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Functions of the major abasic endonuclease (APE1) in cell viability and genotoxin resistance

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Running head: Functions of APE1

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Abstract

DNA is susceptible to a range of chemical modifications, with one of the most frequent lesions being apurinic/apyrimidinic (AP) sites. AP sites arise due to damage-induced (e.g., alkylation) or spontaneous hydrolysis of the N-glycosidic bond that links the base to the sugar moiety of the phosphodiester backbone, or through the enzymatic activity of DNA glycosylases, which release inappropriate bases as part of the base excision repair (BER) response. Unrepaired AP sites, which lack instructional information, have the potential to cause mutagenesis or to arrest progressing DNA or RNA polymerases, potentially causing outcomes such as cellular transformation, senescence, or death. The predominant enzyme in humans responsible for repairing AP lesions is AP endonuclease 1 (APE1). Besides being a powerful AP endonuclease, APE1 possesses additional DNA repair activities, such as 3'-5' exonuclease, 3'-phophodiesterase, and nucleotide incision repair. In addition, APE1 has been shown to stimulate the DNA binding activity of a number of transcription factors through its "REF1" function, thereby regulating gene expression. In the article here, we review the structural and biochemical features of this multifunctional protein, while reporting on new structures of the APE1 variants Cys65Ala and Lys98Ala. Using a functional complementation approach, we also describe the importance of the repair and REF1 activities in promoting cell survival, including the proposed passing-the-baton coordination in BER. Finally, results are presented indicating a critical role for APE1 nuclease activities in resistance to the genotoxins methyl methanesulfonate and bleomycin, supporting biologically important functions as an AP endonuclease and 3'-phosphodiesterase, respectively.

Genotoxic stress can arise from altered cellular metabolism or following exposure to many external agents, including sunlight, ionizing radiation, polycyclic aromatic hydrocarbons (e.g., benzo[*a*]pyrene), aromatic amines (e.g., 2-acetylaminofluorene), aflatoxins, various heavy metals (e.g., cadmium), and numerous therapeutic agents (e.g., X-rays, platins, alkylators, crosslinkers, topoisomerase poisons, and anti-metabolites). One of the more common forms of intracellular stress originates from an imbalance in reactive oxygen species (ROS) formation, typically stemming from defects in mitochondrial respiration or metabolism, and their sequestration by the protective scavenging systems. In conditions of so-called oxidative stress, the elevated ROS can attack nearby intracellular macromolecules, including proteins, lipids, and nucleic acids, potentially giving rise to cellular dysfunction. As damage to one's genetic material can lead to permanent sequence changes or to alterations in DNA and/or RNA metabolism that can promote disease and aging, DNA repair processes have evolved as a major front-line defense against the harmful consequences of genotoxic stress. In the article here, we review the biochemical and structural features of a key DNA repair enzyme, apurinic/apyrimidinic endonuclease 1 (APE1), and present new data that provides important insights into the biological functions of this multifunctional protein.

DNA Damage and Its Consequences

DNA damage can exist in multiple chemical forms. These include simple base modifications, such as uracil or 8-oxoguanine, bulky base modifications like ultraviolet light-induced photoproducts or platinum adducts, DNA strand break interruptions, and linkages that covalently attach the two strands of the helix (so-called interstrand crosslinks). Depending on their chemical composition and structural impact, the various DNA lesions can: (i) alter the coding sequence of the genome, directing mutations during chromosome replication, and/or (ii) adversely affect the progression of replicative DNA or RNA polymerases, leading to fork collapse or transcription arrest. Mutational events are known to underlie cancer etiology, and likely contribute more broadly to disease and aging, while replication fork collapse can drive chromosome instability commonly associated with carcinogenesis, and persistently stalled transcription apparatuses can contribute to premature aging phenotypes, as seen in Cockayne syndrome ([1]).

Apurinic/Apyrimidinic (AP) Sites

One of the more common modifications to occur in DNA is the AP site (Fig. 1). Most AP sites arise due to damage-induced or spontaneous hydrolysis of the N-glycosidic bond that attaches the base moiety to the ribose ring of the phosphodiester backbone. Based on chemical extrapolations, Lindahl and colleagues estimated that >10,000 AP sites will be created in a single mammalian genome per day under normal physiological conditions ([2]). The hydrolytic AP site exists in two major forms, ring-opened and ring-closed (Fig. 1, top). The latter is the predominant form, while the former is susceptible to reactions with amines (such as those present in lysine residues) that can establish a covalent intermediate (i.e, an imine or Schiff-base) and bulky adduct, as seen with many DNA binding proteins (see for instance, [3], [4], [5]). Recent work has shown that AP sites can also generate links between the two strands of DNA by reacting with adenine or guanine in the complementary strand ([6], [7]). Such interstrand crosslinks pose strong impediments to progressing DNA or RNA polymerases, as they prevent unwinding of the DNA duplex. More information on the processing of lethal DNA interstrand crosslinks can be found elsewhere ([8], [9]).

Besides the hydrolytic AP site, abasic sites can exist in oxidized forms, e.g., C1'-oxidized (a.k.a. 2-deoxyribonolactone) or C4'-oxidized (Fig. 1, bottom), produced by free radical-mediated hydrogen abstraction at the C1' or C4' position of the ribose sugar ring ([10]). As with the hydrolytic AP site, oxidized abasic lesions can form covalent crosslinks with several proteins, most notably the major gap-filling DNA polymerase, POLβ ([11]). The mechanisms for coping with large protein-DNA adducts, which clearly would hinder DNA or RNA polymerase progression, are just now becoming better understood ([12], [13]). Lastly, AP sites are generated as enzymatic intermediates during the process of base excision repair (BER), after substrate base release by a DNA glycosylase (see more later).

AP sites have been reported to have very little effect on the overall helical architecture of DNA, although they appear to allow for a more flexible phosphodiester linkage at the site of the lesion, a feature that is likely important to recognition and incision [14]. However, it's noteworthy to point out that the structural effects of AP sites can be influenced by the base opposite and the neighboring bases ([15] and references therein). For example, when the AP site is flanked by a purine on either side, the duplex is more likely to collapse (due to base-stacking), rotating the abasic lesion out of the helix and often extruding the base opposite. A purine opposite, however, is more likely to favor the normal B-DNA form. Notably, this subtle sequence-context variability can have significant, albeit moderate, effects on cleavage rates, a feature that might influence repair efficiency and the molecular consequences ([16],[17]). Moreover, X-ray crystal structures of APE1 (the major mammalian AP endonuclease; see later) in complex with DNA substrates containing various mismatched base pairings flanking an AP site revealed unique DNA conformations that corresponded to a wide range of incision efficiencies ([18]).

As the name implies, an AP site lacks the instructional information provided by the base moiety, and thus, is considered non-coding. Early evidence suggested an A-rule mutational signature upon DNA polymerase bypass of an AP site, particularly in *Escherichia coli* ([19]), but there has been unclarity regarding this topic, as much of the mutagenic outcome of DNA damage has been complicated by the identification of numerous translesion synthesis (TLS) DNA polymerases in eukaryotes. These enzymes seemingly have evolved to permit survival via error-prone (often mutagenic) bypass as opposed to persistent replication arrest and cell death, and don't necessarily follow the same insertion strategy to synthesize past a particular blocking lesion ([20], [21]). At present, what is clear is that AP sites can present major blocks to both DNA and RNA polymerases, but in the former situation, can be effectively bypassed by several TLS polymerases, with bypass leading to single nucleotide substitutions or small insertions/deletions ([22], [23], [24], [25], [26]). With the development of new animal models that permit selective deletion of APE1 and consequent cancer formation (see later), it may now be possible to assess disease-associated mutational patterns in the absence of the repair protein.

