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Characterization of the Role of Acetylated APE1 in DNA Damage Repair and Transcriptional Regulation

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CHARACTERIZATION OF THE ROLE OF ACETYLATED APE1 IN DNA DAMAGE REPAIR AND TRANSCRIPTIONAL REGULATION

By

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Dedicated to my parents Arunima and Susanta Roychoudhury who always motivate me to dream big.

It’s all in the genes.

Acknowledgments

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“It always seems impossible until it’s done.”- Nelson Mandela

Shrabasti Roychoudhury

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Abstract

Characterization of the role of acetylated APE1 in DNA damage repair and transcriptional regulation

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Apurinic/apyrimidinic (AP) sites are the most frequently formed DNA lesions in the genome. The primary enzyme to repair AP sites in mammalian cells is the AP endonuclease (APE1), which functions through the base excision repair (BER) pathway. Mammalian APE1 has a unique N-terminal unstructured tail and has both DNA repair and transcriptional regulatory activities. Our lab discovered that APE1 can be regulated via post-translational acetylation of lysine residues 6, 7, 27, 31, and 32. The role of mammalian APE1 in repair has been extensively studied and well characterized. However, the regulatory role of APE1 acetylation (AcAPE1) in the context of both DNA damage repair and transcriptional regulation has not been elucidated.

We show that APE1 is acetylated after binding to the AP sites in chromatin and that AcAPE1 is exclusively present on chromatin throughout the cell cycle. Positive charges of acetylable Lysine residues in the N-terminal domain of APE1 are essential for chromatin association. Acetylation-mediated neutralization of positive charges of Lysine residues in the N-terminal domain of APE1 induces a conformation change; this, in turn, enhances the AP-endonuclease activity of APE1. In the absence of APE1 acetylation, cells accumulated AP sites in the genome and showed higher sensitivity to DNA-damaging agents. Our study reveals that APE1 acetylation is an integral part of the BER pathway for maintaining genomic integrity.

By mapping genome-wide occurrence of endogenous AP site damages and binding of repair proteins APE1 and AcAPE1, we demonstrate that oxidative base damages predominantly occur in transcriptionally active regions, particularly G-quadruplex (G4) sequences and activation of APE1-mediated BER pathway promotes the formation of G4 structures in the genome. Loss of APE1 or its acetylation abrogates the formation of G4 structures in cells. Acetylation of APE1 enhances its residence time and facilitates transcription factor loading, providing mechanistic insight into the role of APE1 in G4-mediated gene expression. Our study unravels an acquired function of endogenous base damage and AcAPE1-mediated BER in regulating transcription.

Together this study highlights role of AcAPE1 in coordinating potential functional overlap between DNA damage repair activity and transcriptional regulation.

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List of Abbreviations

8-oxoG	8-oxoguanine
5′-dRP	5′-deoxyribose-phosphate
AP	Apurinic/Apyrimidinic
APE1	Apurinic/Apyrimidinic endonuclease 1
AcAPE1	Acetylated APE1
BER	Base excision repair
CD	Circular dichroism
ChIP	Chromatin immunoprecipitation
Cy5	Cyanine 5
DOX	Doxycycline
E. coli	Escherichia coli
FEN-1	Flap endonuclease-1
G4	G- quadruplex
GO	Glucose oxidase
Lig III	DNA ligase IIIα
MEF	Mouse embryonic fibroblast
MMS	Methyl methanesulfonate
MX	Methoxyamine
nCaREs	Negative Ca^{2+} response elements
NLS	Nuclear localization signals
OGG1	8-oxoguanine DNA glycosylase

PCNA	proliferating cell nuclear antigen
PTMs	post-translational modifications
SIM	Structured Illumination Microscopy
SSB	Single-strand breaks
XRCC1	X-ray cross-complementing protein 1

CHAPTER 1

Introduction

Some of the material included in this chapter has been previously published: *Roychoudhury S, Pramanik S, Harris H and Bhakat k. K. "Biochemical and Cellular Assays to Assess the Effects of Acetylation on Base Excision Repair Enzymes". Book chapter; Methods in Molecular Biology. 2019; 1983:191-206,doi: 10.1007/978-1-4939-9434-2_11*

1.1 **Overview of the base excision repair pathway**

The genome is continuously challenged with endogenous and exogenous sources of genotoxic agents such as cellular oxidative metabolism, exposure to chemicals, radiation and cytotoxic drugs, resulting in oxidation, alkylation, and deamination-mediated base damage (1-3). Base excision repair (BER) pathway is the predominant DNA damage repair pathway for processing most of the base damages, single-strand breaks (SSBs), and Apurinic/Apyrimidinic (AP) sites (4). AP sites are reported to be among the most abundant type of DNA damage and can also occur as the result of spontaneous hydrolysis of the N-glycosyl bond (5-7). If left unrepaired, AP sites can exert cytotoxic effects by blocking DNA replication, and transcription, and can result in mutagenesis through base substitutions, insertions, or deletions (8, 9). Due to the high frequency of AP sites in the DNA and potential for promoting deleterious outcomes, in 1970s Lindahl searched for possible repair activities specific for these lesions, discovered series of enzymes and established BER pathway(5).

BER is a highly coordinated, multistep cellular process (10). There are several damage-specific DNA glycosylases that initiates the BER pathway by excising modified DNA base lesions (11). Two different types of lesion-specific DNA glycosylases, monofunctional glycosylases that has only DNA glycosylase activity and bifunctional glycosylases that has both DNA glycosylase and DNA strand cleavage activities have been discovered (12). Monofunctional DNA glycosylase cleaves the damaged base and creates an AP site. Subsequently, AP endonuclease 1 (APE1) recognizes the AP site, cleaves the DNA backbone, and this results in the formation of a one nucleotide gap flanked by 3′-hydroxyl and 5′-deoxyribose-phosphate (5′-dRP) end which is recognized by the downstream enzyme DNA polymerase β (pol β). On the other hand, bifunctional DNA glycosylases, along with base damage removal, incise the DNA backbone to create

a single-nucleotide gap flanked by either a 5′ phosphate and a 3′-α,β-unsaturated aldehyde (termed β-elimination) or 5′-phosphate and 3′-phosphate residues (termed β,δ-elimination). 8-oxoguanine (8-oxoG) DNA glycosylase (OGG1) and the endonuclease III homolog (NTH1) are known to catalyze β-elimination, creating "dirty ends". Following their activity, the 3′-α, β-unsaturated aldehyde is excised by APE1 to generate a "clean" 3′-hydroxyl end, which is the same product resulted from monofunctional DNA glycosylase and APE1 action (13). After APE1's action, Pol β hydrolyzes the 5′dRP moiety and fills the single nucleotide gap by incorporating a complementary base (14, 15). Finally, DNA ligase IIIα (Lig III) and X-ray cross-complementing protein 1 (XRCC1) together seals the remaining nick in the DNA backbone (16). This pathway is known as short-patch BER (**Fig. 1**) (17). Alternatively, when the 5′-dRP residue is oxidized or reduced and resistant to excision by Pol β, series of (typically 2 to 8) nucleotides into the single-nucleotide gap is added, generating a 5′-DNA flap structure (18). This flap is excised by flap endonuclease-1 (FEN-1) in a proliferating cell nuclear antigen (PCNA)-dependent process. Finally, DNA ligase I (Lig I) in the presence of PCNA seals the remaining nick in the DNA backbone completing long-patch BER pathway (19). Overall, BER relies on sequential recruitment and coordinated actions of multiple proteins via a series of transient repair complexes that assemble at the site of the DNA lesion (20).

