, Apn1 binds a particular region on chromosome II that is more vulnerable to oxidative stress-related genome destabilization [18], suggesting

that the repair of DNA damage is prioritized here to prevent genome destabilization in this region of the genome. The results presented here demonstrate a potential relationship between oxidative DNA damage repair and chromosomal instability where the base composition and gene activity, and potentially chromatin state, may influence the location and frequency of oxidative DNA damage-induced chromosomal changes.

2. Materials and Methods

2.1. Yeast Strains and Culture Conditions

Standard yeast cell culture conditions utilizing either YPD (yeast extract, peptone, dextrose) or YP-Galactose (2%) culture medium as described previously [21]. Yeast cell transformation was performed using the lithium acetate method, as described previously [22].

2.2. Strain construction

Parental strain DSC0320 (*MATa [lys2::Alu-DIR-LEU2-lys2D5'] ade5-1 his7-2 leu2-3 112 trp1-289 ura3-52*) was isolated as a haploid spore of the diploid strain hDNP16 [18] . Strain DSC0436 (*MATa [lys2::Alu-DIR-LEU2-lys2D5'] ade5-1 his7-2 leu2-3 112 trp1-289 ura3-52 TRP1 GAL1*-TAP-*APN1*), which contains a construct with the *GAL1* promoter and the tandem affinity purification (TAP)-tag integrated directly upstream of the chromosomal *APN1* coding sequence (P_{GAL1}-TAP- *APN1*), was constructed as follows: a P_{GAL1}-TAP-*APN1* fragment was PCR amplified with primers APN1TAPf (AAACACAAAACGCAACATTAATAAGCTTTTGG CATATCGGAACCATCGTAGAACAAAAGCTGGAGCTCAT) and APN1TAPr (AATTT GTATTTCGAGACAGCAGATCTAACAAAGCTAGGTGTCGAAGGCATCTTATCG TCATCATCAAGTG) using plasmid pBS1761 [23] as a template. Strain DSC0320 was transformed with the resulting PCR fragment. Correct integration of the P_{GAL1}-TAP construct at the *APN1* locus was confirmed by PCR with primers APN1chkTAPf (TTCTGGGAACTTGAACGTGGAATT) and TRP1TAPchk (CGTGGTACAGTTGAAGGACATCATC).

2.3. H_2O_2 Cytotoxicity Analysis

Yeast cell cultures were prepared by inoculating a 35 mL culture with cells of strain DSC0436, incubating at 30°C to OD_{600} = ~0.8, and then splitting into three, 5 mL aliquots. Cultures were harvested, and cells were washed twice in sterile water and resuspended in either 5 mM or 35 mM H_2O_2 solution or mock solution containing no H_2O_2. Samples were incubated for 90 minutes at 30°C. Cells were harvested, washed twice with sterile water and resuspended in 5 mL sterile water. Appropriate dilutions of cells were plated onto YPD in duplicate, and numbers of viable colonies were assessed after two days of incubation at 30°C. Percent survival was calculated based on the number of colonies that grew from cultures exposed to H_2O_2 versus the number from mock-treated cultures. Results are the average of at least 4-6 independent experiments.

2.4. Chromatin Immunoprecipitation

Chromatin immunoprecipitation experiments were carried out as previously described [24] with the following modifications. A 350 mL culture inoculated with cells from strain DSC0436 was grown to OD600 ~0.8, and then split into three, 100 mL aliquots. Each of the three cultures was exposed to a different concentration of H_2O_2 (including a "mock" exposure) as described above. Harvested cell pellets were stored at -80°C until used. Following glycine quenching, cells were washed with sterile water. The chromatin solution was incubated overnight with anti-TAP antibody (Thermo). The chromatin-antibody mixture was incubated with protein A agarose beads (Invitrogen) for 2 h at room temperature. Cross-link reversal was performed in the absence of proteinase K. Three independent experiments were performed for each experimental condition. One sample from the 0.5 mM condition was used for assay optimization. Thus, there were

three biological replicates for 0 mM and 35 mM conditions, and there were two replicates for the 0.5 mM condition.

2.5. ChIP-chip procedure

ChIP DNA was labeled and hybridized to the Affymetrix *S. cerevisiae* Tiling Array 1.0 according to the Affymetrix Chromatin Immunoprecipitation Assay Protocol (http://www.affymetrix.com/support/technical/manuals.affx), with the following modifications. For the PCR amplification of immunoprecipitated DNA, the DNA was first amplified using the GenomePlex Single Cell Whole Genome Amplification Kit (Sigma-Aldrich). The amplified DNA was then purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich). Next, the purified DNA was amplified again using the GenomePlex WGA Reamplifications Kit (Sigma-Aldrich), and then purified as just described. The tiling array was then scanned according to the Affymetrix ChIP Assay Protocol.

2.6. ChIP-chip Data Analysis

Normalization of the data was carried out using the loess method within the Starr R package. The Affymetrix probe annotations for the yeast 2003 build were used for data analysis. Ratios of probe intensities were then calculated for each condition, again using the Starr package.

For each of the three different experimental conditions, enriched regions, or "peaks," of Apn1 binding were characterized using the Cmarrt R package with the normalized ratios generated via the Starr package as inputs. The replicates that produced peaks include two from the 0 mM condition, one from the 0.5 mM condition,

and three from the 35 mM condition. Thus, data from a total of six replicates from the three experimental conditions were further analyzed.

The average GC-content (the fraction of a sequence made of G and C residues) was determined for the Apn1 ChIP-chip peaks for each of the three experimental conditions. These averages were compared against a genomic background distribution of average GC-content generated via simulation analysis using the overlap between 10,000 sets of random genomic loci.

Apn1 ChIP-chip peaks were intersected with annotated genes from the genome browser UCSC sacCer2 version of the *S.cerevisiae* genome. The significance of overlap between Apn1 peaks and gene sequences was determined using the same 10,000 random sets as described above.

Results

3.1. Assessment of H_2O_2-induced Cell Killing

We set out to identify Apn1 binding sites across the yeast genome to represent the occupancy of the base excision repair activity under normal and oxidative stress conditions. We expressed N-terminal tandem affinity purification (TAP)-tagged Apn1 from the galactose-inducible *GAL1* promoter integrated directly upstream of the TAP-*APN1* construct at the chromosomal *APN1* locus for two reasons. First, though Apn1 is present at a higher concentration (copy number) than the other yeast BER proteins, there are only ~7,000 copies of Apn1 per cell [25, 26] , and galactose-induced overexpression would increase the likelihood of identifying Apn1 genomic target sites. Secondly, as no prior knowledge of the genomic localization of Apn1 exists, we employed previously published ChIP methods used for the identification of the DNA target sites of TAP-tagged yeast DNA binding proteins [24].

In order to assess the relationship between oxidative stress level and localization of BER machinery in the genome, we employed hydrogen peroxide (H_2O_2) to induce different levels of DNA damage. As a biologically relevant endpoint for the level of induced DNA damage in yeast cells, we measured cell survival following exposure of cells to H_2O_2 [15]. We utilized two doses of H_2O_2 and a “mock” exposure condition. Measurement of cytotoxicity following exposure to oxidative stress revealed that exposure to 0.5 mM H_2O_2 for 90 minutes resulted in 80% survival (Fig. 1), while exposure to 35 mM H_2O_2 resulted in 32% survival, compared to the mock condition. Based on these levels of cell killing we regarded exposure to 0.5 mM H_2O_2 as mild stress,

and exposure to 35 mM H_2O_2 as high stress. We regarded mock exposure as the normal (no stress) cellular condition.

3.2. Identification of Apn1 Binding Sites Across the Yeast Genome

The enrichment of genomic DNA in our ChIP DNA samples was assessed by microarray analysis with the Affymetrix *S. cerevisiae* Tiling Array 1.0, as described in "Materials and Methods." Peaks of Apn1 binding were identified using the Cmarrt algorithm based on the normalized ratios produced by the Starr software package. Each ChIP experiment was performed in triplicate, but one sample from the 0.5 mM experimental condition was utilized for microarray quality control and optimization and thus was not hybridized to the DNA chip. Two replicates from the no stress conditions, one replicate from the mild stress condition, and all three replicates from the high stress conditions produced peaks.

3.3. Genome-wide Apn1 Binding is Influenced by Oxidative Stress

Under normal conditions, we identified 916 Apn1 binding peaks across the genome (Fig. 2). Under mild and high stress conditions we identified 242 and 211 peaks, respectively. There were few peaks common to multiple experimental conditions and only three peaks common to all conditions. These data, along with the cytotoxicity results, suggest that the three experimental conditions represent three different cellular responses to three different levels of oxidative DNA damage. This is in line with our previous work showing that exposure to similar H_2O_2 doses, "mock" and high, caused undetectable and substantial levels, respectively, of repair protein-detectable DNA damage [15].