BER Overview

BER is the major pathway for coping with base damage, abasic sites, and various single-strand break interruptions (Fig. 2). In brief, classic BER can be broken down into five major steps, although the precise order of the steps may vary due to protein concentration variability, efficiency of the different reactions, and the nature of the DNA intermediates: (i) base excision by a DNA glycosylase, (ii) incision at the resulting AP site, (iii) clean-up of the 5' and/or 3' terminal end(s), (iv) replacement of the damaged and excised nucleotide, and (v) nick ligation to complete the process. In mammals, there are eleven DNA glycosylases that remove any number of inappropriate bases from DNA, including uracil (the spontaneous product of cytosine deamination) and 8-oxoguanine (one of the many oxidatively modified

base lesions) ([27]). Besides the monofunctional DNA glycosylases (e.g., UNG, MPG), which only operate to excise base substrates, bifunctional DNA glycosylases can cleave the phosphodiester backbone at the AP site product, creating a strand break with a 3'-α,β-unsaturated aldehyde (β-elimination; OGG1, NTH1) or 3'-phosphate (β,δ-elimination; NEIL1, NEIL2). It's unclear, however, how much the AP lyase activities of the bifunctional enzymes are at play in cells, since they are quite poor enzymatically. Regardless, in situations involving an intact AP site (left behind by either type of DNA glycosylase), the predominant pathway appears to be cleavage by the major AP endonuclease, APE1 (Fig. 2, right). Following APE1 incision, the primary gap-filling DNA polymerase, POLβ, inserts the missing nucleotide and excises the remaining 5'-abasic fragment (deoxyribose phosphate). The final nick is sealed by a complex of X-ray cross-complementing protein (XRCC1) and DNA ligase 3 α (LIG3α). There exist variations of the BER pathway, including a strand-displacement, long-patch mechanism, but the reader is directed to additional reviews for a more thorough description of the BER system, including its prominent role in both nuclear and mitochondrial DNA repair ([28], [29], [30], [31], [32]).

Structure, Mechanism and Biochemical Functions of APE1

APE1 is a 318 amino acid, multifunctional protein that participates in DNA repair and transcriptional regulation. Indeed, APE1 was independently purified and subsequently cloned based on its ability to: (i) process DNA damage, such as AP sites and 3'-DNA modifications, and (ii) stimulate the DNA binding activity of several transcription factors, such as the AP-1 heterodimer ([33], [34], [35], [36]). This latter function led to the nomenclature "redox effector factor 1" (a.k.a., REF1), one of the proteins many aliases. APE1 shares notable sequence homology to *E. coli* exonuclease III (Xth), an enzyme with AP endonuclease, 3'-phosphodiesterase, and 3'-5' exonuclease activities ([37]). The conservation occurs mainly in the C-terminal ~260 residues of APE1, which make up the nuclease core of the mammalian protein (Fig. 3A). The presumably acquired N-terminal region of APE1 appears to direct intranuclear localization, refine nucleic acid interactions, mediate protein associations, and participate in the aforementioned REF1 function (reviewed in [38], [39]).

X-ray crystallographic studies have revealed that APE1 (residues 43-318) belongs to the phosphoesterase superfamily of enzymes that contain a common four-layered α/β sandwich structural core (Fig. 3B), showing clear similarity to other nucleases (i.e., Xth, DNase I, RNase H, and LINE-1) and also several functionally unrelated proteins (i.e., IP5P, Ccr4p, CdtB, NOCT) ([40], [41]). Key elements of the APE1 core include distinct loop regions and a unique active site that allows APE1 to specifically recognize, bind, and slide along a DNA strand in search of an AP site primarily through interactions with the DNA phosphate backbone ([42], [43]).

Crystal structures of APE1 complexed with AP site analogue [THF (tetrahydrofuran)]-containing duplex DNA have revealed that the protein kinks the DNA by ~35° and flips the AP site out of the double helix and into the hydrophobic active site binding pocket (comprised of residues Phe266, Trp280, and Leu282) to facilitate complex formation. Once in the active site, the AP site is stabilized in position for cleavage of its 5'-phosphate backbone by residues contained within several loop regions that recognize the AP-DNA ([44], [45]). Specifically, APE1 is proposed to stabilize the flipped-out AP site via a double-loop penetration mechanism, involving interactions of loops α11 and α5 with the minor and the major grooves, respectively. With the AP site positioned into the APE1 active site, an orphan base is left in the opposing strand of the double helix. Side chain Arg177, of loop α5, acts as a surrogate base by intercalating into the major groove and forming a base-stacking interaction ([45]). Importantly, this

interaction is specific to product DNA and thus corroborates biochemical data indicating Arg177 specifically enhances product binding while only moderately affecting substrate binding ([46], [47]). An additional loop region (α8) interacts with the major groove on the 3' side of the AP site, and along with the hydrophobic pocket, has been shown to dictate substrate specificity as demonstrated by site-specific APE1 variants ([48], [49]).

Prior studies have shown that amino acids Glu96, Asp210, and His309 are critical to the enzymatic nuclease activity of APE1 ([50], [51]). The reported data are consistent with a catalytic mechanism that involves in-line nucleophilic attack of the AP site phosphorus atom by a nucleophilic water molecule that is highly coordinated through hydrogen bonding to Asn212 and Asp210 [52]. This attack is facilitated by charge neutralization afforded by two hydrogen bonds to the phosphate non-bridging oxygens from Tyr171 and His309. A single Mg^{2+} is coordinated by Asp70, Glu96, and a water that is in contact with the non-bridging oxygen of the phosphate. Crystal structures imply a pentacovalent intermediate that is stabilized by Mg^{2+} and key active site contacts ([45], [53]). Moreover, it has been proposed that during catalysis, the metal shifts to coordinate both the phosphate non-bridging oxygen and the newly generated O3' ([45]). Importantly, the interactions mentioned here appear to be fundamental for APE1 catalysis, as their independent elimination via site-directed mutagenesis has been shown to significantly diminish catalytic activity (in some cases, by >4-orders of magnitude).

As mentioned above, APE1 participates as a central component of the BER response (Fig. 2). In particular, following base excision by a substrate-selective DNA glycosylase, APE1 cleaves at the resulting AP site intermediate to generate a strand break with a 3'-hydroxyl priming group and a 5'-deoxyribose phosphate (5'-dRP). Based on biochemical and structural studies, APE1 has apparently evolved not for enzymatic efficiency, but to optimize pathway efficiency by remaining bound to its incised product to facilitate "hand-off" to the next enzyme in the pathway, POLβ, in a process referred to as "passing-the-baton". This model is supported by the evidence that mutation of the Arg177 residue in APE1 leads to improved turnover of the enzyme ([44]). However, clear cellular evidence of the significance of this mechanism has not be reported.

Besides its robust AP endonuclease activity, APE1 has been demonstrated to remove 3'-mismatched or damaged nucleotides through its 3' to 5' exonuclease function; excise obstructive 3'-lesions, such as α,β-unsaturated aldehydes, phosphoglycolates, and phosphates; initiate repair of bulky oxidative base lesions via a process called nucleotide incision repair (NIR); and degrade damaged RNA molecules as a potential cleansing mechanism (Fig. 3B) (reviewed in [31], [54]). Of note, both biochemical and structural data indicate that unique active site contacts (i.e., Phe266) play a key role in APE1 3' end processing activities ([55], [56]). In addition to its various nuclease and redox regulatory activities, APE1 has been shown to operate as an integral component of transcription complexes, exhibiting the ability to even control its own gene expression ([57]).

As mentioned above, APE1 is often referred to by the alias REF1, due to its ability to stimulate the DNA-binding activity of several transcription factors through modulation of their redox status ([33]). Transcription factors in which APE1 has been shown to activate via this mechanism include AP-1, NFκβ, HIF1α, and p53 ([58]). The APE1 redox activity can be disabled by mutation of residue Cys65 to Ala; however, because APE1 lacks the C-X-X-C motif common to most redox regulatory proteins for the formation, isomerization, and reduction of disulfide bonds, the precise mechanism it employs to activate transcription factor activity remains enigmatic ([59], [60], [61]). There is some evidence

indicating Cys93 plays a role in the APE1 redox reaction, but both Cys65 and Cys93 are buried in the protein structure and are positioned too distant from one another to form a disulfide bond. Regardless, a model in which Cys65 acts as the nucleophile for reduction of the disulfide bond in the targeted transcription factor, and Cys93 serves as the resolving reside, has been proposed ([62]). In this model, APE1 must undergo a large conformational change or become partially unfolded to reveal a third Cys residue in order to facilitate the reaction. Further work is clearly required to determine the precise mechanism of the REF1 function of APE1 ([63]).

Effect of Amino Acid Substitutions on APE1 Structure-Function

As an extension of the early structure-function work, studies have since identified a set of strategic, site-specific mutations that uniquely affect the different proposed activities of APE1 (Table 1). We hypothesized that these variants would offer a means to evaluate the biological contribution of the different functions of the protein using a previously described complementation approach (see later). However, before determining their cellular complementation effectiveness, we sought to compare the structures of the different APE1 variants to gain insight into their potential biochemical and structural properties.