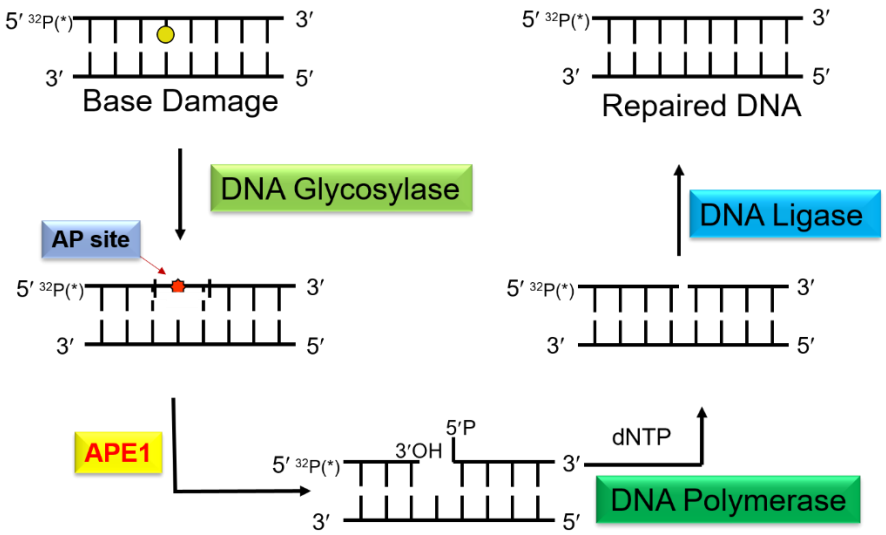


Figure 1: Schematic of the BER pathway. Base excision repair (BER) is initiated by a damage specific DNA glycosylase that excises the damaged base to create an AP site, which is then incised by APE1 creating a DNA SSB flanked by 3′-hydroxyl and 5′-dRP ends. Pol β cleaves the 5′-dRP moiety and simultaneously adds a single correct nucleotide into the one-nucleotide gap. Finally, the DNA SSB ends are sealed by the XRCC1-Lig IIIα complex.

1.2 **Overview of APE1 biology**

Human apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional protein playing a pivotal role in not only the removal and repair of numerous DNA lesions through its endonuclease function, but also as a redox activator of numerous transcription factors, including Egr1, NF-κB, p53, and HIF1a and as a direct transcription regulator through its N-terminal tail domain (7, 21, 22). Although functionally independent, all three functions are important for cell survival (7, 23).

In the early 1990s, the transcript encoding the human AP endonuclease (apurinic/apyrimidinic endonuclease 1 (APE1/APEX1)) was cloned by the Demple, Hickson, and Seki groups (24). Surprisingly, around the same time, human APE1 was independently identified by Curran and colleagues as the major nuclear protein (termed REF-1) to simulate the DNA-binding activity of the AP-1 (Fos/Jun) transcription factor complex (25, 26). This activation was shown to be mediated through reduction of a conserved cysteine residue within the DNA binding domain of the target protein and was also observed with factors such as NF-κB, Myb, and members of the ATF/CREB family (22, 26-28). Soon after, Okazaki's group demonstrated that APE1 could directly bind to negative Ca^{2+} response elements (nCaREs) and modulate gene expression. Thus, acting as a direct trans-acting factor (29). Of note APE1-nullizygous mice are early embryonic lethal, and that no APE1-null mouse embryo fibroblast (MEF) line could be established (30).

The human *APE1* gene is located on chromosome 14q11.2 and ubiquitously expressed in all tissue and cell types from a housekeeping-like promoter (7). It encodes a protein of 318 amino acids (theoretical molecular weight of 35.5 kDa) (7). Unlike its *E.coli* counterpart Xth, mammalian APE1 has acquired a unique, unstructured, highly positively charged 60-amino acid region in the N-terminal, which also houses the

consensus nuclear localization signals (NLSs) (31) . While the C-terminal part of the protein is highly conserved, the N-terminus is not. Mammalian APE1 has highly conserved (>90%) N-terminus, this region is almost always absent in other organisms. X-ray diffraction and site-directed mutagenesis have revealed that APE1 is composed of a rigid globular C-terminal nuclease domain and a highly disordered N-terminal domain (32, 33). The N-terminal domain is responsible for the transcription regulatory activity of APE1 and is thought to additionally mediate alternative APE1 functions and/or its protein-protein interactions, whereas the C-terminal domain is responsible for DNA binding and backbone cleavage activity (34, 35).

1.2.1 **Biochemical functions of APE1:**

APE1 as a nuclease: APE1 is known as a skilled nucleic-acid surgeon, exhibiting endonuclease, 3′ phosphodiesterase, 3′ to 5′ exonuclease, and RNA cleavage activities (36). Most well-characterized role of APE1 is that it cleaves the DNA phosphodiester backbone at the 5′ termini of the AP site, generating a nick in the DNA with 3′ hydroxyl and 5′ dRP termini (37). There is a consensus that the D210 and H309 amino acid residues have critical functions in the hydrolytic reaction chemistry, and E96 plays a role in divalent metal coordination (32, 34). Other important residues include N68, D70, Y171, N212, D283, and D308, which are generally conserved throughout the diverse members of the phosphoesterase superfamily (32). Experiments using human whole cell extracts have shown that APE1 functions in the rate-limiting step of the BER pathway (38). The pre-steady state kinetic description of strand incision at AP sites by APE1 is that rapid catalysis is followed by slow product release. This rapid catalysis is vital for genomic stability given the prevalence of AP sites in the genome, while the slow catalysis step has been proposed to conceal cytotoxic BER intermediates during DNA-

damage processing and facilitate coordination with the next enzyme in the BER pathway.