The observed changes in Apn1 localization suggests that certain regions may be more susceptible to exogenous H_2O_2-induced DNA damage under particular levels of oxidative stress. Of the experimental conditions for which the microarray analysis produced peaks, the average width of each Apn1 binding site was the same across all samples (Fig. 2B).

3.4. Apn1 Preferentially Binds GC-rich Sequences

Several investigations have shown that guanine and cytosine bases are common and mutagenic targets of oxidative damage [27, 28]. Thus, we assessed the GC-content of Apn1 binding regions as described in "Materials and Methods." For each of the three experimental conditions, the GC-content of the Apn1 peaks was found to be significantly higher than the genomic background (Table 1). These results suggest that BER preferentially targets certain GC-rich genomic regions, which we predict may be highly susceptible to mutagenic damage including oxidation and alkylation of guanine and spontaneous loss of guanine bases [28].

3.5. Oxidative Stress Level Influences the Intragenic Content of Apn1 Binding Sites

The overlap of ChIP-chip peaks with intragenic sequences was determined as described in "Materials and Methods." Under both normal conditions and high stress conditions, Apn1 peaks were significantly depleted of intragenic sequences. Mild stress conditions produced peaks that were significantly enriched for intragenic sequences (Table 2), which may suggest that three different responses to different levels of oxidative stress are being observed. This is in line with a previously identified genotoxic stress response in yeast where the the amount of DNA damage within the genome

dictates the cellular level of ROS signaling molecules (superoxide: $O_2^{\bullet-}$), low, medium or high [29].

3.6. Apn1 Occupies a Genomic Hotspot of Oxidative DNA Damage-induced Rearrangements

In our previous studies we identified a substantial number of chromosomal rearrangements following accumulation of chronic oxidative DNA damage in yeast [18]. Mapping the breakpoints of these rearrangement events revealed hotspots that emerge in the absence of excision repair (BER^-/NER^- strain). One such hotspot was mapped to a 50 kb region on chromosome II. We predicted that the oxidative-stress induced chromosomal rearrangement hotspots represent regions that are prioritized for repair, as destabilization occurs in the absence of the major BER and NER enzymes. Thus, we expected that Apn1 binding sites would map to this region. Under normal conditions, three Apn1 peaks mapped to the breakpoint hotspot region (Table 3), and one Apn1 binding peak from each of the oxidative stress conditions mapped to the breakpoint hotspot region.

4. Discussion

To our knowledge, this is the first study mapping the genomic binding sites of an excision repair protein on a genome-wide level. A major advantage of this study was our unbiased approach whereby we identified binding sites of a protein for which no prior information regarding genomic binding localization was available. We have identified the genome-wide binding sites of Apn1 under different levels of oxidative stress, and have also identified underlying features, the locations of which may influence the genomic localization of BER enzymes. The hypotheses based on our data that will be tested in future studies are described here.

A major finding from our studies was, regardless of the oxidative stress level, CG content was enriched within peaks of Apn1 binding. Gs and Cs are thought to be the most common targets of mutagenic base damage. A previous study in human cells revealed that 8-oxoG is unevenly distributed within the genome in a pattern that correlates with single nucleotide polymorphisms in the human genome [30]. Thus, the enrichment of Gs and Cs within Apn1 binding peaks suggests that BER promotes genomic stability by preferentially protecting regions with a higher content of bases that are more susceptible to oxidative attack. Additionally, GC rich regions of the genome in yeast have a more open and extended chromatin conformation than AT rich regions of the genome [31], suggesting that chromatin state may be an important determinant for localization of damage in addition to repair.

Under normal conditions (Fig. 3A), there were approximately 1,000 Apn1 binding peaks across the genome (more than expected by chance). There was a significant enrichment for intergenic sequences within these binding sites, which indicates that Apn1

normally binds mostly non-gene sequences. One might expect there to be a preference for repair of damage occurring in the protein coding regions of the genome, but there are other mechanisms in place to handle the subset of the damage occurring in genes. For example, oxidative damage, namely the well-studied and mutagenic 8-oxo-guanine, was shown to be subject to transcription-coupled repair (TCR) in *E. coli* [32], a sub-pathway of NER that preferentially repairs the transcribed strand of actively-transcribed genes [33]. It is possible that under normal conditions this pattern of genome-wide Apn1 binding represents a division of labor for DNA repair where TCR and BER of spontaneous damage are deployed to active and intergenic regions, respectively, with the caveat that TCR will not be active within genes that are not being expressed. To test this model, we could perform ChIP under the same experimental conditions but with a TCR protein, such as Rad26, as the target protein and compare the Apn1 and Rad26 genomic binding maps.

Under mild oxidative stress conditions (Fig. 3B), there is an enrichment for intragenic sequences within Apn1 binding peaks. This indicates a preferential targeting of coding regions of the genome for repair under this level of oxidative stress. There was also a substantial decrease in the number of peaks (from ~1000 to ~250). One might expect that an increase in oxidative stress, and thus an increase in DNA damage, would lead to an increase in the number of Apn1 peaks. However there are several possibilities in line with these data. Fewer Apn1 peaks may indicate oxidative damage to Apn1 (since Apn1 expression is not inducible by DNA damaging agents [34]), leading to a proteolytic degradation [35] and a smaller pool of Apn1 molecules available for DNA repair. Apn1 peaks under these mild oxidative stress conditions may represent regions highly

prioritized for repair via BER specifically, and other DNA damage management pathways handle the damage in other regions no longer covered by BER. The amount of DNA damage under these conditions may also exceed the capacity of BER to repair it, leading to other pathways such as recombination to act on the DNA to promote cell survival, at the expense of increased genomic instability. On a technical note, that there are fewer peaks in the mild stress conditions versus the no stress condition may be a reflection of the fact that only a single replicate for the mild conditions versus two replicates for the normal condition were analyzed. The number of peaks for each condition may be directly influenced by the number of replicates.

Under high oxidative stress conditions, the number of peaks following H_2O_2 treatment also decreased substantially (~290 peaks) compared to the untreated conditions, and the Apn1 binding peaks fall primarily within intergenic regions. It is unclear whether cell survival is a relevant endpoint at which to capture physiologically meaningful interactions between Apn1 and genomic DNA because the majority of the cells eventually die and the number of living cells at the time of crosslinking is unknown. To better understand how high oxidative stress affects the Apn1 genome-wide DNA binding pattern, our futures studies will aim to assess DNA repair kinetics following exposure to H_2O_2, via the comet assay for yeast genomic DNA damage detection [36], as a more relevant biological read-out with which to correlate our Apn1 ChIP-chip results.

The fact that Apn1 binds to the chromosome II region where the oxidative stress related rearrangement breakpoints occur is in line with our hypothesis that certain genomic regions are prioritized for repair via BER to prevent chromosomal rearrangements and genomic instability. However, further analysis needs to be done

regarding whether other Apn1 binding sites correlate with sites of oxidative damage-induced fragility.

Overall, the results presented here allow for the development of an initial working model describing a genomic map of the BER pathway; the localization of Apn1 across the genome is influenced by the level of genotoxic stress and is also dictated by the susceptibility of the underlying sequence to certain types of DNA damage. Under normal conditions, genomic Apn1 localization is mainly intergenic and is mainly intragenic under moderate oxidative stress conditions. This suggests a regional occupancy of DNA repair, which may be dependent on the amount of DNA damage present. The localization of repair proteins to particular regions, e.g. chromosome II breakpoint hotspot, and GC rich regions may represent a prioritization of repair in regions more susceptible to oxidative damage-induced genetic instability. These studies also provide a framework for further exploration of mechanisms of oxidative DNA damage repair and factors affecting oxidative stress-induced genome destabilization. Further, identification of such features in yeast can be used to facilitate identification of similar regions in human premalignant lesions as a predictive screening tool for prognosis or anti-cancer treatment [37].

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Table 1. GC content in Apn1 binding peaks

Experimental Condition	Average GC-Content within Peaks*		p-value
	Expected	**Observed**	
0 mM	0.3833	0.433	$<10^{-5}$
0.5 mM	0.3833	0.401	$<10^{-5}$
35 mM	0.3833	0.402	$<10^{-5}$

*Assessment of the peak GC-content significance was determined via simulation analysis as described in "Materials and Methods".

Table 2. Intragenic and intergenic content in Apn1 binding peaks.

Experimental Condition	Total number of Apn1 binding sites	Apn1 binding sites overlapping with genes*		p-value
		Expected	Observed	
0 mM	979	719	541	$<10^{-5}$
0.5 mM	246	181	217	$<10^{-5}$
35 mM	292	214	165	$<10^{-5}$

*Assessment of the overlap significance was determined via simulation analysis as described in "Materials and Methods".