To establish structures for a few of the proteins, we employed a previously described expression system ([56]) and X-ray crystallographic method ([45]). Structures of untagged Cys65Ala (the REF1 mutant) and Lys98Ala (the NIR mutant) engaged with an AP endonuclease product (i.e., the cleaved AP site) showed high similarity to wild-type (WT) APE1, with very little change in the orientation of the DNA, protein backbone, or sidechains in response to either mutation (Table 2; Fig. 4). In the case of Cys65Ala (Fig. 4A), the neighboring residue Trp83 is slightly shifted (1.4 Å); however, it is likely that the absence of the key reducing cysteine residue, and not this modest shift, results in the abolition of redox activity in response to the mutation (see [62] and references therein). While the Lys98Ala structure does not show any structural perturbation in response to the mutation (Fig. 4B), the hydrogen bonding interaction between Lys98 and the carboxyl group of Asp70 observed in the WT structure is lost. Given the generally normal structural features of the Lys98Ala mutant, it's not complete clear how the substitution would selectively interfere with the NIR activity as reported ([64]).

Structures of APE1 containing the mutation Asp210Asn or Phe266Ala have been previously reported in the literature. A catalytically dead double mutant enzyme (Asp210Asn Glu96Gln) demonstrated the structural consequence of Asp210Asn to be in its altered coordination of the nucleophilic water and fellow catalytic triad reside Asn68 ([45]). Consistent with its critical role in the APE1 nuclease reaction, substitution of Asp210 renders the protein essentially inactive ([51]). In a separate study, a structure of Phe266Ala APE1 bound to DNA containing a C/T mismatch at the 3' end of a nick (a 3' exonuclease substrate) revealed an enlarged active site pocket, allowing the mismatched C to occupy an alternate conformation in which it has rotated ~120° relative to WT ([56]). These data support the observation that a Phe266Ala substitution enhances the enzyme's 3'-5' exonuclease function ([48]).

To our knowledge, there are no reported structures of either Arg177Ala or Asp70Ala. However, based on structures of WT APE1 and complementary biochemical studies, the functional roles of these two residues have been described in detail (see also Table 1). In particular, Arg177 contributes to BER coordination (see earlier), while Asp70 has been implicated in binding the divalent metal ion ([45], [65]), and substitution of this residue with Ala has been reported to enhance the 3'-phosphodiesterase activity

of the protein ([66]). Lastly, any structural consequence of the Lys6Arg/Lys7Arg mutations remains enigmatic, as the N-terminal region of APE1 has yet to be crystallized due to its inherent disorder.

In addition to the many mechanism-based mutations that have been introduced into APE1 (see above), evidence indicates that several missense substitutions, both germline and disease-associated, exist in the protein within the general population. While it's unclear for many of the amino acid variants whether they exhibit altered function, the polymorphic variants Gln51His (~3% frequency), Ile64Val (~0.5%) and Asp148Glu (~45%) have been demonstrated to display WT properties in a number of functional and structural tests ([67]). Recent work, however, has found that one tumor-associated variant (Arg237Cys) identified in a single endometrial cancer patient may lead to impaired biochemical and cellular activity ([68], [69]). This observation suggests that defects in the function of APE1, either germline or somatic, could contribute to disease susceptibility, although profound deficiencies in APE1 at conception are likely to be incompatible with life (see next).

Backdrop on APE1 Biology

Early work demonstrated that germline deletion of both alleles of APE1 in mice leads to embryonic lethality, indicating a requirement for the protein in multicellular organismal development ([70], [71]). More recent efforts have also found that sufficient depletion of APE1 in cells (around 60% or more) results in apoptotic death ([72]). The essential nature of APE1 has made investigations into the biological role of the protein and its various biochemical activities complicated. And while useful cell models have been reported using knock-down strategies (see for instance, [73]), this approach is complicated by the residual, endogenous WT APE1 protein that remains. Nonetheless, beyond the cell growth complications noted above, evidence indicates that defects in APE1 function give rise to profound sensitivity to alkylating agents, such as methylmethane sulfonate (MMS) and the chemotherapeutic drug temozolomide, and to a lesser extent, increased sensitivity to oxidizing agents (such as hydrogen peroxide), ionizing radiation, the radiomimetic bleomycin, and the chain-terminating nucleoside analog, β-L-dioxolane-cytidine (clinically known as troxacitabine). These findings are consistent with a major role for the AP endonuclease function of APE1, and a biologically important role for its 3'-repair activities (reviewed in [74]). We note that there are reports describing functions of APE1 in cellular processes such as antibody diversification, RNA metabolism, and the granzyme A (GzmA)-activated cell death response, which are described in greater detail elsewhere ([75], [76], [77]).

The recent creation of floxed APE1 mouse models has permitted the first glimpse into the consequences of APE1 absence on organismal physiology and health. In the first report describing an APE1 tamoxifen-inducible, Cre-recombinase, conditional knockout mouse, the protein was found to be vital for protecting both gray and white matter from the oxidative stress induced by transient focal cerebral ischemia, and for functional recovery of the central nervous system after mild stroke injury ([78]). Subsequent analysis examining the consequences of whole-body, conditional-deletion of APE1 revealed that gene inactivation before weaning (i.e., around post-natal day 7-12) resulted in profound growth impairment and animal death ([79]). APE1 gene deletion later in life (around week 6 post-weaning) caused a more subtle phenotype, which became more evident around 8-months of age and included several premature aging characteristics, such as loss of hair, reduced wound healing, and increased senescence. The most recent paper, where APE1 was selectively deleted in the brain by crossing APE1-floxed animals with mice that express the Cre recombinase via the brain-specific promoter Nestin, found that mutant animals appeared normal at birth (despite embryonic gene inactivation), yet experienced

rapid and profound brain-wide degenerative changes that coincided with the change to respiratory oxygenation and led to death within a few months ([80]). Moreover, loss of APE1, even in a heterozygous state, along with inactivation of the tumor suppressor p53 resulted in a dramatic increase in cancer susceptibility, specifically for glioblastoma and medulloblastoma. These more targeted studies are generally consistent with past work that has found that haploinsufficient animals, relative to controls, exhibit an increase in spontaneous mutagenesis and cancer development ([81], [82]). What is still unclear from the current analysis, however, is the precise contribution of the different functions of APE1 to the pathology observed.

Biological Significance of the Different APE1 Functions

Several studies have investigated the biological contributions of the different functions of APE1, focusing mainly on their role in preserving cell viability. Three independent efforts, using variations on the functional complementation theme, have found that the repair nuclease activity of APE1 is essential in averting apoptotic cell death of APE1-depleted/deficient cells ([72], [83], [73]). Findings regarding the REF1 function are less clear, with two of the studies indicating no obvious role in preserving cell growth, yet the other reporting a critical contribution ([73]). The work of Izumi et al ([83]) also described an important function for the transcriptional regulatory activity of APE1 via its N-terminal lysine residues (Lys6 and Lys7). However, in that work, they did not demonstrate expression of the mutant protein, raising doubt about the conclusions drawn, and Vascotto et al ([73]) has since reported near WT rescue of cell viability with the same Lys6/7Arg mutant protein. As mentioned earlier, we developed a complementation strategy to more comprehensively investigate the biological significance of the different functions of this multifunctional protein in an APE1-deficient background, using a set of APE1 mutants that are uniquely altered in a particular activity (Table 1). We acknowledge, however, that although we have taken steps to ensure reproducibility and accuracy of the results, our method of transient overexpression needs to be kept in mind when interpreting results herein.

Before carrying out the complementation work, we examined the effect of the different mutations on protein localization and stability when expressed in mammalian cells. As our complementation studies were to be conducted with GFP-tagged fusion proteins, we were able to easily monitor intracellular distribution using fluorescence confocal microscopy after transfection of the different plasmid DNA constructs. As shown in Fig. 5A, when expressed in HeLa cells, GFP alone exhibited the typical pan staining throughout the cell, whereas most APE1 GFP fusion proteins displayed nuclear localization similar to that of the WT protein. The exceptions were ΔN20 (lacking the 20 N-terminal residues) and Lys299Ala/Arg301Ala, proteins designed to address issues related to nuclear ([84]) and mitochondrial ([85]) localization, respectively. However, given the lack of a defined localization pattern for these two APE1 variants (Fig. 5A), we excluded them from further analysis, as any results would be difficult to interpret with confidence. As seen with the HeLa cells, the remaining APE1 mutants when expressed in the TM-Cre APE1$^{fl/fl}$ MEFs exhibited a nuclear localization pattern similar to that observed for WT APE1 (Fig. 5B). Moreover, examining the fluorescence intensity of each protein as a means of assessing protein expression and stability revealed that each APE1 mutant was present at a steady-state level that was similar to the WT protein (Fig. 5C). The findings in total, including the current X-ray structure data (see earlier), support the conclusion that the site-specific mutations have no gross effect on protein conformation and that the different mutants are expressed at comparable levels in the target mouse cells.