APE1 as a Redox-signaling factor: Besides serving in a crucial role in the maintenance of genome stability through its endonuclease activity, APE1 also acts as a master regulator of cellular response regulating redox signaling activity (22). APE1; also known as REF-1, reduces oxidized cysteine residues of specific transcription factors as part of their transactivation process. REF-1 regulates numerous transcription factors, including nuclear factor kappa B (NF-κB), STAT3, HIF-1α, AP-1, p53 (39-42). Although the repair function has been conserved from *E. coli* to humans; the redox signaling function is observed only in mammals (43). Among seven Cystine (Cys) residues APE1/Ref-1, three of the Cys residues, C65, C93, and C99, are sufficient for its redox activity (43). The mechanism involves a redox cycle with potential formation of intermolecular disulfide bonds with the protein target (44, 45). Structural studies demonstrated that APE1 exists in both native and partially unfolded conformations (46). The partially unfolded state of APE1 represents the redox active intermediate of the enzyme, which can be targeted by the APE1 redox inhibitor APX3330 (formerly E3330) (47). APX3330 stabilizes the unfolded state causing buried Cys residues such as C65 and C93 to be exposed and facilitates disulfide bond formation. This disulfide bond formation results in inactivation of APE1 and a decrease in interaction with downstream transcription factors effectively causing them to be inactive. APX3330 has shown to be a selective inhibitor of REF-1/APE1 redox activity with minimal cytotoxic effect and moved to a successful clinical trial (48, 49). APE1/ REF-1 is viewed as a critical node in many important signaling pathways and thus is a prime target for anticancer therapy (39).

Transcription regulatory function of APE1: Okazaki's group first established a Redox independent transcription regulatory role of APE1 as they identified APE1 as one of the

proteins that bind to negative calcium response elements (nCaRE) complex in the human PTH gene promoter and regulate PTH expression (29). Subsequently, a nCaRE-B sequence, identical to that in the PTH promoter, in the human renin gene has been identified (50). Involvement of this element and binding of APE1 in Ca^{2+}-mediated repression of renin gene expression has been shown by promoter-driven reporter assay system in cells. Interestingly, Jayaraman *et al*. showed that recombinant APE1 can stimulate DNA binding of full-length p53, which was further strongly stimulated in the presence of DTT, indicating that APE1-mediated activation of P53 might be independent of its function as a redox activator (51). In a separate study Egr-1, a transcription factor with tumor suppressor function was shown to stably interacts with APE1 and treatment with H_2O_2 strongly stimulates their association and increases Egr-1's DNA-binding activity in cells to regulate expression of many genes, including p53 and PTEN (52). APE1's interaction with HIF-1α and p300 was found to be critical for the assembly of the hypoxia-inducible transcriptional complex on the hypoxic response element (HRE) in the VEGF gene promoter in the rat pulmonary artery endothelial cells (53). Gray *et al*. showed that APE1 along with CBP/p300 associates with STAT3 and HIF-1α at the VEGF promoter, where they form an active transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinoma (54). Our studies highlighted that APE1-YB1 complex binds to MDR1 gene promoter and activates gene expression (55). Importantly, redox-inactive Cys65Ser and Cys138Ser APE1 mutants behaved same as WT APE1 in modulating YB-1-mediated MDR1 promoter activity, suggesting that APE1's redox activity is not involved in MDR1 activation. Together, all these studies demonstrating redox-independent transcriptional function of APE1 in modulating HIF-1α, Egr-1, and YB-1 activities established the third role of APE1 as a trans-acting factor. APE1's presence in diverse trans-acting

complexes requires interaction with diverse partners, which can be possible through its N-terminal unstructured tail domain. Various studies have observed deletion of N-terminal 30 aa or neutralizing positive charge of Lysine residues abrogates protein-protein interaction and affects gene expression (31, 56, 57). However, the detailed molecular mechanism by which APE1 regulates gene expression as a co-transcription factor is still unclear.

1.2.2 **Post-translational regulation of APE1 by Acetylation:**

The activity of a single protein with pleiotropic functions could be "fine-tuned" via post-translational modifications, including phosphorylation, acetylation, ubiquitination, and methylation, in order to coordinate specific biological activities. Research on disordered regions of proteins have revealed that due to the lack of structure and increased malleability, disordered regions can act as a hub for modulating protein interactions and are highly susceptible to post-translational modifications (PTMs) (58). Given the multiple functions, it is not surprising that APE1 is post-translationally modified in vivo (59). The cross talk between different post-translational modifications and different function of APE1 is yet to be fully characterized. Increasing evidence suggests that many of the proteins that participate in BER can be acetylated, as determined by in vitro analysis of purified proteins or cell extracts and in vivo analysis (**Fig. 2**) (60-64). Acetylation provides increased specificity and efficiency to the BER pathway. Acetylation can alter the binding characteristics, turnover rates, subcellular localization, and the overall efficacy of the target protein.