Table 3. Apn1 binding peaks overlapping oxidative stress-related fragile sites

Peaks at 0 mM	
start	***stop***
193581	193826
194968	195032
235945	236222
Peaks at 0.5 mM	
start	***stop***
189012	189096
Peaks at 35 mM	
start	***stop***
192125	192161

Breakpoint region start/stop coordinates: 184,091-237,421

Figure 1.

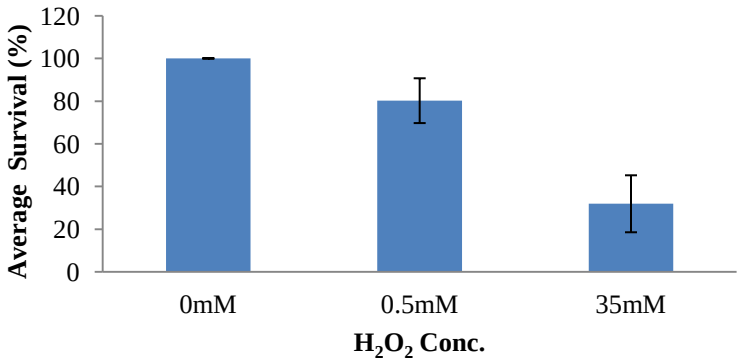
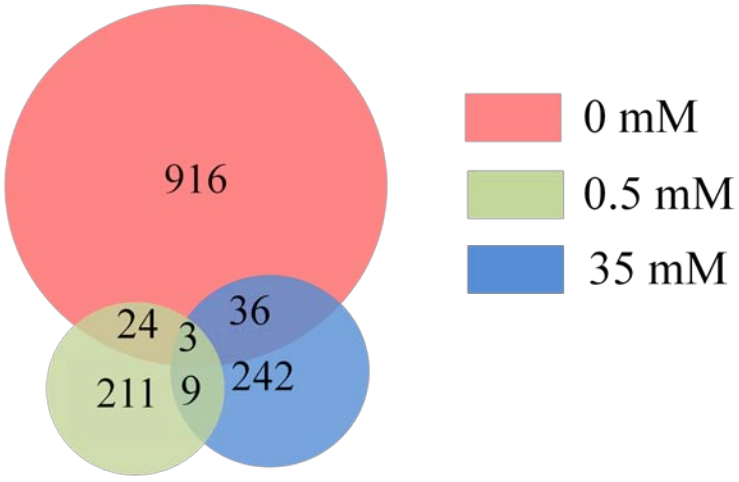


Figure 1. H_2O_2-induced cytoxicity analysis. Yeast cell cultures were incubated with different concentrations of H_2O_2 in sterile H_2O, including a mock exposure condition. Cells were washed and plated on complete medium to determine viable cell numbers.

Figure 2.

A)


916
24
3
36
211
9
242
0 mM
0.5 mM
35 mM

B)

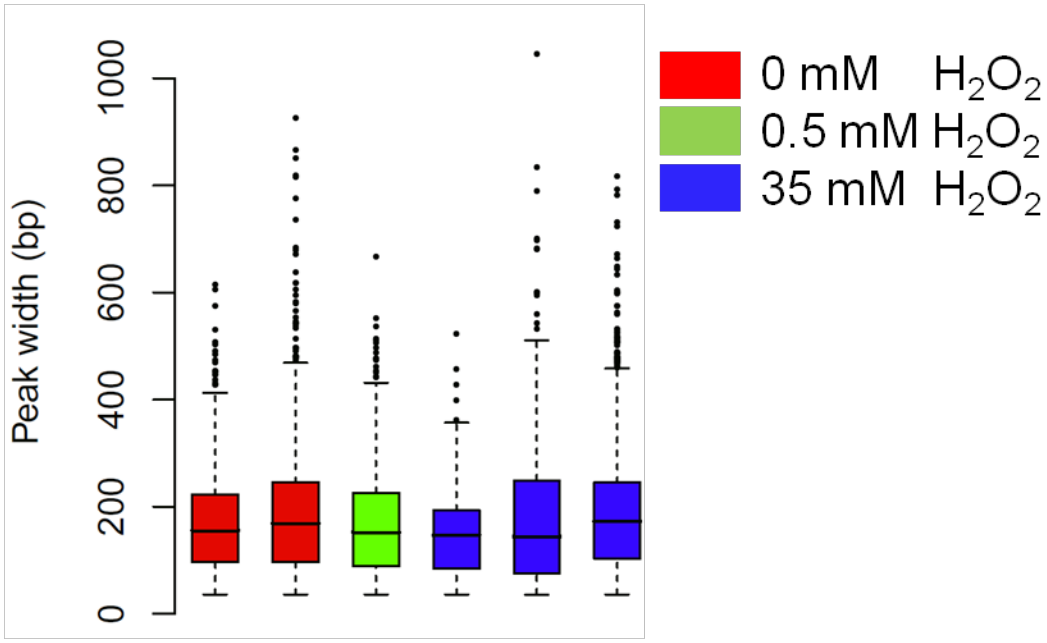
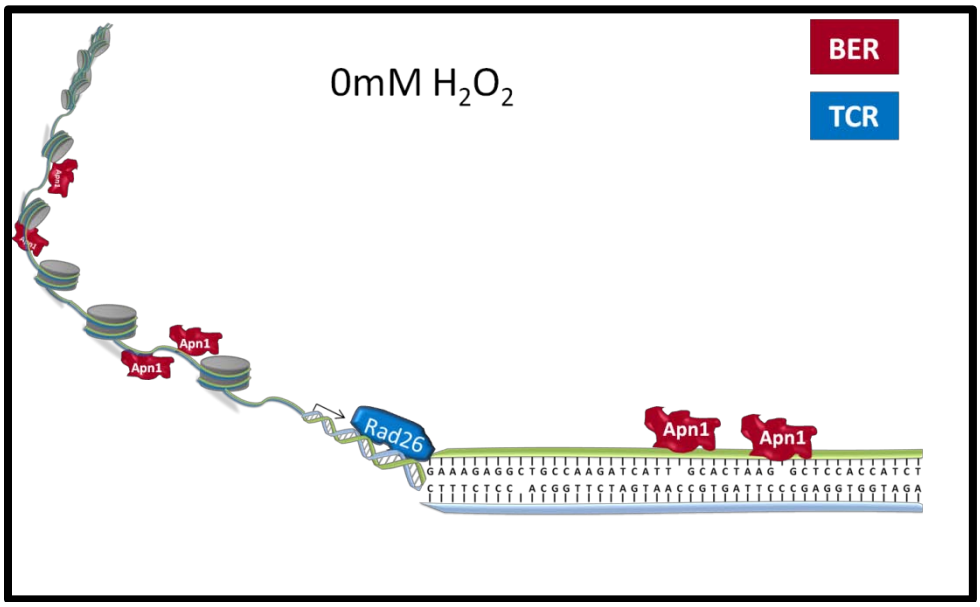

Peak width (bp)
0
200
400
600
800
1000
0 mM H2O2
0.5 mM H2O2
35 mM H2O2

Figure 2. Characteristics of Apn1 binding peaks. A) Numbers of Apn1 binding peaks. Overlaps between the numbers of Apn1 binding peaks under no oxidative stress (red), mild (0.5 mM H_2O_2) oxidative stress (green), and high (35 mM H_2O_2) oxidative stress (blue). **B)** Apn1 binding site sizes. The average peak with was determined for each of the experimental conditions for which there was a significant number of peaks.

Figure 3.