We have described previously the complementation strategy ([69]), which we have outlined in Fig. 5D. In brief, we used mouse embryonic fibroblasts (MEFs) that harbor floxed APE1 alleles ($APE1^{fl/fl}$) and a tamoxifen-inducible Cre recombinase expression cassette (TM-Cre). In our experimental paradigm, we first transfect TM-Cre $APE1^{fl/fl}$ MEFs with a designated plasmid (i.e., vector or specific APE1 construct), briefly select for the plasmid with hygromycin, and then incubate the cells with TM to induce deletion/inactivation of both endogenous APE1 alleles. We can then monitor cell viability (replicative lifespan) over time or determine genotoxin sensitivity of the MEFs as a function of the complementing plasmid.

Repair and REF1 Function Contribute to Cell Viability

It has been demonstrated that mammalian cells deficient for APE1 have an abbreviated replicative lifespan (see earlier). To address the role of the different APE1 functions in facilitating cell survival, we assessed viability over time following transfection of the indicated plasmid and subsequent inactivation of the endogenous *APE1* alleles. As shown in Fig. 6A, APE1-deficient TM-Cre $APE1^{fl/fl}$ MEFs harboring the GFP vector control survived for only ~5 days, whereas the WT-complemented cells grew for >14 days. The acetylation-defective Lys6/7Arg, the 3'-repair-enhanced Asp70Ala, and the NIR-defective Lys98Ala mutants all exhibited viability patterns similar to WT-complemented cells; there was a potential slight increase in survival of the Phe266Ala exonuclease-enhanced mutant. Notably, the Asp210Asn nuclease-dead and the Arg177Ala passing-the-baton mutants provided little rescue of cell growth, while the Cys65Ala REF1 mutant displayed intermediate complementation of cell viability. These data clearly support a major role for BER efficiency in supporting cell viability under normal growth conditions, as well as an important role for APE1's transcriptional regulatory (REF1) function in promoting cell proliferation.

Contribution of Different APE1 Nuclease Activities to Genotoxin Resistance

We next examined the contributions of the different functions in the context of exogenously-induced DNA damage. As seen previously for other cell types (reviewed in [74]), the vector-complemented APE1-deficient MEFs were far more sensitivity to the alkylator MMS than the WT-complemented counterparts (Fig. 6B). Moreover, only the nuclease-dead (Asp210Asn) and, less so, the pathway coordination (Arg177Ala) mutant showed impaired complementation of MMS sensitivity. Since MMS is known to generate a high level of AP sites, these data specifically point to a critical role for the AP endonuclease function of APE1 in coordinating repair and preventing damage-induced cell death. The other activities of APE1, i.e., its transcriptional regulatory, NIR or 3'-processing functions, play a minor role at best in the alkylation survival response.

To more directly interrogate the 3'-repair functions of APE1, we explored sensitivities to oxidative stress-inducing agents, which are more inclined to generate oxidative DNA strand breaks harboring 3'-blocking lesions, such as phosphates or phosphoglycolates, as well as oxidative base lesions, including those potentially processed by NIR ([86]). A clear increased sensitivity was observed for the vector-complemented APE1-deficient TM-Cre $APE1^{fl/fl}$ MEFs in comparison to the WT controls for bleomycin and paraquat, but much less so for menadione (Fig. 6C and unpublished observation). Focusing on the first two agents, we expectantly observed that the nuclease-dead mutant (Asp210Asn) was unable to rescue sensitivity to both oxidizing agents (Fig. 6C). The Cys65Ala (REF1) and Lys98Ala (NIR) mutants were generally similar to WT in both paradigms, whereas the Asp70Ala appeared similar to WT in the case of paraquat, but more effective than WT at promoting cell survival in the case of bleomycin. These

data indicate APE1 plays an important role in oxidative stress resistance, with its 3'-repair functions operating prominently, and support a particularly significant function in 3'-phosphoglycolate (a prominent product of bleomycin attack) excision ([87], [88]).

Closing Thoughts

AP sites are frequent, non-coding DNA lesions that can block RNA or DNA polymerases or lead to mutagenic by-pass. Such molecular events can ultimately alter cellular behavior, and in extreme cases, lead to apoptosis, senescence or transformation. The major protein in mammals assigned to cope with AP sites in both the nuclear and mitochondrial genomes is APE1. Besides being the predominant AP endonuclease, APE1 also maintains 3'-repair activities, an NIR function, and the ability to regulate transcription factor binding activity via its REF1 function. While many of these activities have been well described *in vitro* (although in some cases remain inadequately defined mechanistically), given the inviability of cells or animals lacking APE1, the contribution of the different functions of the protein to biology has been more difficult to ascertain. Using a combination of structural and functional complementation approaches, we have added new insights to the existing molecular picture of the multifunctional protein APE1.

There is broad consensus within the current data that the conserved nuclease function of APE1, most likely its AP endonuclease activity, is critical to cell viability, and likely plays a central role in the embryonic lethality observed upon germline deletion of the gene in mice. Notably, when comparing the Arg177Ala mutant to the Asp210Asn mutant (Fig. 6A), it's not only the nuclease activity that is critical for efficient cell growth under normal conditions, but also coordination within the BER pathway. The fact that the Arg177Ala mutant played a lesser role in protecting against MMS-induced cell death (Fig. 6B), in comparison to its complementation efficiency under normal growth conditions, may suggest that active coordination is most important when DNA damage levels are low and effective communication is more necessary due to the low levels of the proteins downstream of APE1 (e.g., POLβ). Our results also indicate that accumulating DNA strand-breaks can be toxic (in addition to unrepaired AP sites) and that there are not effective backup repair mechanisms for abasic sites in mammals, despite the presence of another Xth ortholog (APE2) or the evidence that pathways such as nucleotide excision repair can handle such damage ([89], [90]). Nevertheless, there are reports of mammalian cells surviving severe depletion or inactivation of APE1, presumably relying upon compensatory mechanisms that are presently unknown ([91], [17]).

Collectively, the current results support an important biological role for the acquired REF1 function of APE1. Our studies, in particular, indicate a specific contribution to normal cell survival, yet no role in genotoxin stress resistance. This finding is consistent with a function for the REF1 activity in maintaining essential gene regulatory networks, particularly of immediately early genes ([80]), without directly carrying out DNA damage repair. Numerous studies have indeed supported significant roles for the REF1 function in various biological responses, with REF1 proving to be a promising target in various treatment efforts, such as those involving the killing of cancer cells (reviewed in [58]).

The collective data are also consistent with an important biological role for the 3'-phosphodiesterase activity of APE1, certainly in the context of oxidative stress or a relevant genotoxin exposure, most notably to the radiomimetic bleomycin that creates 3'-phosphoglycolates (see for instance [92]). The significance of the 3'-5' exonuclease is less clear, although there is a hint that an enhanced function in this capacity might improve cell growth, possibly by reducing polymerase errors ([93]) . Conversely, we

found no clear indication for a biological contribution of the transcriptional regulatory (Lys6/7Arg mutant) or NIR functions of APE1. While our analysis is by no means exhaustive, our data imply either highly specialized roles for these activities or limited significance overall. Further investigations using the developed system within will go a long way towards addressing the remaining issues, as will the creation of animal models designed to alter one function or another.

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Figure Legends

Figure 1. Chemical form(s) of the hydrolytic and oxidized abasic sites. Hydrolytic AP sites (top) are in an equilibrium mixture of ring-closed [racemeric β- and α-hemiacetals (2-deoxy-d-erythro-pentofuranoses), left] and ring-open (aldehyde, center; hydrated aldehyde, right) forms. *In vitro* experiments indicate that the hemiacetal forms predominate, in roughly equal number, with ~1% aldehyde forms being present. The C4'- and C1'-oxidized AP sites are shown at the bottom, along with their alternative forms where relevant.