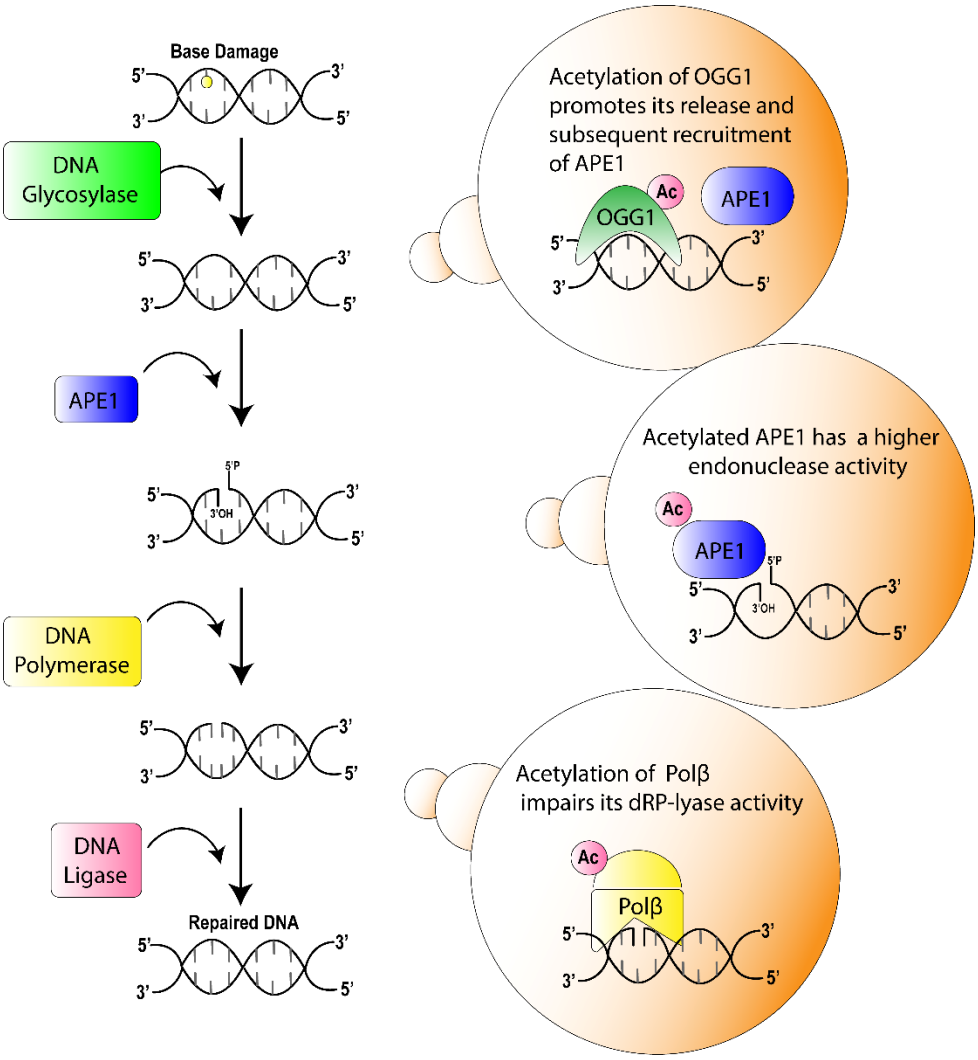


Figure 2: A schematic representation of steps involved in the BER pathway. Acetylation of multiple enzymes facilitates sequential processing of BER intermediates.

Our lab has discovered that Lysine 6 and 7 residues in mammalian conserved N - terminal domain of APE1 can undergo acetylation by the histone acetyltransferase p300 both in vitro and in vivo (56, 65). Mass spectroscopic analysis of in vitro acetylated APE1 (AcAPE1) could not detect diacetylated molecule suggesting possible due to steric effects of acetyl groups attached to ε-amino groups during the second acetylation. Thus, either Lys6 or Lys7 but not both can be acetylated in the same molecule. Generation of affinity-purified AcAPE1-specific antibody using a human APE1 peptide with acetylated Lys6 and showed that the AcAPE1 antibodies are highly specific for AcAPE1, and do not cross-react with at least 25-fold excess unmodified APE1 (55). Moreover, this antibody recognizes ectopic FLAG-tagged WT APE1, but not non-acetylable K6R/K7R APE1 in cell extracts confirming its specificity for AcAPE1.Importantly, the presence of endogenous AcAPE1 in cells was confirmed using this antibody. Although the first 20 amino acid residues contain the consensus nuclear localization signal (NLS) sequence (MPKRGKK) that includes the acetylation sites, acetylation of K6 and K7 of APE1 is not involved in its nuclear localization (66). Importantly, APE1 acetylation stimulates the formation of the nCaRE-B complex at PTH promoter, activates Egr-1 dependent PTEN expression, and promotes YB-1 binding at MDR1 promoter (55, 56, 67, 68). *H. pylori* infected gastric cells induces AcAPE1 that promote binding at nCARE element and regulation of Bax gene (69). Later, Tell group in collaboration with our lab discovered that APE1 can also be acetylated at lysine residues 27, 31, and 32 along with 6 and 7 and acetylation is regulating transcription regulatory function (62). They showed, Lys 27, 31, and 32 acetylation is dependent on Lys 6 and 7. However, cellular dynamics between acetylation sites, spatiotemporal regulation, and functional importance of

acetylation sites is not clear. Of Note, HDCA1 and SIRT1 is responsible for APE1 deacetylation in cells (70, 71).

AcAPE1 levels are enhanced in primary tumor tissues of diverse background (57, 71). Our lab discovered that acetylation APE1 (AcAPE1) enhances endonuclease activity and regulates transcription of genes including, Multi-Drug Resistance gene (MDR1), via loading of transcription factors (67). Importantly, both the DNA repair domain and acetylable Lys6 and 7 residues of APE1 are essential in cell proliferation and survival in conditional APE1-nullizygous mouse embryo fibroblast (MEF) cells, (72). Furthermore**,** a recent study showed that the absence of acetylation at Lys 6/7 sites in APE1 or its DNA repair function resulted in telomere fusion and mitotic defects (73). Given the key role of APE1 in the repair of AP sites, which inhibits replication and transcription, the importance of the DNA repair function of APE1 is not surprising. However, the essentiality of the acetylable Lys residues for mitotic progression and cell proliferation were unexpected.

Acetylation of APE1 protects APE1 proteolysis, by a serine protease, following residue lysine (Lys) Lys6 and/or Lys7 and after Lys27 and Lys31 or Lys32 at its N-terminus in the tumor tissue (57). The N-terminal domain of APE1 and its acetylation modulates its AP-endonuclease activity and is important for regulation of the expression of hundreds of genes and essential for sustained cell proliferation and/or survival (57, 71). Elucidating the mechanisms by which N-terminal acetylation modulates the essential DNA repair and transcriptional regulatory functions *in vivo* that promote cell survival and cell proliferation is extremely important to understand the role of AcAPE1 in tumorigenesis. It is possible that acetylation-mediated conformational change in the disordered N-terminal segment (encompassing 40 aa residues), modulates protein-

protein interaction and thus regulates its DNA repair and co-transcription regulatory function.

1.3 **Objectives of the dissertation:**

The role of mammalian APE1 in repair has been extensively studied and well characterized. However, the regulatory consequences of APE1 acetylation in the context of both DNA damage repair and transcriptional regulation has not been elucidated.

The overall objectives of my dissertation research are,

- Investigate the spatiotemporal sub-cellular and genome-wide distribution of AcAPE1 and non-modified APE1.
- Characterize unique and divergent role of AcAPE1 to that of non-modified APE1, which can help to understand the role of AcAPE1 in cell proliferation.
- Elucidating the role of AcAPE1 in the BER pathway.
- Delineate the molecular mechanism by which AcAPE1 regulates gene expression.
- Investigate potential functional overlap between DNA damage repair activity and transcriptional regulation coordinated by AcAPE1.

This dissertation research aims to characterize the role of AcAPE1 in mammalian cells.

CHAPTER 2

Human AP-endonuclease (APE1) is acetylated at DNA damage sites in chromatin and acetylation modulates its DNA repair activity

The materials presented in this chapter has been previously published: Roychoudhury S, Nath S, Song H, Hegde M, Bellot L, Mantha A, Sengupta S, Ray S, Natarajan A and Bhakat k.K. "Human AP-endonuclease (APE1) is acetylated at DNA damage sites in chromatin and acetylation modulates its DNA repair activity". Molecular and Cellular Biology, MCB, DOI:10.1128/MCB.00401-16.