A)



B)

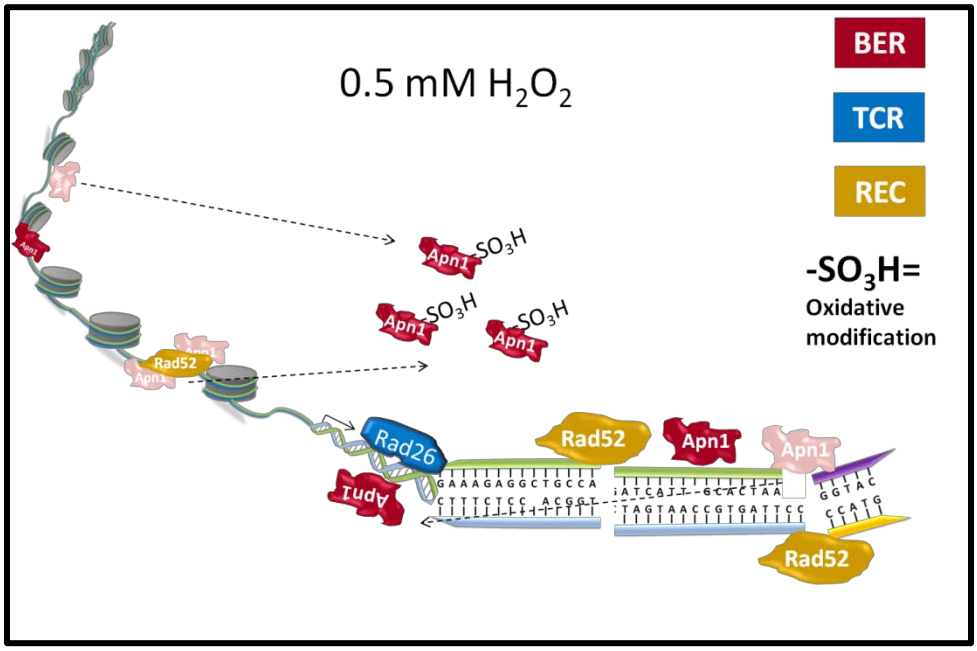


Figure 3. Model:A pn1 genomic occupancy. A) Apn1 binds to ~900 genomic sites under normal conditions. The Apn1 binding sites are significantly enriched for sequences containing cytosine and guanine, and are significantly depleted for intragenic sequences

under normal cellular conditions. **B)** Following exposure to 0.5 mM H_2O_2, the number of genomic Apn1 binding sites decreases to ~250. Under these conditions, Apn1 binding sights are also significantly enriched for sequences containing cytosine and guanine, and are significantly enriched for intragenic sequences.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Research Issues Explored in this Dissertation

Base excision repair (BER) is a critical cellular pathway responsible for the removal of many types of small, helix-nondistorting mutagenic and cytotoxic DNA lesions. Such DNA damage is continuously generated, occurring both spontaneously and following exposure to environmental DNA damaging agents. Specific genetic defects in BER proteins, including MUTYH, UNG and DNA polymerase β, have been linked to the development of human pathologies [1-3]. Though epidemiological analyses have shown association between SNPs in BER genes with disease development [4], the molecular basis for impaired BER capacity has only been experimentally validated for a few of these repair gene variants [4]. Thus, a relationship between BER dysfunction and disease etiology is largely unexplored.

Previous studies demonstrated that in *Saccharomyces cerevisiae*, elevated levels of unrepaired oxidative DNA damage, a major class of damage handled by BER, cause the acquisition of large-scale chromosomal aberrations at substantially high frequencies [5]. Such genomic instability is a common feature of cancer cells that enables the development of tumors [6, 7]. A relationship between ROS and chromosomal instability has also been observed in various other systems [5, 8-13], suggesting that similar mechanisms of oxidative stress-induced genomic instability may occur in mammalian cells.

Discussion of Major Findings

A major goal of the present studies was to delineate mechanisms by which the BER pathway protects the cell against genetic instability using two major experimental

approaches. The first approach presented in chapter 2, explored BER biology on a structure-function level. Important findings include: 1) An *APN1* mutation affecting amino acid 156, which is outside the well-characterized functional domains of Apn1 (e.g. active site [14], nuclear localization sequence [15]), substantially compromises cellular DNA repair capacity; 2) Overexpression of the Apn1 protein variant V156E, predicted to be catalytically deficient based on homology modeling, rescued functional DNA repair activity; 3) Increasing or decreasing the normal steady-state expression level of Apn1 compromises cellular DNA repair capacity; 4) Differences in structure-function relationships between then yeast Apn1 and *E. coli* endo IV orthologs; 5) While proteolysis of wild type Apn1 occurs through proteasome-mediated degradation, V156E is degraded by a proteasome independent mechanism, which results in V156E half-life that is three times shorter than that of wild type Apn1.

With the second approach, we explored BER biology at the genome-wide level via a discovery-based chromatin immunoprecipitation approach. Important findings include: 1) Oxidative stress induces changes in patterns of BER distribution across the genome; 2) Apn1 binding sites are enriched for C and G nucleotides; 3) Under normal conditions and high levels of oxidative stress, Apn1 binding sequences are enriched in intergenic regions, and under low levels of oxidative stress, Apn1 binding sequences are enriched in intragenic regions; 4) Apn1 binding sequences overlap with a hotspot of large-scale rearrangement breakpoints on chromosome II. Overall, our results suggest that the localization of repair machinery to sites of DNA damage is influenced by the oxidative stress level and the propensity of the underlying DNA to be damaged by

oxidizing agents. The results suggest that BER is prioritized in certain regions to prevent oxidative damage-induced chromosomal instability from occurring.

Study of yeast Apn1 reveals novel aspects of AP endonuclease structure-function relationships

In chapter 2 of this thesis we explored and revealed novel aspects of structure-function relationships for a class of proteins critical to repair via the BER pathway, the AP endonucleases. We used the major yeast AP endonuclease Apn1 as a model for our studies. The main goal of our work was to identify mechanisms by which relevant physiological disruptions in AP endonuclease activity affect cellular repair capacity in yeast as a model for similar repair defects in mammalian systems.

We performed a random mutagenesis screen at the endogenous *APN1* locus to identify changes in Apn1 amino acid structure to help better understand the genetic changes that may negatively impact DNA repair capacity in the human functional homolog APE1. Such changes include missense SNPs and mutations associated with various diseases including cancer and amyotrophic lateral sclerosis [4, 16, 17].

We reported the results of a pilot screen from which ~30 MMS sensitive mutants were identified, nine of which represented synonymous or missense substitutions. In addition to the V156E substitution, we identified the nuclear localization signal substitutions T340S [15] and L343M as well as the active site substitutions D192G [14], H195L, and D242G, some of which were previously described. The identification of these functional mutations shows that our screen is an efficient way to study structure-function relationships of yeast DNA repair proteins. Seven of these nine mutants harbored from 2-10 mutations total, indicating that additional optimization of the

mutagenic PCR protocol needs to be performed before scaling up the screen to identify and characterize additional mutants. Because the Apn1 structure has not yet been solved, an important component of our analysis was the use of information from the well-characterized structure and biochemistry of the *E. coli* homolog of Apn1, endonuclease IV (endo IV), to make predictions to be tested in the yeast studies.

The Apn1 V156E missense mutation disrupts DNA repair capacity by an unanticipated mechanism.

Based on homology to a previously characterized amino acid variant in the *E. coli* homolog, endo IV V143E [18], the V156E substitution identified in the screen was expected to cause Apn1 catalytic deficiency. Instead, the impairment of cellular DNA repair capacity caused by the V156E substitution occurred due to decreased steady-state protein levels, not to any detectable impairment in catalytic activity.

In summary, we found that the decreased cellular levels of the mutant protein correlated with a concomitant decrease in AP incision activity within cell lysates and that overexpression of the Apn1 V156E variant at normal steady-state levels resulted in restoration of *in vitro* incision activity within cell lysates and *in vivo* cellular repair function. We determined that V156E mutant had a half-life of ~30 minutes compared to ~90 minutes for wild type Apn1. And while protein turnover for wild-type Apn1 is regulated via the proteasome, we found Apn1 V156E was not subject to proteasomal regulation. The thermostability of both Apn1 variants within cellular lysates was found to be the same as there were no differences in sensitivity to heat treatment with respect to AP site incision activity.

Taken together, the data suggest that the substitution of valine for glutamic acid at amino acid position 156 in yeast Apn1 results in a structural defect that we hypothesize affects proper protein folding kinetics. If folding into the correct functional conformation is inefficient, protein quality control mechanisms target aberrant folding intermediates and unstable proteins for degradation to prevent formation of toxic protein aggregates [19]. The similar stabilities of the wild type and mutant proteins as detected in thermal stability assays support a model for a defect in protein folding kinetics rather than intrinsic instability of the mutant protein structure. In *apn1-V156E* mutant cells, correct folding might only occur for a fraction of synthesized peptides at any given time, allowing the accumulation of only a small subpopulation of Apn1V156E polypeptides that can fold into the stable immunoreactive and enzymatically active conformation.

Steady-state Apn1 levels are an important determinant of cellular DNA repair capacity.

We showed that changes in steady-state Apn1 protein levels have substantial effects on cellular repair capacity in yeast. Based on our *in vitro* AP incision assays, different steady-state levels of protein correlated with the amount of cleavage activity detected, where lower protein levels resulted in decreased cleavage activity. In *in vivo* MMS sensitivity assays, lower levels of Apn1 protein correlated with increased sensitivity, where the degree of sensitivity of *apn1-V156E* mutant was intermediate between wild type and *apn1Δ*. This is consistent with the intermediate level of antibody-detectable Apn1 V156E protein and AP incision activity within cell lysates. Overexpression of wild-type Apn1 also correlated with increased sensitivity to MMS,

which suggests that increased cellular Apn1 activity, in the presence of DNA damage from an endogenous source, can be mutagenic.