Figure 2. AP site formation and repair. The three primary mechanisms for the generation of genomic AP sites are shown at the top, including BER-initiated via the activity of a monofunctional or bifunctional DNA glycosylase. Following AP site formation, the predominant repair process involves APE1 incision, creating a strand break with a 3'-hydroxyl (OH) and 5'-dRP (right). At some low frequency, bifunctional DNA glycosylases can cleave at the AP site via β-elimination (left) or β,δ-elimination (center) to generate a strand break with a 3'-α,β-unsaturated aldehyde or 3'-phosphate (P), respectively, and a 5'-P. The single-strand breaks are then processed by the depicted enzyme(s) to create 3'-OH and 5'-P single-nucleotide gap intermediates. Shown is single-nucleotide gap-filling, executed by DNA POLβ, and nick ligation by an XRCC1/LIG3α complex.

Figure 3. Key features of APE1. (A) Linear comparison of human APE1 and *E. coli* EXO III proteins. The redox domain of APE1 is shown in orange, and the nuclease domains of APE1 and EXO III are shown in green. Key catalytic and functional residues in APE1 are indicated; see also Table 1. **(B) APE1 structural fold and biochemical functions.** X-ray structure of the APE1 core displaying its conserved four-layered α/β sandwich structural fold (center). The major reported biochemical functions of the protein are indicated in red, with either sub-activities or protein/gene targets indicated below or in parentheses, respectively.

Figure 4. X-ray structures of mutant APE1 proteins bound to incised product DNA. (A) Overlay of C65A (green) with the WT APE1 protein (magenta, PDBID: 5DFF). **(B)** Overlay of K98A (cyan) with the WT APE1 (Magenta, PDBID: 5DFF). Panels on the right show a zoomed-in view of the mutation.

Figure 5. APE1 protein intracellular localization and expression. HeLa cells **(A)** or TM-Cre $APE1^{fl/fl}$ MEFs **(B)** were transfected with the indicated plasmid vector, allowed to grow for 48 hr, and then photographed using confocal microscopy for the GFP signal. Representative images of GFP fluorescence are shown. **(C)** TM-Cre $APE1^{fl/fl}$ MEFs were transfected and photographed as above. Captured images were then analyzed by marking a defined spot in the nucleoplasm and quantifying GFP intensity. Shown are the averages and standard deviations of the raw intensity values from at least 6 individual cells from three independent experiments. **(D) Overview of complementation strategy.** TM-Cre $APE1^{fl/fl}$ MEFs are transfected with one of the different GFP-containing vectors. Fibroblasts are then placed under hygromycin B selection for 3 days (to enrich for cells carrying the plasmid), before being exposed to TM at an optimized concentration (5 μM) for 96 hr to inactive both endogenous *APE1* alleles. Cells are subsequently monitored for replicative lifespan or genotoxin sensitivity. A more detailed protocol can be found in ([69]).

Figure 6. Complementation effectiveness of WT and mutant APE1 proteins. (A) Replicative lifespan. TM-Cre $APE1^{LoxP/LoxP}$ MEFs were transfected with the indicated plasmid construct, put under selection and treated with TM (see Figure 5D), and then monitored for viability over time (every day) in culture

using the Cell Counting-8 assay (Dojindo, Rockville, MD). Averages and standard deviations shown represent 4 biological experiments, each done in duplicate. **(B) Cell survival after MMS exposure.** Following TM treatment (see above), TM-Cre *APE1*$^{fl/fl}$ MEFs harboring the designated plasmid were treated with the indicated dose of MMS for 1 hr. Survival (relative to the untreated control) was determined 48 hr after MMS exposure as noted in panel A. Averages and standard deviations shown represent 4 biological experiments, each done in duplicate. **(C) Cell survival after bleomycin or paraquat exposure.** Relative survival was determined as in panel B, except exposure to bleomycin was for 1 hr and paraquat was for 4 hrs. For these survival assays, averages and standard deviations represent at least 4 data points from 4 biological replicates.

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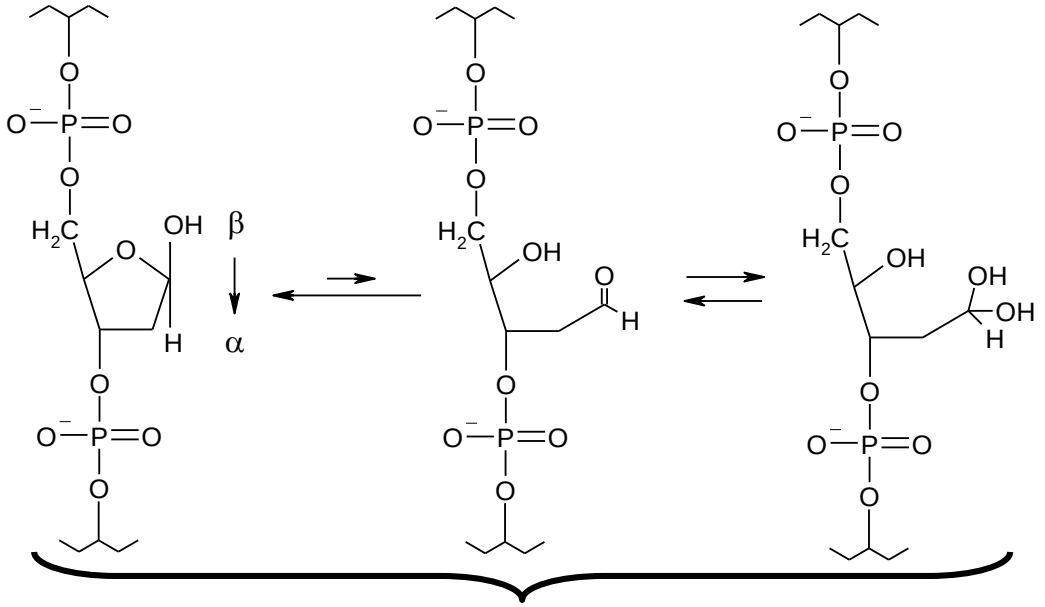
Table 1. APE1 Mutants. See text for further details.

Mutant	Biochemical Consequences	Structural Consequences	Function Targeted	Possible Complementation Outcome	Observed Complementation Outcome
Lys6Arg/ Lys7Arg	Impaired protein acetylation; impaired transcriptional regulation	Unknown	Transcription regulation	Impaired acetylation will result in defective transcriptional regulation of target genes, cellular dysfunction and possibly inviability	No effect
Cys65Ala	Impaired REF1 function	Similar to WT; removal of reducing catalytic cystine residue	REF1	REF1 defect will result in an impaired transcriptional regulation and reduced cellular viability	Reduced cell viability
Asp70Ala	Enhanced 3'-phosphodiesterase activity	Unknown	3'-Phosphodiesterase	Improved 3'-phosphodiesterase activity will lead to increased resistance to oxidative stress and bleomycin	Enhanced bleomycin resistance
Lys98Ala	Reduced NIR activity	Similar to WT; loss of hydrogen bonding between Asp70	NIR	Reduced NIR activity will lead to increased sensitivity to oxidizing agents and base damage accumulation	No effect
Arg177Ala	Impaired BER coordination	Unknown	Passing-the-baton	Reduced BER coordination will permit survival, but lead to increased damage accumulation and sensitivity to relevant DNA-damaging agents, such as MMS	Reduced cell viability and MMS resistance
Asp210Asn	Inactivation of nuclease activity	Altered coordination of the nucleophilic water and fellow catalytic triad residue Asn68	All nuclease activities	Absence of nuclease-competent protein will lead to cellular inviability	Reduced cell viability and genotoxin resistance
Phe266Ala	Enhanced 3'-5' exonuclease activity	Larger active site and 120° rotation of 3'-mismatched base	3'-5' Exonuclease	Increased exonuclease function will reduce single nucleotide mutation frequency	Possibly enhanced cell viability

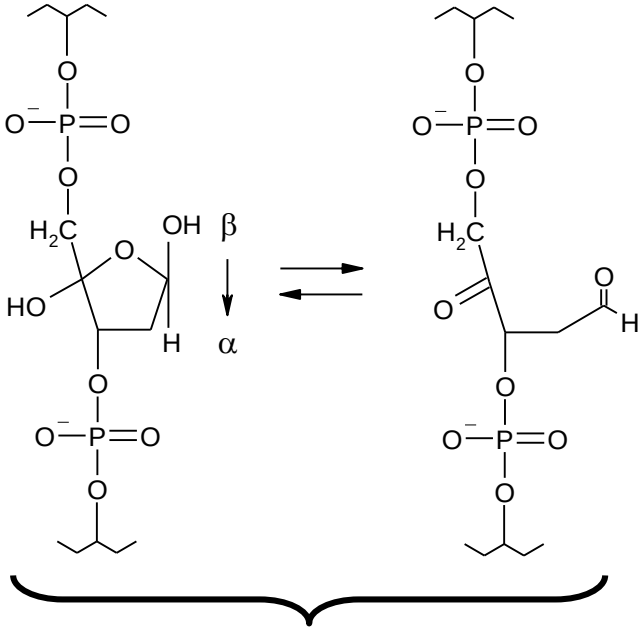
Table 2. Data collection and refinement statistics of mutant APE1:DNA product complexes with abasic (THF) containing DNA.