2.1 **Introduction**

Common forms of DNA damage in the genome are apurinic/apyrimidinic (AP) sites (5, 74). AP sites can be generated either spontaneously through water-mediated depurination or depyrimidination or after removal of oxidized and modified bases by DNA glycosylases (5). Thousands of such AP sites are daily generated in the genome of a human cell (74). These noninstructional AP sites are mutagenic and can inhibit DNA replication and transcription (9, 75) . The primary enzyme to repair AP sites in mammalian cells is the AP endonuclease (APE1), which functions through the Base Excision Repair (BER) pathway (76, 77). Human APE1 is a ubiquitous and multifunctional protein (76). It was originally discovered as a DNA repair enzyme playing a central role in the repair of spontaneously generated AP sites and oxidative and alkylated DNA damages in the genome via the BER pathway (4, 24, 77). Apart from its DNA repair function, APE1 functions as a redox activator of many transcription factors (TFs), as well as a direct transcriptional co-regulator of many genes (31, 40).

APE1 is essential for embryonic development and for cell viability and or proliferation in cultures (26, 72, 78). Unlike its *E. coli* prototype Xth, Human APE1 is unique in that it has an N-terminal disordered 42 amino acid (aa), and has both DNA repair and transcriptional regulatory activities (31). In previous studies, we discovered that APE1 can be acetylated (AcAPE1) at multiple Lysine (Lys) 6 and Lys 7 residues in the N-terminal domain, and that acetylation modulates the transcriptional co-regulatory activity of APE1 (56, 67). Moreover, Dr. Tell's group in collaboration with us found that other Lys residues (Lys,27,31,32 &35) in the N-terminal domain of APE1 can be modified by acetylation and these Lys residues modulates nucleolar localization and BER activity of APE1(62). We have recently shown that tumor tissue of diverse cancer types have elevated levels of AcAPE1 (57). APE1 was also shown to be ubiquitynated at Lys 24,25

and 27 residues (79). Further, using conditional APE1-nullizygous mouse embryo fibroblasts (MEF), we showed that acetylable Lys6 and Lys7 residues of APE1 are essential for cell survival (72). The acetylation sites are conserved in most mammalian APE1(31), suggesting that evolutionary conservation or neutralization of the basicity of these Lys residues by acetylation in the N-terminal domain has essential biological functions. Over the last 20 years, the mechanisms by which AP sites are repaired by APE1 *in vitro* via BER pathway have been extensively investigated (15, 32, 80-82). However, it is largely unknown how APE1 repairs AP sites in mammalian cells.

In this study, we show that APE1 is acetylated after binding to the AP sites in the chromatin and that AcAPE1 is exclusively associated with chromatin throughout the cell cycle. Further, our study revealed the key role of positive charges of the acetylable Lys residues for nuclear localization of APE1 and its binding to chromatin. APE1 acetylation induces a conformation change in APE1 which enhances the AP-endonuclease activity of APE1 and its interaction with downstream BER proteins. Our study shows that acetylation of APE1 plays a crucial role in the repair of AP sites and oxidative and alkylated base damages in the genome and thus promotes cell survival and proliferation.

2.2 **Materials and methods**

Cell lines, plasmids, siRNAs, transfection and treatments: Human embryonic kidney HEK-293 (ATCC # CRL-1573) and inducible APE1-downregulated HEK-293T^{APE1siRNA} cells were cultured in DMEM-high glucose medium (Thermo Fisher scientific) with 10% fetal calf serum (FCS; Sigma) and antibiotic mixture of 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco-BRL) as described previously (67). Human Colon cancer HCT116 (ATCC #CCL-247) was grown in MaCoy 5A medium (Thermo Fisher scientific). Generation of HCT116 cells stably expressing APE1-shRNA or control shRNA were

described previously (83) . hTERT-immortalized human foreskin fibroblast BJ-5ta (ATCC #CR-4001), Normal lung fibroblast IMR-90 (ATCC# CCL-186), Lung adenocarcinoma A549 (ATCC# CCL-185), cells were cultured in DMEM-low glucose medium (Thermo Fisher scientific) with FCS and antibiotics. All cell lines were authenticated by STR DNA profiling on August, 2015 by Genetica DNA laboratories, Burlington, NC. Mutation of Lys residue (K6,7,27,31 and 32) singly or in combination to arginine or to glutamine or to Alanine in APE1-FLAG-tagged pCMV5.1 plasmid were generated using a site-directed mutagenesis Kit (Agilent, Stratagene) following manufacturer's protocol. Exponentially growing HCT116 cells stably expressing APE1-shRNA cells were transfected with wild type (WT) APE1, K6,7,27,31,32 to arginine (K5R) or to glutamine (K5Q) mutants expression plasmids. In another set of experiment, HEK-293T^{APE1siRNA} cells were treated with Doxycycline (1ug/ml) for 5 days to knockdown the APE1 levels then cells were transfected with FLAG-tagged WT APE1 or K5R or K5Q or N-terminal 33 amino acid deleted (NΔ33) mutants APE1 as described elsewhere (55, 67, 84, 85). Expression plasmid Adenovirus EIA12S and the mutant E1A (2-36 amino acid deletion) were described earlier (86). Cells were transfected using Lipofectamine 2000 (Invitrogen) and harvested after 48 hrs. Methoxyamine, Glucose oxidase and Methyl Methanesulfonate were obtained from Sigma.

Immunofluorescence and Proximal Ligation Assay (PLA): Different types of cells (HEK-293, HCT116, A549, BJhTERT, IMR-90) were grown on coverslips. After transfection with indicated plasmids cells were fixed with 4% formaldehyde (Sigma) for 20 minutes and permeabilized with 0.2% Triton X-100 (Sigma) containing blocking solution (goat serum, glycine, sodium azide) for 1 hour. Slides were incubated overnight at 4°C with the primary antibodies, washed three times with PBS, and incubated with the secondary antibodies Alexa Fluor 488 anti-mouse IgG (Life technologies, 1:500) or

Alexa Fluor 594 anti-rabbit IgG (Life Technologies; 1:500) at room temperature for 1 h. After washing three times, slides were mounted on Vectashild-DAPI containing media (Vector Laboratories). Primary antibodies used for immunofluorescence studies were mouse monoclonal anti-APE1 (1:100; Novus Biologicals; # NB100-116), anti-AcAPE1 (1:50; (55)), Anti-FLAG(1:50; Sigma; #F3165), Anti-p300(1:100; Activemotif; #61401), Anti-OGG1 (1:50), Anti-Histone H3 (1:100; Santa Cruz; #sc10809), Anti-H3K27 aetylated histone (1:100; Milipore; #05-1334), Anti-Ligase III (1:50; Novus Biologicals; #NB100-152) Anti-Lamin B (1:50; Abcam; #ab16048) . PLA was performed following the manufacturers protocol (Duolink® In Situ – Fluorescence PLA technology, Sigma). Numbers of signal for co-localization of these two molecules were calculated using the Duolink PLA software. For the quantification at least 50 cells nuclei were counted for each experiment, and SDs from three independent experiments were calculated. Images were acquired by fluorescence microscope, 63X-oil (Zeiss LSM 510) and Structured Illumination Microscopy (SIM) was done with a Zeiss Elyra PS.1 Microscope (Carl Zeiss) by using a 63× objective with NA of 1.4. To measure colocalization, ImageJ was used to measure Manders colocalization using the JaCoP plug-in.