In agreement with our findings, heterologous expression of yeast Apn1 in lung epithelial cells showed a similar pattern: the higher the expression of *APN1*, the greater the sensitivity of cells to the DNA damaging agent bleomycin [20]. Such imbalances in other BER proteins are also mutagenic, such as overexpression of the Mag1 DNA glycosylase, which increases spontaneous mutation rates by several hundred-fold due to accumulation of AP sites in the genomic DNA [21]. Thus, normal cellular levels of Apn1, and BER proteins such as Mag1 and others, must be maintained for effective DNA repair. These observations provide an explanation for the relatively low steady-state levels of yeast DNA repair proteins. Apn1 is expressed at ~7,000 molecules per cell [22, 23], and other BER proteins such as Ung1 and Ntg2 are expressed at ~4800 and 125 molecules/cell, respectively [23]. This is in contrast to common housekeeping proteins like PGK (phosphoglycerate kinase), which is expressed at 314,000 molecules/cell [23]. Maintaining low steady-state protein levels, and thus proper balance of repair activities, could be one mechanism by which yeast cells preserve genetic stability and prevent accumulation of toxic repair intermediates.

The phenotypes resulting from missense mutations in many disease genes is caused by decreased cellular levels of proteins [24, 25], similar to the molecular phenotype observed for the Apn1 V156E variant. Some examples include the NF2 gene in CNS tumors [26], the RDH12 gene in retinal degeneration [27], and PHGDH gene in serine biosynthesis defects associated with various pathologies [28]. Most human studies attempting to identify molecular mechanisms underlying the role of repair protein defects

in reduced repair capacity are in an *in vitro* setting using purified enzymes. As demonstrated by our studies, this is a limitation because disease-associated missense mutations/ SNPs that occur in APE1 could affect repair capacity, not by affecting enzymatic activity or DNA binding, but by decreasing AP endonuclease levels. This indicates that structural integrity and/or efficient protein folding (or other such aspects of protein biology) are as important for maintaining DNA repair capacity as intrinsic catalytic function, DNA binding ability or other activities carried out by a properly folded, functional repair protein.

Several recent *in silico* studies have used computational simulations to predict the impact of amino acid substitutions (identified in public domain databases) on the structure and function of APE1. Two variants (with no currently known disease associations) common to multiple investigations of APE1 SNPs, I64T and P311S, are hydrophilic substitutions for hydrophobic residues buried within the protein hydrophobic core, similar to the Apn1 V156E substitution. These residues are not part of the active site and are predicted to affect protein structure by obstructing stability and folding. Both of these outcomes could potentially lead to decreased cellular protein levels. We believe that decreased levels of a repair protein is an important biological outcome that should be assessed for repair variants shown to be associated with disease, especially for those that do not otherwise exhibit repair defects *in vitro* [16, 17].

Differences in structure and function relationships: yeast Apn1 and E. coli Endo IV

As mentioned previously, we utilized information from studies of *E. coli* endonuclease IV to make predictions about *apn1* yeast mutants. Endo IV and Apn1 share

41% amino acid identity, including 100% identity at 9 metal binding residues important for enzymatic activity. In addition, cross-species complementation experiments demonstrated that heterologous expression of endo IV can rescue the repair defects of Apn1-deficient yeast mutants [29], and vice versa [30]. We reasoned that results from analysis of the homologous *E. coli* mutation V143E [18] could provide clues as to the particular molecular defect caused by the V156E substitution in yeast. Contrary to our prediction, these homologous mutations resulted in molecular phenotypes that differed for the *E. coli* and *S. cerevisiae* orthologs. Endo IV V143E caused decreased cellular protein levels and 60-fold reduction in enzymatic activity, while Apn1 V156E exhibited decreased cellular levels but no apparent decrease in enzymatic activity. This discrepancy could be a reflection of differences in cellular environments when comparing certain processes as they occur in a eukaryotic cell versus a prokaryotic cell, such as protein quality control mechanisms, which ensure that aberrant protein products do not harm the cell. Despite the fact that many of the protein quality control components (especially chaperone proteins) are evolutionarily conserved from bacteria to humans [31], these pathways may handle mutant proteins differently with respect to folding into the correct functional conformation. This proposed difference could lead to the difference cellular and molecular phenotypes observed in our studies. However, this would not explain the differences in *in vitro* activity and DNA binding assays between purified recombinant endoIV and purified recombinant Apn1, as both proteins were harvested in *E. coli* [14, 18].

The observed differences in the molecular and cellular functions of Apn1 and endo IV protein variants may reflect intrinsic structure-function differences between the

two proteins. Previous studies characterizing homologous amino acid substitutions in active site residues of Apn1 and endo IV [14, 18] revealed that *in vitro* biochemical properties (incision activity and DNA binding) of Apn1 E158G and Apn1 D192G differed from the homologous endo IV E145G and D179G variants, respectively. Such differences may be dictated by other non-conserved amino acids within the protein structures such as would occur with the evolutionary acquisition of additional sequences at the amino and carboxy termini housing the sequences for mitochondrial and nuclear targeting, respectively. Again, this could be a reflection of differences between protein activities in a eukaryotic versus prokaryotic cellular context.

Genome-wide Mapping of Oxidative-stress induced Apn1 Binding Site Redistribution

We developed a novel approach to mark genomic regions undergoing BER using chromatin immunoprecipitation (ChIP) by exploiting the major yeast AP endonuclease, Apn1, as a representative BER protein to map genome-wide BER occupancy at genomic regions undergoing BER. Importantly, there was no prior information about specific binding sites to which excision repair proteins localize during the repair process. Although ChIP approaches have traditionally been used to map the DNA interactions for proteins with predictable binding sites (e.g transcription factors, histones), our studies add Apn1, an excision repair enzyme, to the list of proteins for which detailed, genome-wide DNA binding patterns have been determined. Mapping the base excision repair process has provided a missing link between oxidative DNA damage and chromosomal instability, two important hallmarks of cancer [7, 32].

Genomic localization of Apn1 changes under different levels of oxidative stress.

We captured Apn1-DNA interactions across the genome under different levels of oxidative stress. Based on the level of cell killing induced by different concentrations of H_2O_2, the experimental conditions were designated as normal (0 mM), moderate stress (5 mM, 20% cell killing), and severe stress (35 mM, 68% cell killing). In retrospect, we are uncertain whether the peaks identified for the severe stress condition represent true Apn1-DNA binding or represent passive interactions between the DNA and Apn1. Since the majority of the cells die in this condition, we may not be observing repair-specific binding.

Apn1 binding sites were found to be enriched for G and C nucleotides, regardless of the level of oxidative stress (Fig. 2). Several lines of experimental evidence indicate that G and C nucleotides are the most common sites of mutagenic, oxidative attack in double-stranded DNA [33-41]. Thus, the data suggest preferential targeting of the most mutagenic types of DNA damage by base excision repair in order to prevent mutagenic outcomes.

One common, mutagenic and extensively-studied DNA lesion resulting from oxidative attack is 8-oxoG. Results from a recent study, in which microscopy and chromatin fractionation were used, showed that following oxidative stress, the BER machinery specific for the repair of 8-oxoG is recruited to regions of open chromatin and that open chromatin conformation is required for efficient DNA repair [42]. This is in support of a growing body of literature regarding the relationship between chromatin conformation and BER, whereby the efficient BER of DNA damage requires DNA free of nucleosomes [43-46]. In addition, GC content is generally higher in regions of more

open chromatin conformation [47]. Thus, Apn1 binding sites identified in our study may represent specific regions of chromatin that open up, or remain open, and are targeted often for repair under different levels of oxidative stress.

Sequences within genes were found to be significantly enriched or depleted within Apn1 binding sites, depending on the cellular oxidative stress level. Under normal conditions, Apn1 binds primarily to intergenic sequences and thus genes may be protected from spontaneous oxidative damage by other DNA damage management mechanisms, such as transcription-coupled repair (TCR) (Fig. 1A). Alternatively, if there are regions that are more susceptible to DNA damage they may be more susceptible to large-scale changes if oxidative damage is allowed to accumulate there, most Apn1 molecules will go there in the majority of cells. Under moderate levels of oxidative stress, Apn1 binds primarily intragenic sequences, and there are also substantially fewer Apn1 binding sites (Fig. 1B). This suggests that BER capacity may be decreased due to induction of an amount of damage that overwhelms the ability of BER proteins to repair it; this decreased capacity for repair by BER may be due in part to direct oxidative damage of BER proteins, rendering them unable to repair the DNA. This result may also represent a pooling of resources (employing several repair pathways) for repairing mutagenic oxidative DNA damage to protect regions of the genome that encode functional gene products under increased oxidative stress conditions.