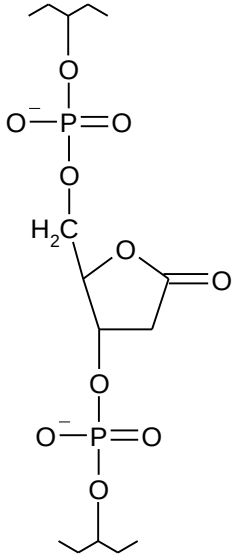
	C65A	K98A
Data collection		
Space group	P1	P1
Cell dimensions		
a, *b*, *c* (Å)	43.59,60.46,71.92	44.35,60.49,72.62
α, β, γ (°)	83.69,79.47,88.74	83.72,79.66,88.46
Resolution (Å)	25-2.15	25-2.1
R_{meas} (%)	0.152 (0.798)	0.084 (0.551)
$CC_{1/2}$	0.528	0.667
I/σ_I	7.81 (1.08)	17.2 (2)
Completeness (%)	99.2 (96.1)	99.8 (98.2)
Redundancy	3.6 (2.1)	3.9 (2.1)
Refinement		
Resolution (Å)	2.09	2.09
No. reflections	36463	58444
R_{work}/ R_{free}	0.1862/0.2445	0.1713/0.2272
No. atoms		
Protein	4321	4323
DNA	836	836
Water	290	292
B-factors (Å^2)		
Protein	33.28	30.64
DNA	43.12	41.04
Water	36.06	30.52
R.m.s deviations		
Bond length (Å)	0.009	0.008
Bond angles (º)	1.091	1.025
PDB ID	6P94	6P93

*Highest resolution shell is shown in parentheses



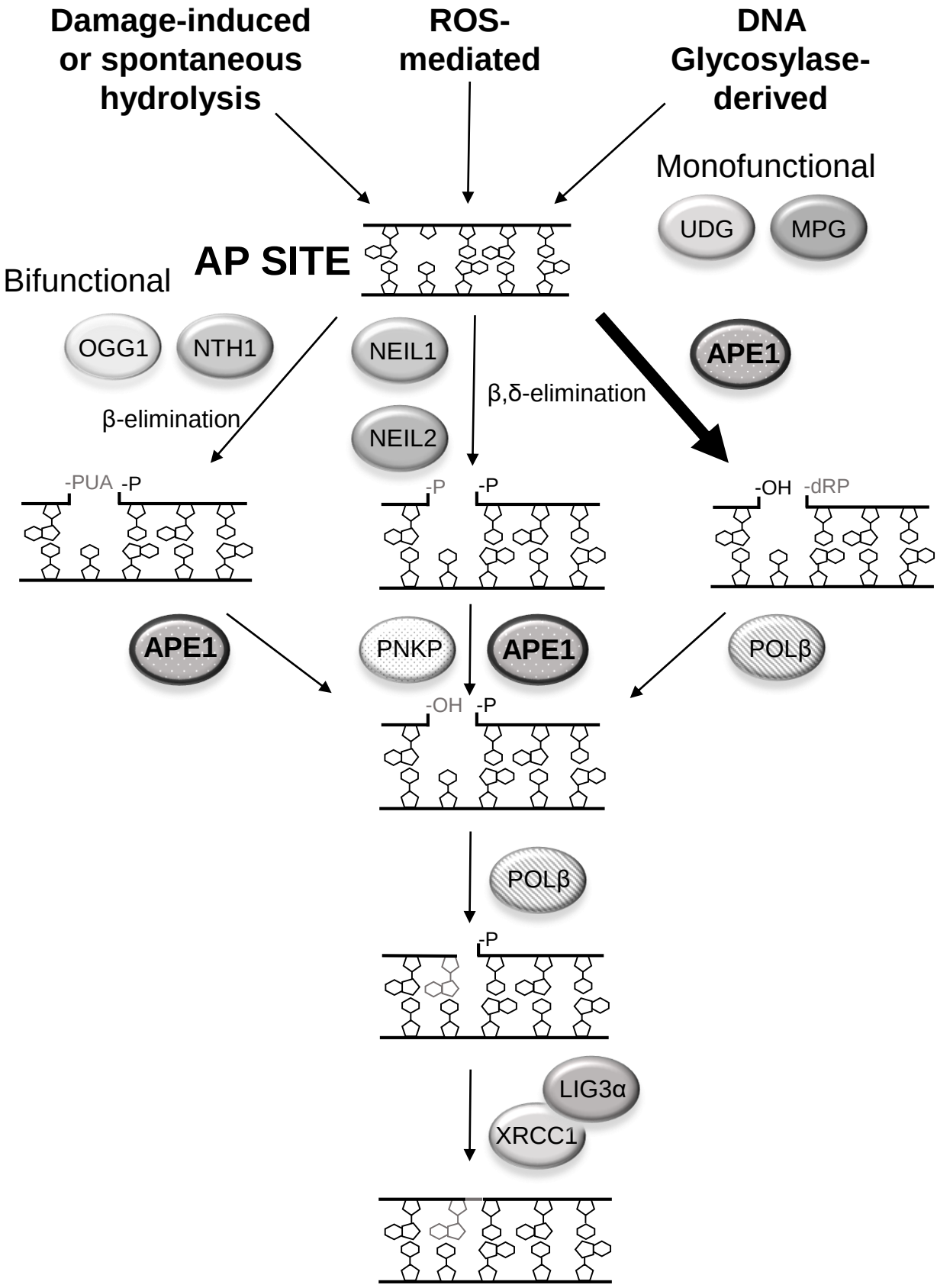
Hydrolytic AP Site





C4’-Oxidized AP Site

C1’-Oxidized AP Site


Damage-induced or spontaneous hydrolysis
ROS-mediated
DNA Glycosylase-derived
Monofunctional
UDG
MPG
AP SITE
Bifunctional
OGG1
NTH1
NEIL1
NEIL2
APE1
β-elimination
β,δ-elimination
-PUA
-P
-P
-P
-OH
-dRP
APE1
PNKP
APE1
POLβ
-OH
-P
POLβ
-P
LIG3α
XRCC1