Isolation of cytoplasmic, nuclear and chromatin fractions and Western blot analysis: Cells were lysed in cytosol extraction buffer (Tris-HCl pH8 10mM, Sucrose 0.34mM, $CaCl_2$ 3mM, $MgCl_2$ 2mM, EDTA 0.1mM, DTT 1mM, Nonidet P-40 0.1%, Protease inhibitor cocktail). After centrifugation, the supernatant was collected as cytoplasmic fraction. The pellet was then dissolved in nuclear extraction buffer (HEPES pH 7.9 20mM, EDTA 3mM, Glycerol 10%, Potassium acetate 10mM, Magnesium chloride 1.5mM, DTT 1mM, Nonidet P-40 0.5%, Protease inhibitor cocktail) and collected as nuclear fraction. Finally, the pellet was dissolved in chromatin extraction buffer

(HEPES 150Mm, Mgcl2 1.5Mm, potassium acetate 150mM, Glycerol 10%, Protease inhibitor cocktail), 4 U nuclease (DNase and RNase) was added and incubated for 30 minutes at 37ºC. After centrifugation the supernatant was collected as chromatin fraction. The whole cell lysates were prepared with cold lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1 mM EDTA and protease inhibitor cocktail buffer tablet (PI; Roche Diagnostics) resolved by SDS/PAGE. Various primary antibodies (Ab) used are mouse monoclonal α-APE1 (Novus), α-FLAG (sigma), α-HSC70 (B6-Sc7298, Santa Cruz Biotechnology), α-α-Tubulin (Sigma: # T6199) Abs, AcAPE1, mSin3a (Santa Cruz; #sc994).

Apurinic/apyrimidinic (AP) endonuclease activity assay: A 43-mer oligonucleotide containing AP site analog tetrahydrofuran (THF) at nucleotide 31 (Midland Corp) was 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase as described previously (31, 87, 88). Following annealing to the complementary strand with an opposite THF, the duplex oligomer was purified by gel filtration column (Chroma Spin TE 10; Clontech). This THF-containing duplex oligomer was incubated with recombinant WT-APE1 or recombinant AcAPE1, prepared as described earlier (55, 67) at 37º C for 3 min during which the reaction rate was linear in a 15 µL reaction mixture containing 50 mM Tris-HCl pH 8.5, 50 mM KCl, 1mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 100 µg/mL bovine serum albumin. The reaction was stopped with 10 µl 80% formamide/40 mM NaOH containing 0.05% xylene cyanol, followed by heating at 95°C for 5min. The samples were ran in a denaturing gel electrophoresis in 20% polyacrylamide containing 8M urea to separate the substrate oligomer from the cleaved product. The gels were dried and the radioactivity was quantitated by phospho-imager analysis in a Storm system (Molecular Dynamics). The kinetic parameters K_m and k_{cat} were calculated by incubating 33 pM enzyme at 37°C for 3 min with substrates at various concentrations (0-160 nM).

The enzyme kinetics data were fitted by nonlinear least-squares regression to obtain V_{max} and K_m with the Michaelis–Menten equation using Sigma plot software.

AP site measurement assay: After endogenous APE1 downregulation in HEK-293T^{APE1siRNA} cells with Dox treatment, the cells were transfected with WT or mutant APE1 expression constructs, as described above. 48 hrs. post transfection, cells were treated with or without glucose oxidase for 30 min and total genomic DNA was isolated by Qiagen Dneasy kit following manufacturer's protocol. AP sites were measured using aldehyde reactive probe (Dojindo Laboratories) according to manufacturer's protocol.

UV fluorescence: The recombinant APE1 or AcAPE1 protein solution in PBS buffer (pH 7.5) were excited at 280 nm at 25ºC, emission was monitored at 300-450nm. The average spectrum was obtained from triplicate measurement.

Chromatin Immunoprecipitation assay and ChIP-on-Western: Cross-linked chromatin was immunoprecipitated with anti-AcAPE1 antibody and p21 promoter directed ChIP analysis was carried out essentially as described previously (85). ChIP-on-Western was performed as described (89) using anti-OGG1.

Colony forming assay: Generation of HEK-293T^{APE1siRNA} cell line stably expressing APE1 siRNA from a doxycycline-inducible promoter HEK-293T (HEK-293T^{APE1siRNA}) cells were described earlier (67). HEK-293T^{APE1siRNA} cells were treated with doxycycline (Sigma; 1 µg/ml) for 5-6 days to knockdown endogenous APE1 and transfected with expression plasmids containing WTAPE1 or different APE1 mutants. Equal numbers (Approximate 500) of cells plated on 60 mm plates were treated with or without glucose oxidase (100 ng/ml for 30 min). After washing fresh medium was added and cells were

allowed to grow for two weeks until visible colonies appear. The colonies were fixed with 100% methanol, stained with Giemsa staining solution (1:50) and counted. HCT116 cells stably expressing APE1 shRNA were transfected with WTAPE1 or APE1 mutants. 48 hrs after transfection approximately 500 cells on 60-mm dishes were treated with various doses of Methyl Methanesulphonate, MMS (0.5, 1, 1.5 and 2mM) for 1hrs and then washed and fresh medium was added to grow for two weeks until visible colonies appear. HCT116 cells expressing control shRNA used as a control.

2.3 **Results:**

AcAPE1 is exclusively associated with chromatin throughout cell cycle.