Apn1 Binding Sites within the Chromosome II Oxidative Stress Related Breakpoint Region

The localization of BER on chromosome II was of particular interest because of a previously identified hotspot of oxidative DNA damage-induced chromosome breakage on this chromosome [5]. These sites may represent regions of prioritized DNA damage management by BER as the main defense against genome destabilization due to breakage and error-prone repair by homologous recombination. Thus, we hypothesized that Apn1 binding would correlate with the location of breakpoints. We identified Apn1 binding peaks in all three experimental conditions overlapped with the breakpoint hotspot region, which supports our hypothesis that the repair of oxidative DNA damage is prioritized in certain regions over others. More analysis needs to be performed to determine whether this overlap is biologically meaningful. Nonetheless, these results set the stage for future studies to determine why such regions are particularly susceptible to chronic oxidative damage-induced destabilization. Our previous studies revealed an especially high amount of repetitive DNA in these regions. Our data suggests that the efficient repair of DNA in such regions may prevent rearrangements in these regions as a result of aberrant recombination “repair” processes.

How does DNA damage ultimately translate into rearrangements?

Although BER is the primary pathway responsible for repairing oxidative DNA damage, in certain contexts, the nucleotide excision repair and mismatch repair pathways can target the same lesions for removal. This may occur at a low level when BER is functioning normally, and may be increased if cells are deficient in BER or if the amount

of damage exceeds the capacity for BER to repair it [48]. Under conditions where the capacity for excision repair mechanisms (BER, NER and MMR) to handle the damage is exceeded, the cell will employ tolerance mechanisms, such as recombination and translesion synthesis, which promote cellular survival, but do so at the expense of increased genetic instability. The pattern of chromosomal breakpoints resulting from large-scale rearrangements in yeast cells harboring chronic oxidative damage [5] implies that particular regions are more prone to DNA damage or that the handling of damage in a particular genomic context is prioritized, compared to repair in the rest of the genome. Although there are many studies correlating oxidative damage with large scale genome destabilization [5, 8-11], the molecular mechanisms underlying such oxidative damage-induced genomic instability are not yet known. The results presented in chapter three suggest that certain regions are prioritized for base excision repair to prevent the damage from being handled by recombination in an aberrant repair process. Importantly, our results show that these regions and the underlying genomic landscape can be identified by mapping the localization of the major proteins that are responsible for their repair.

Biological implications for future studies

Part I

Implications for assessing DNA Repair Capacity in Humans

A major experimental approach for assessing the defects in human AP endonuclease APE1 function is the computational prediction of the molecular effects of missense SNPs identified in humans based on high resolution structure of APE1 and sophisticated *in silico* analyses [16, 49]. It has been proposed that alterations in repair proteins most likely to affect repair capacity will occur in catalytic or protein-protein

interaction domains [50]. Our results indicate that amino acid substitutions outside known functional domains that cause less functional protein to accumulate can have substantial negative effects on cellular repair capacity and should be prioritized for experimental validation of function. We hypothesize that the two population variants I64T and P311S, predicted to cause APE1 structural defects, exhibit decreased steady-state protein levels. This suggests that the assessment of cellular protein levels should be included in the repertoire of molecular tests employed to understand the impact of these variants on repair capacity and disease association.

Mutations identified in a subset of patients with amyotrophic lateral sclerosis (ALS), a fatal disease of the neuromuscular system, potentially play a role in disease pathology since the same mutations were not found in healthy individuals in these particular studies [17]. *In vitro* biochemical characterization of several ALS-associated APE1 variants revealed wild type levels of AP site incision activity and DNA binding activity, and three variants represent non-conservative changes that were predicted to cause local structural changes based on molecular modeling [16]. The only hypotheses posed by investigators studying these mutations were that the substitutions either affected functional protein-protein interactions or that the substitution variants are dominant-negative molecules. However, decreased levels of APE1 protein and decreased AP site incision activity were also detected in patient samples [17]. As our results have shown that decreased AP endonuclease levels due to a single amino acid substitution can lead to substantially decreased repair capacity, we propose that these APE1 mutations may play a role in ALS pathology by missense mutation-mediated degradation.

Assuming the proposed mechanism of a role for the enzymatically proficient APE1 variants in ALS pathogenesis, can these mutant proteins be targeted for therapy? A class of compounds that selectively and reversibly stabilize protein structures, called pharmacological chaperones (PCs) [51, 52], may be of use. There are two proposed mechanisms of action for PCs: 1) Stabilization of mature protein conformation and protection against thermal instability [51]; and 2) Binding to immature protein and promoting proper, efficient folding [52]. Experimentally, PCs could be used within an ALS model system to test whether there truly is a correlation between APE1 levels and phenotype presentation. If so, investigators could further develop particular compounds for therapy. Typically, agonists or antagonists of particular proteins are good candidates for PCs with structural/folding defects leading to decreased protein levels [53]. Several research groups have developed APE1 inhibitors to target APE1 overexpression in cancer [54], and these compounds may also be candidates as PCs for experimental studies to develop therapies for APE1 disease associated mutations or SNPs proven to have a folding and/or stability defect leading to decreased steady-state protein levels and concomitant decrease in repair capacity.

Expression Level Determines DNA Repair Capacity

Our data from studies in cell lysates indicate the mutant V156E protein is as thermal labile as wild type and that decreased cellular protein levels result from a folding defect. The *in vitro* biochemical analysis on purified Apn1 enzymes would provide a more straightforward line of support for these conclusions. Such analysis would allow for direct comparisons of the kinetic properties of wild type and mutant proteins. This would allow us to rule out other defects that could potentially lead to increased protein turnover

such as disrupted protein-protein interactions. Also, *in vitro* folding and thermostability assays would also allow us to validate the results of our experiments using cellular lysates.

Homologous Missense Mutations Reveal Differences in Yeast Apn1 and E. coli Endo IV Structure-function Relationships

Apn1 and endo IV are considered orthologous due to the degree of amino acid identity (41%) and the amino acid conservation within functional domain, including the 9 metal binding, catalytic residues (Fig. 1). In addition, cross-species complementation studies confirm their functional conservation. Protein orthologs, which, derived from a common ancestral protein, evolve under different selective pressures [55]. Thus, while the residues vital for conserved enzymatic function remain the same for endo IV and Apn1 (Fig. 1), the 3-dimensional topological network in which the conserved residues reside and interact with other residues [56, 57] could be different. While the V156 residue is conserved in yeast (Fig. 1), we hypothesize that the local interaction network of V156 may not include an actual contact with E158, although the homologous interaction occurs in the *E. coli* enzyme. If this prediction is true, it may explain why the substitutions of Apn1 V156/ endo IV V143 impact the enzymatic activity of endo IV, but not Apn1.

Homology modeling is a useful technique for developing limited hypotheses due to the provisional nature of the 3D structures produced. Based on the *E. coli* endo IV-templated Apn1 homology model produced in our studies, V156 was predicted to physically interact with catalytic residue E158 and the V156E substitution was predicted to abolish this interaction, leading to decreased enzymatic activity. Our molecular and functional data do not support this prediction. The question then arises as to how closely

the endo IV structure actually approximates the Apn1 structure. Production of a high resolution Apn1 structure could help confirm our prediction regarding amino acid network differences between Apn1 V156 and endo IV V143. Comparison of structures could provide insight into how amino acid interaction networks have evolved and aid in determining if the observed cellular and biochemical differences result from obvious structural differences.

Part II

Future directions

Our initial experimental approach took into account a technical consideration that we felt needed to be addressed to increase the likelihood of capturing DNA-protein interactions by ChIP. As mentioned, Apn1 is expressed at relatively low levels. Also, the interaction of Apn1 with the genomic DNA is expected to be transient [41], especially compared to other DNA binding proteins such as histones [58]. Thus, we overexpressed a TAP (tandem affinity purification)-tagged version of Apn1 for the ChIP analysis. We reasoned that overexpression may increase the likelihood of capturing native Apn1-DNA interactions via ChIP, with the caveat that some of the identified binding sites may not represent normal interactions. As both protein overexpression and epitope tagging introduce artificial and potentially confounding factors into the analysis, once candidate Apn1 binding sites to be validated are identified in preliminary studies, we propose the use of native expression conditions using either a TAP-tagged version of Apn1 or anti-Apn1 antibodies to detect native enzyme for future ChIP experiments. One way to identify candidate sites is to focus on Apn1 binding site regions present in multiple biological replicates for particular experimental conditions.