A

APE1

1 C65 E96 D210 H309 318

Lys6/7Arg
Transcription Regulation

Asp70Ala
3'-Phosphodiesterase

Cys65Ala
REF1

Lys98Ala
NIR

Arg177Ala
BER Pathway Coordination

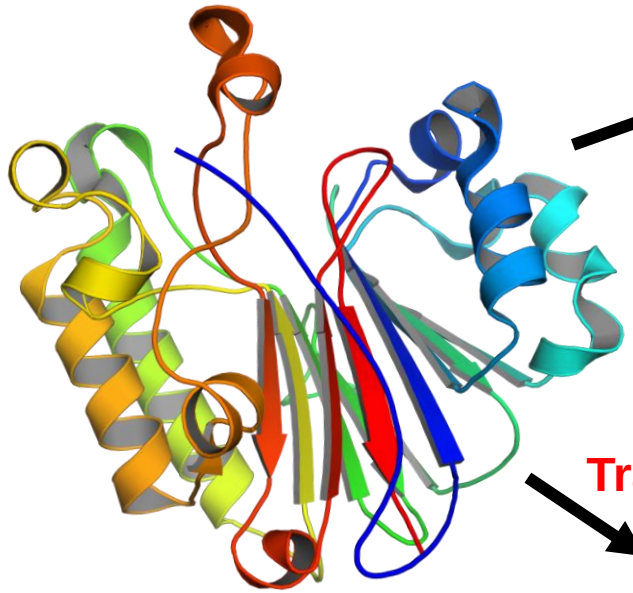
Asp210Asn
General Nuclease

Phe266Ala
3' to 5' Exonuclease

Exo III
46.3% Similarity

1 268

B



REF1 Regulatory Function

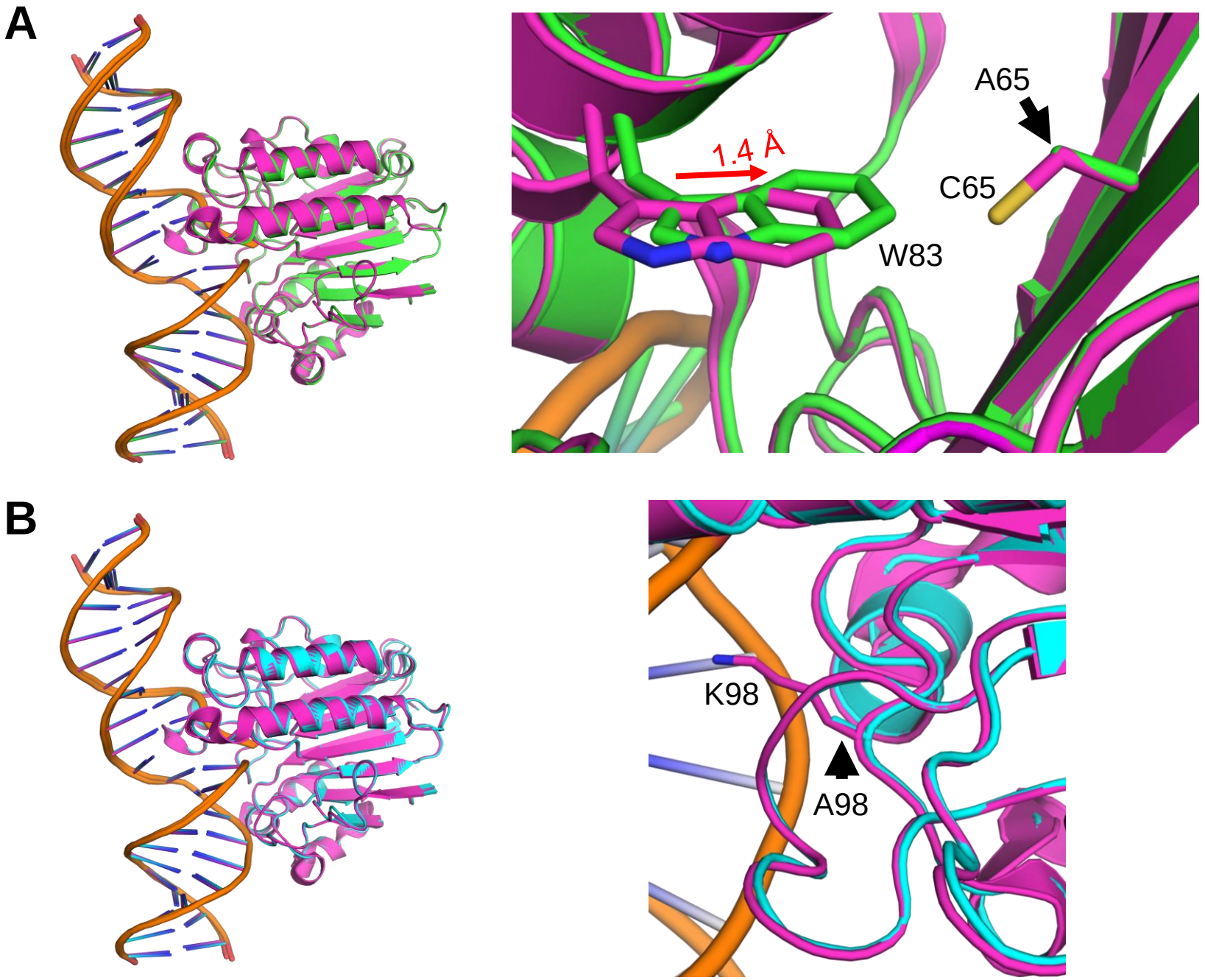
(AP-1, p53, NF-κB, etc.)