We investigated the sub-cellular localization of AcAPE1 using our previously characterized AcAPE1 antibody (Ab) (55, 67). We showed earlier that this AcAPE1 Ab is highly specific for recognizing APE1 species acetylated at the N-terminal Lys6 residue and does not cross-react with 50-fold excess of unmodified APE1 (55). Moreover, this Ab was unable to recognize ectopic APE1 molecules with mutated Lys6 residues (31). Confocal microscopy and super resolution (110 nM) 3D Structured Illumination Microscopy (SIM) data revealed AcAPE1 staining to be strictly nuclear, whereas unmodified APE1 was observed both in the nucleus and cytoplasm in human normal lung fibroblasts (IMR90) cells, hTERT-transformed diploid BJ fibroblast cells, as well as human lung adenocarcinoma A549 cells (**Fig. 1A,1B and 1D**). Using chromatin marker histone H3 or active enhancer marker H3K27Ac Abs, we found that AcAPE1 is present on chromatin (**Fig. 1C**). Furthermore, Structured Illumination Microscopy (SIM) revealed that AcAPE1 is exclusively localized in the chromatin **(Fig. 1B**). As chromatin can be easily observed during cell division in mitosis, we examined AcAPE1 localization in

mitotic cells. AcAPE1 was found to be exclusively localized to the condensed chromatin at all stages of mitosis, from prometaphase to telophase in both fibroblast cells and in cancer cells (**Fig. 1D and 1E**). Exclusive association of AcAPE1 on chromatin was also confirmed by Proximal Ligation Assay (PLA) using APE1 or Histone H3 and AcAPE1 Abs (**Fig. 1G)**. Our data show higher PLA signal localized on DAPI. Consistent with this, biochemical extraction of proteins with different salt concentration demonstrated a higher proportion of AcAPE1 in high salt fractions at different stages of cell cycles (**Fig. 1F**). We demonstrated earlier that p300 is the primary acetyltransferase for acetylating APE1 (67). We observed that AcAPE1 co-localizes with p300 only on chromatin (**Fig. 1H**). Furthermore, overexpression of E1A12S, which was shown to bind p300 and inhibit its HAT activity (86), significantly reduced AcAPE1 staining, but not APE1 staining in cells (**Fig. 1I**), further confirming the specificity of our AcAPE1 Ab. Overexpression of E1A deletion mutant 2-36, which cannot bind to p300, had no effect on AcAPE1 staining (**Fig. 1I**). Together these data suggest that APE1 is acetylated by p300 and that AcAPE1 is exclusively associated with chromatin in cells.

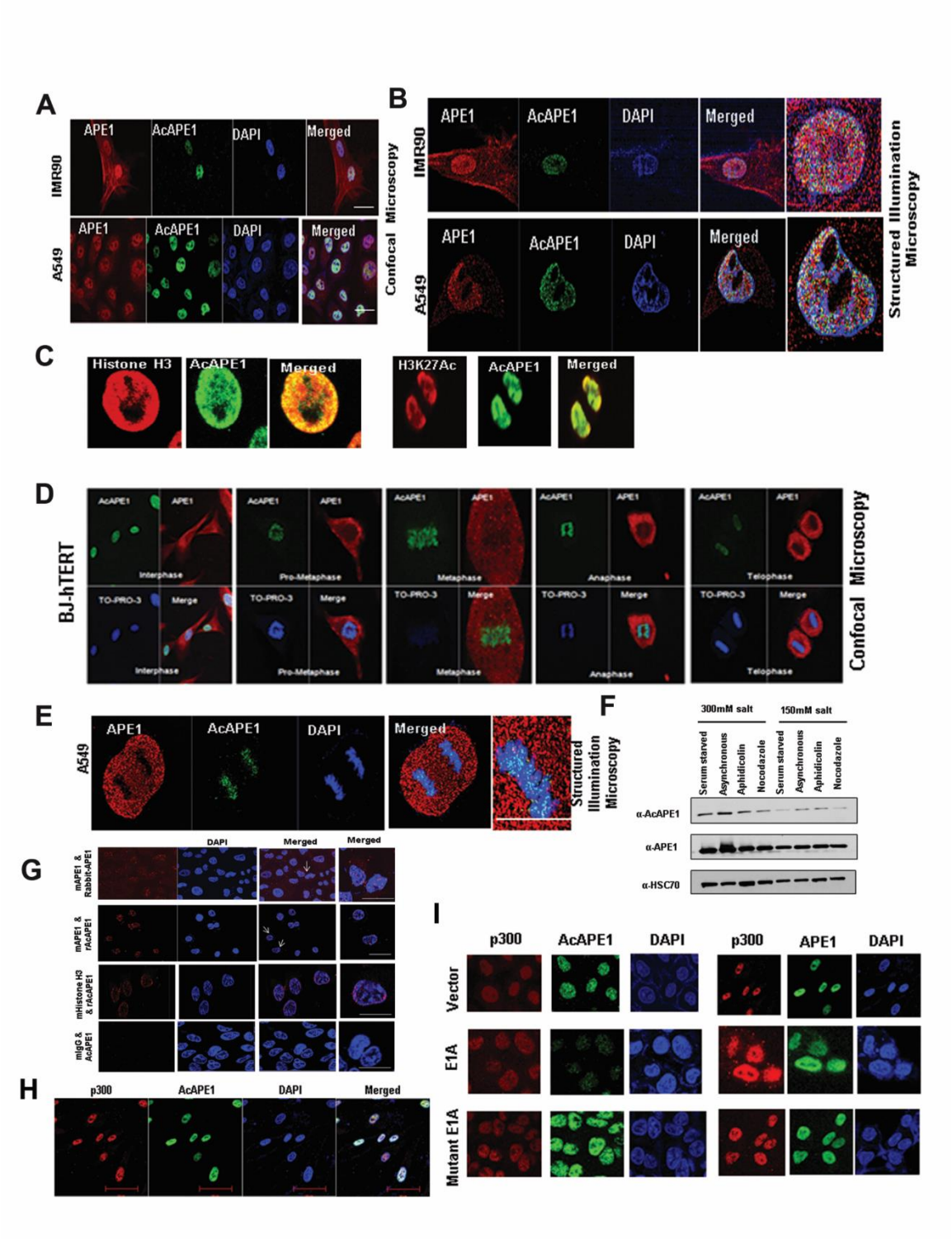
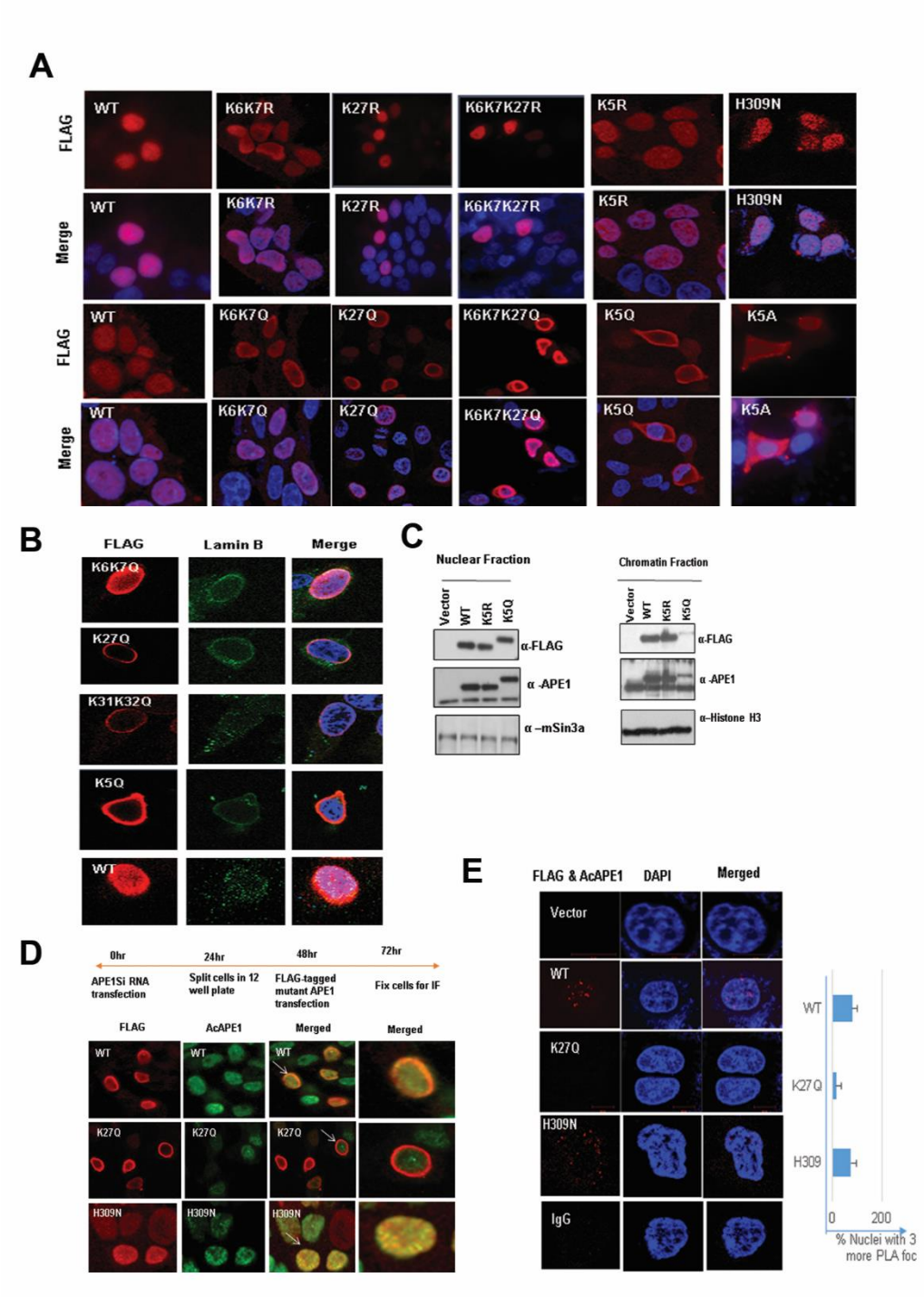