There was not a sufficient amount of DNA in our ChIP samples to directly apply the immunoprecipitated DNA to the tiling arrays for DNA microarray analysis. Thus, the protocol was modified to include a whole genome amplification step, which produced enough DNA to proceed to the microarray analysis step. The lack of a sufficient quantity of DNA was also revealed by the relatively low number of peaks for several of the biological replicates. For example, one of the three replicates for the 0 mM condition only produced three peaks, and this replicate will not be used for further statistical analysis of the current data. To remedy this issue, in the future we can increase the amount of ChIP DNA by increasing the volume of yeast culture to increase the number of nuclei, and thus the number of Apn1-DNA interactions probed. Other factors that affect yield of ChIP DNA include cell lysis efficiency, immunoprecipitation efficiency and efficiency of chromatin sonication. We can also optimize these steps to hopefully eradicate both the whole genome amplification step and lack of sufficient peaks for thorough bioinformatics analysis.

Alternative Experimental Approaches

Dominant Negative Apn1 Protein as an Experimental Tool

An intriguing experimental approach was previously employed by investigators who isolated a dominant negative form of the major human AP endonuclease APE1 to study DNA repair mechanisms [59, 60]. Relevant features of this mutant include lack of nuclease activity, higher binding affinity for DNA than wild type enzyme, and the interference with the repair function of wild type APE1. Thus, we propose to generate a dominant negative allele of Apn1 to effectively increase the binding of target DNA damage sites to aid in capturing sites of BER to address the transient nature of Apn1-DNA interactions. The dominant negative APE1 enzyme contains amino acid

substitutions of two active site residues. Single amino acid substitution mutants disrupting enzymatic activity and increasing the DNA binding affinity of Apn1 have been reported [14]. Creating double mutants similarly to the APE1 dominant negative mutant may be a good starting point for the generation of similar Apn1 mutant protein. Using such a molecular tool for ChIP could be done without overexpression or epitope tagging of Apn1.

Assessment of DNA Repair Kinetics

After 90 minutes of exposure to H_2O_2, a relatively unstable molecule, we are unsure to what extent the DNA damage has been repaired, if at all. If damage was repaired to an appreciable extent at this time point, we may not be effectively identifying all Apn1 binding sites. At the time this project was started, there were no known reliable methods to detect oxidative DNA damage level in the yeast genome. In a recent report, investigators used an experimental approach called a comet assay to measure the kinetics of genomic DNA repair following exogenous exposure to the oxidizing agent H_2O_2 [61]. Based on their measurements, we estimate that perhaps 75% of the H_2O_2-induced damage may be repaired at the time crosslinking is performed in our ChIP protocol. In order to choose the best time of exposure to H_2O_2 for our experiments, we could perform a comet assay to estimate the kinetics of DNA repair to correlate with our experimental set-up.

Genomic Instability as a Predictive Biomarker for Response to Chemotherapy

It has been proposed that the underlying genomic instability in tumor cells could be used to design more effective cancer treatments that selectively target cancer cells over normal cells [62]. Thus, combined with standard clinical procedures, genetic

instability within at-risk loci could be used to predict tumorigenicity in potentially cancerous lesions, and could be used as a screening tool to provide the most effective cancer treatment regimen.

Concluding Remarks

Overall, results reported in this dissertation demonstrate how studies in a simple model eukaryotic system can provide important insights for elucidating function of disease-associated hAPE SNPs and mechanisms of DNA-damage induced genomic instability, an important hallmark of human cancer [7, 32].

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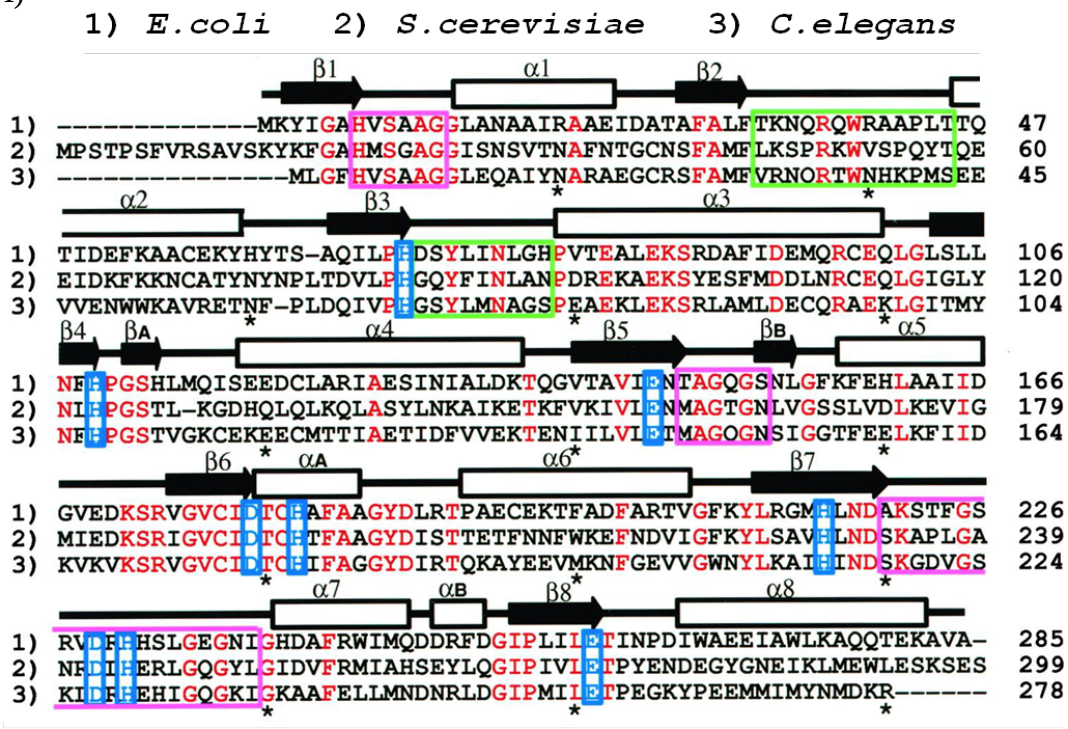
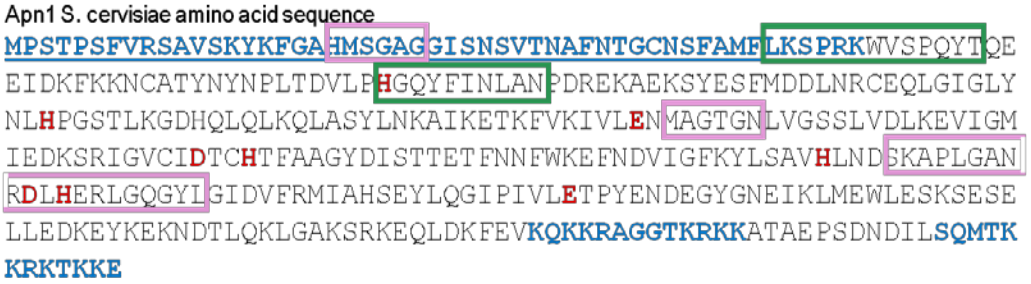
Figure 1.

A)


1) E.coli 2) S.cerevisiae 3) C.elegans
β1 α1 β2
1) ----------MKYIGAHVSAAGGLANAAIRAAEIDATAFALFTKNQRQWRAAPLTTQ 47
2) MPSTPSFVRSAVSKYKFGAHMSGAGGISNSVTNAFNTGCNSFAMFLKSPRKWVSPQYTQE 60
3) ----------MLGFHVSAAGGLEQAIYNARAEGCRSFAMFVRNQRTWNHKPMSEE 45
α2 β3 α3
1) TIDEFKAACEKYHYTS-AQILPHDSYLINLGHPVTEALEKSRDAFIDEMQRCEQLGLSLL 106
2) EIDKFKKNCATYNYNPLTDVLPHGQYFINLANPDREKAEKSYESFMDDLNRCEQLGIGLY 120
3) VVENWWKAVRETNF-PLDQIVPHGSYLMNAGSPEAEKLEKSRLAMLDECQRAEKLGITMY 104
β4 βA α4 β5 βB α5
1) NFHPGSHLMQISEEDCLARIAESINIALDKTQGVTAVIENTAGQGSNLGFKFEHLAAIID 166
2) NLHPGSTL-KGDHQLQLKQLASYLNKAIKETKFVKIVIENMAGTGNLVGSSLVDLKEVIG 179
3) NFHPGSTVGKCEKEECMTTIAETIDFVVEKTENIILVIETMAGQGNSIGGTFEELKFIID 164
β6 αA α6 β7
1) GVEDKSRVGVCIDTCHAFAAGYDLRTPAECEKTFADFARTVGFKYLRGMHLNDAKSTFGS 226
2) MIEDKSRIGVCIDTCHTFAAGYDISTTETFNNFWKEFNDVIGFKYLSAVHLNDSKAPLGA 239
3) KVKVKSRVGVCIDTCHIFAGGYDIRTQKAYEEVMKNFGEVVGWNYLKAIHINDSKGDVGS 224
α7 αB β8 α8
1) RVDRHHSLGEGNIGHDAFRWIMQDDRFDGIPLILETINPDIWAEEIAWLKAQQTEKAVA- 285
2) NRDIHERLGQGYLGIDVFRMIAHSEYLQGIPIVLETPYENDEGYGNEIKLMEWLESKSES 299
3) KIDRHEHIGQGKIGKAAFELLMNDNRLDGIPMILETPEGKYPEEMMIMYNMDKR------ 278