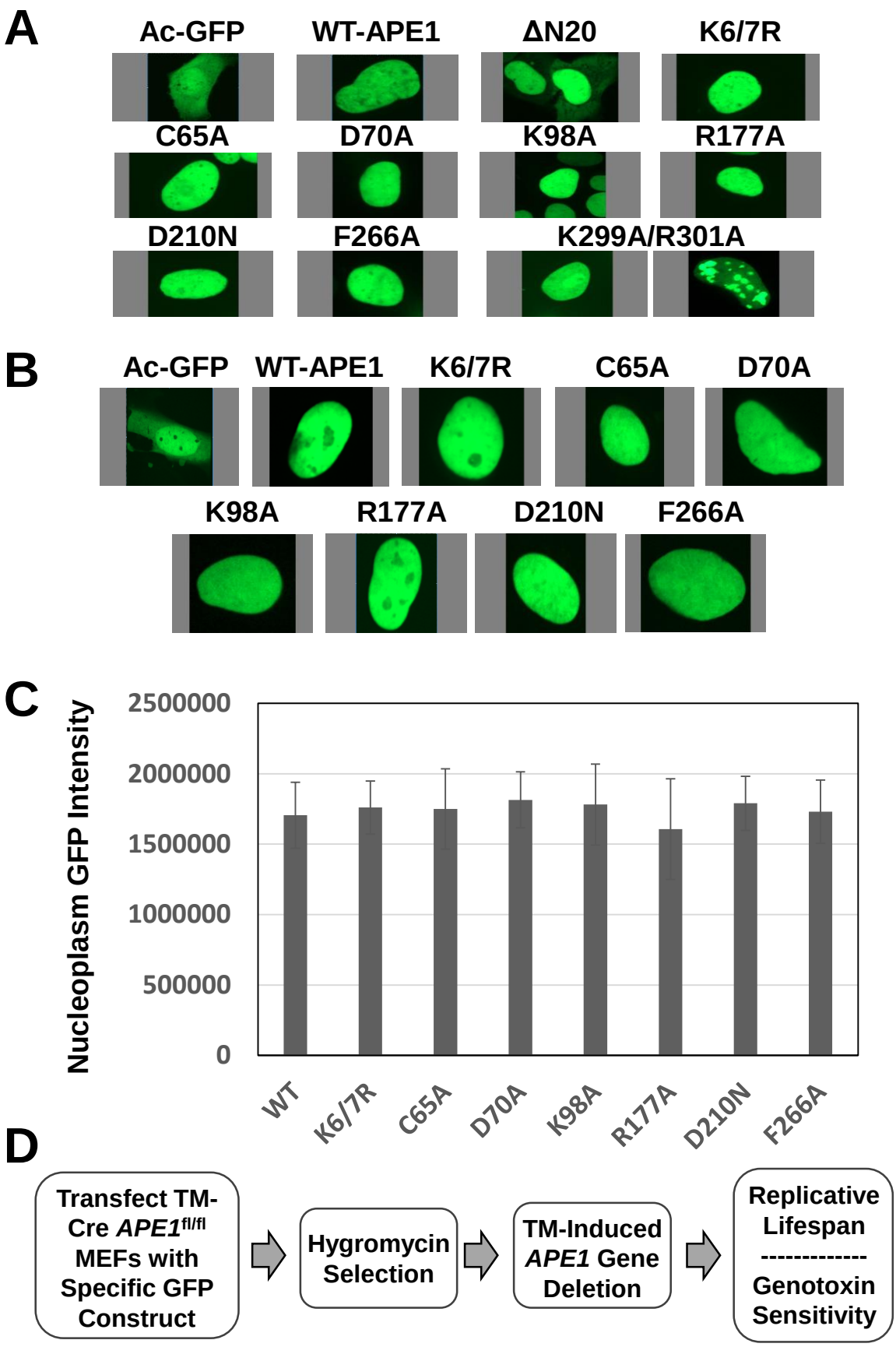
Nuclease Function

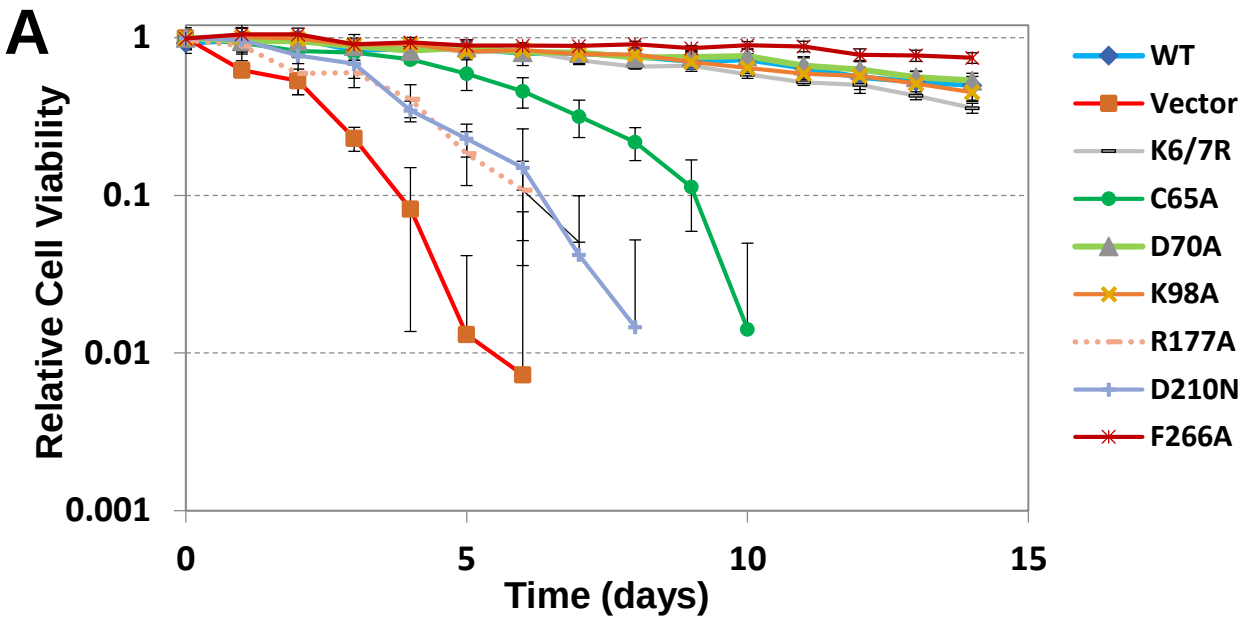
- AP Endonuclease (BER)
- 3′-Phosphodiesterase
- 3′ to 5′ Exonuclease
- Nucleotide Incision Repair (NIR)
- RNA Metabolism

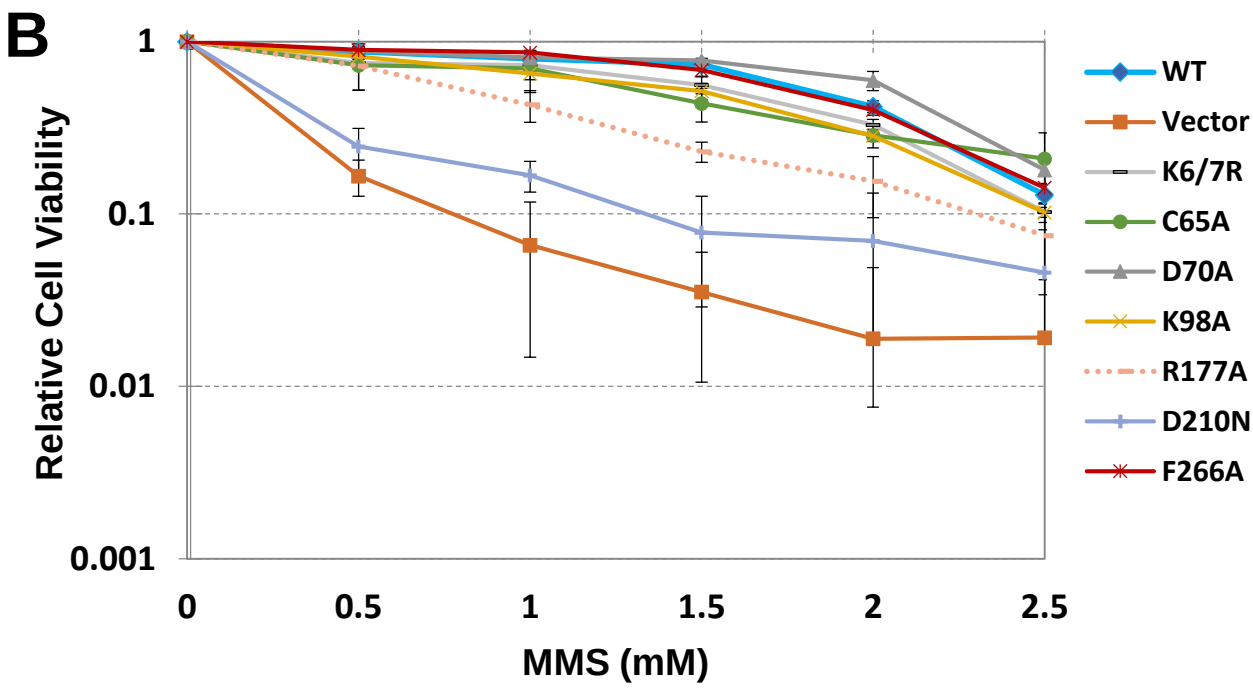
Transcriptional Regulatory Function

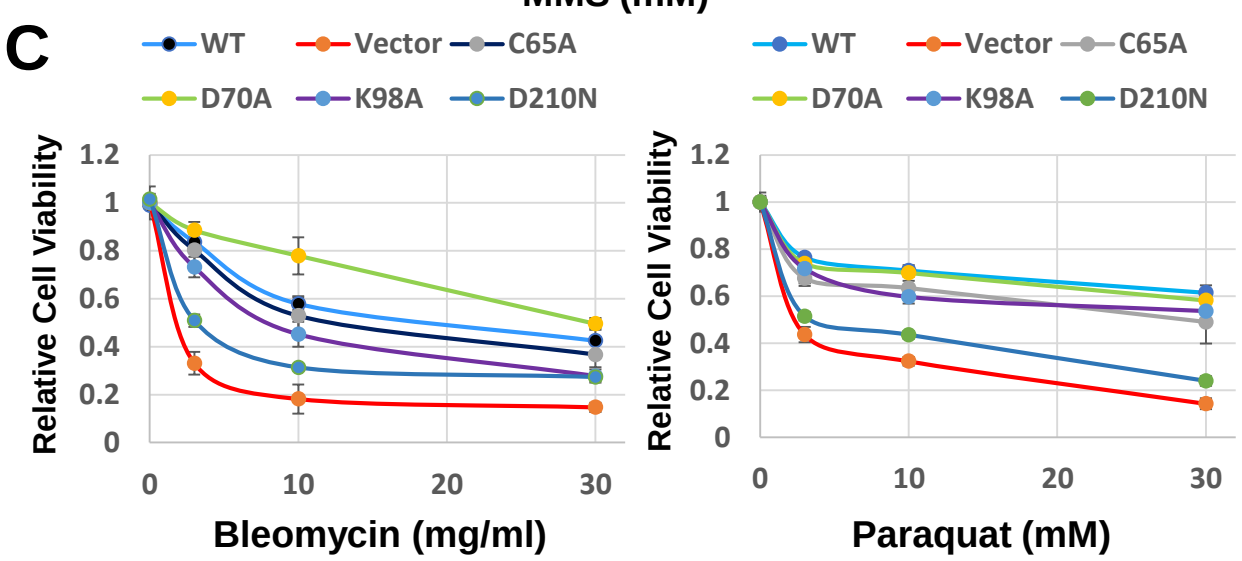
(nCaRE, VEGF, RARE, etc.)


A
1.4 Å
A65
C65
W83
B
K98
A98


A
Ac-GFP
WT-APE1
ΔN20
K6/7R
C65A
D70A
K98A
R177A
D210N
F266A
K299A/R301A
B
Ac-GFP
WT-APE1
K6/7R
C65A
D70A
K98A
R177A
D210N
F266A
C
Nucleoplasm GFP Intensity
2500000
2000000
1500000
1000000
500000
0
WT
K6/7R
C65A
D70A
K98A
R177A
D210N
F266A
D
Transfect TM-Cre APE1fl/fl MEFs with Specific GFP Construct
Hygromycin Selection
TM-Induced APE1 Gene Deletion
Replicative Lifespan
Genotoxin Sensitivity


A
Relative Cell Viability
1
0.1
0.01
0.001
0
5
10
15
Time (days)
WT
Vector
K6/7R
C65A
D70A
K98A
R177A
D210N
F266A


B
Relative Cell Viability
1
0.1
0.01
0.001
0
0.5
1
1.5
2
2.5
MMS (mM)
WT
Vector
K6/7R
C65A
D70A
K98A
R177A
D210N
F266A


C
WT
Vector
C65A
D70A
K98A
D210N
Relative Cell Viability
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
Bleomycin (mg/ml)
Paraquat (mM)