B)


Apn1 S. cervisiae amino acid sequence
MPSTPSFVRSAVSKYKFGAHMSGAGGISNSVTNAFNTGCNSFAMFLKSPRKWVSPQYTQE
EIDKFKKNCATYNYNPLTDVLPHGQYFINLANPDREKAEKSYESFMDDLNRCEQLGIGLY
NLHPGSTLKGDHQLQLKQLASYLNKAIKETKFVKIVLENMAGTGNLVGSSLVDLKEVIGM
IEDKSRIGVCIDTCHTFAAGYDISTTETFNNFWKEFNDVIGFKYLSAVHLNDSKAPLGAN
RDLHERLGQGYLGIDVFRMIAHSEYLQGIPIVLETPYENDEGYGNEIKLMEWLESKSESE
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KRKTKKE

C)

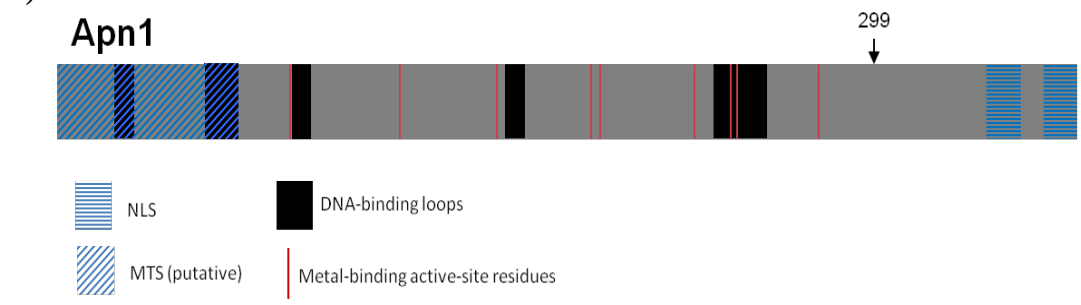
Apn1
299
NLS
DNA-binding loops
MTS (putative)
Metal-binding active-site residues

Figure 1. Apn1 Protein Structure. **A)** Amino acid sequence alignment of 1) *E. coli* , 2) *S. cerevisiae*, and 3) *C. elegans* Endo IV highlighting for this family of AP endonucleases. Conserved residues between species (red), metal-binding active site residues (blue), and functional enzyme motifs, including minor groove and phosphate-binding loops (boxed in green and magenta, respectively). Above the sequences, hollow tubes indicate α helices, black arrows show β strands, and solid lines show the loops that connect the secondary structure elements. Below the sequence, black asterisks mark every twentieth position in the *E. coli* sequence. *The sequence alignment for *S. cerevisiae* Apn1 is cut-off at residue 299, but the primary sequence actually extends to 367 amino acid residues. Adapted from Hosfield [63]. **B)** Apn1 amino acid sequence. Metal-binding active site residues (red), mitochondrial targeting signal (blue underlined), nuclear localization signal (blue), minor groove specific DNA binding loops (pink boxes), phosphate specific DNA binding loops (green boxes). **C)** Schematic of Apn1 protein structure. Unspecified amino acids (grey), nuclear localization sequences (blue striped boxes), mitochondrial targeting signal (blue hashed boxes), DNA-binding loops (black boxes), metal binding active site residues (red lines). The arrow and number above it indicate where the amino acid alignment between yeast Apn1 and *E. coli* endo IV ends in panel A.

Figure 2.

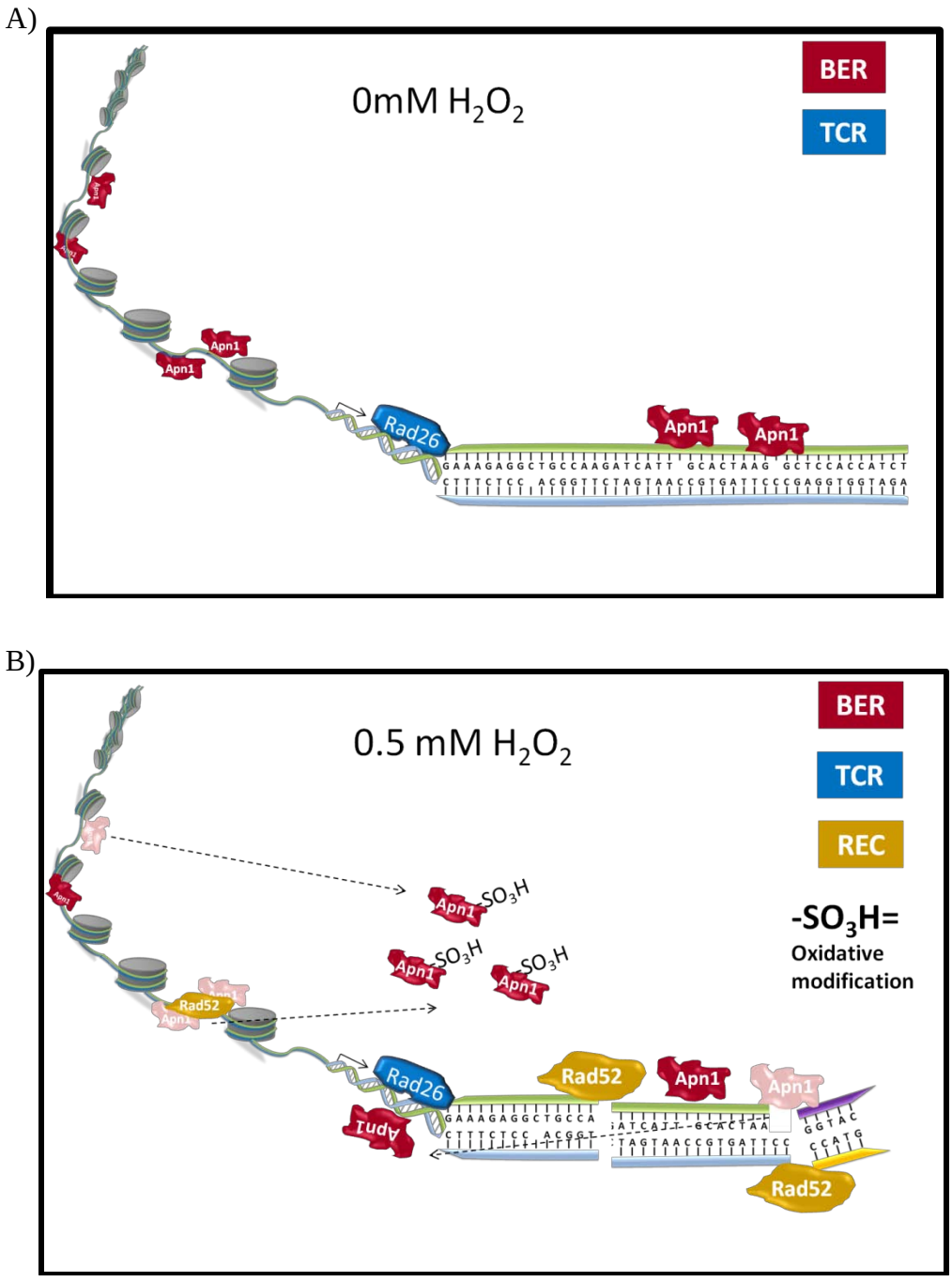

Figure 2. Model: Apn1 genomic occupancy. **A)** 0 mM (normal) conditions. Apn1(base excision repair [BER]) binds at ~1,000 sites across the genome under normal cellular conditions. Apn1 "prefers" GC rich sequences, presumably because Cs and Gs are

thought to be most susceptible to mutagenic oxidative damage. Apn1 binding sites contain “few” intragenic sequences, potentially because other pathways, such as transcription-coupled repair (TCR, Rad26), can handle the repair of active regions of the genome. **B)** Following exposure of cells to 5.0 mM H_2O_2, Apn1 occupancy decreases to ~250 binding sites across the genome. Under this condition, Apn1 still prefers GC rich sequences, but Apn1 peaks contain mostly intragenic sequences. We hypothesize that the decrease in Apn1 binding sites may reflect direct oxidative damage to Apn1 molecules (SO3H=sulfonic acid, irreversible damage product to protein sulfhydryls [64]), rendering them unable to repair the DNA. This same reasoning applies for other repair proteins. In this case, we predict that other damage management pathways, such as recombination (REC), serve to backup and cooperate with BER in the repair of oxidative DNA damage.