Figure 1: AcAPE1 is exclusively associated with chromatin and remains bound to the condensed chromosomes. (A & B) Asynchronous normal lung fibroblast IMR-90 cells and lung adenocarcinoma A549 cells were immunostained with α-APE1 and α-AcAPE1 Abs, and counter stained with DAPI and visualized by Confocal microscopy and

3D Structured Illumination Microscopy (SIM). (**C**) Co-localization of AcAPE1 with Histone H3 or active enhancer- specifichistone marker, H3K27Ac**. (D)** BJ-hTERT cells were serum starved for 72 hrs, and then fixed at different time points. Cells were immunostained with α-APE1 and α-AcAPE1 Abs, and counterstained with α-TO-PRO-3 iodide Ab**.** (**E**) Mitotic A549 cells were immunostained with α-APE1 and α-AcAPE1 and visualized by 3D SIM. (F) BJ-hTERT cells were either serum starved for 72 hrs (G0/G1), or treated with nocodazole (mitotic), or aphidicolin (G1/S synchronized cells) or untreated and whole cell extracts were isolated using 150mM or 300mM salt containing lysis buffer. Western blot analysis for α-APE1 and α-AcAPE1 levels was performed. α-HSC70 was used as loading control. (G) Proximal Ligation assay was performed with α-APE1 mouse (mAPE1) & α-APE1rabbit (Rabbit-APE1), α-mAPE1& α-rAcAPE1(rabbit-AcAPE1) and α-rAcAPE1& α-mHistone H3 (mouse-Histone H3) to confirm the chromatin association of AcAPE1. Mouse IgG (mIgG) & α-rAcAPE1 were used as a control. At least 50 cells were counted for PLA foci. (H) Co-localization of p300 and AcAPE1 on chromatin (DAPI). (I) HCT116 cells were transfected with E1A and mutant E1A (mE1A) and 48 hours after transfection Immunofluorescence assay (IF) was performed. Cells were immunostained with α-p300 and α-APE1 or α-AcAPE1 and counterstained with DAPI.

Positive charges of acetylable Lys residues but not their acetylation are essential for chromatin-binding of APE1.

To test if acetylation of APE1 is essential for association with chromatin, we generated several site-specific acetylable Lys mutants and performed localization studies using immunofluorescence (**Fig. 2A**) and biochemical fractionation assays (**Fig. 2C**). We found that mutations of Lys6,7 or Lys27, or Lys6,7,27 or all five acetylable Lys (Lys6,7,27,31&32, (K5R)) residues to non-acetylable Argnine, which maintains positive charges but cannot be acetylated, did not affect the chromatin association of APE1 (**Fig. 2A**). Surprisingly, neutralization of positive charges of Ly6,7 or Lys27 or Lys6,7,27 or all five acetylable Lys residues to Glutamine (K5Q) or Alanine (K5A) had a drastic effect on the chromatin binding of APE1 (**Fig. 2A**), and showed perinuclear localization of APE1 **(Fig. 2B)**. Our biochemical fractionation assay shows that neutralization of positive charges of these Lys residues significantly affected chromatin association of APE1 (**Fig. 2C**). We also used APE1 H309N mutant which was shown to be catalytically (AP-endonuclease activity) inactive, but can bind to an AP site substrate (90). We found that the H309N mutant can associate with chromatin similarly as WT APE1 (**Fig. 2A**).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FLAG
Merge
WT
K6K7R
K27R
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H309N
K6K7Q
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K5Q
K5A
B
FLAG
Lamin B
Merge
K6K7Q
K27Q
K31K32Q
K5Q
WT
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Figure 2: Positive charges of acetylable Lys residues but not their acetylation are essential for chromatin-binding of APE1.**(A)** Cells expressing different Lys site-specific APE1 mutants were immunostained with α-FLAG Ab (upper panel) and counterstained with DAPI (lower panel). **(B)** Subcellular localization of acetylation sites mutants were analyzed by immunostaining; cells expressing different Lys site-specific APE1mutants were stained with α-FLAG Ab, α-Lamin B Ab and counterstained with DAPI **(C)** Western blot analysis of soluble nuclear and chromatin extracts for FLAG-tagged WT and mutant APE1 levels in cells ectopically expressing these proteins with α-FLAG; α-APE1 and α- Histone H3 and α-msin3a as a control. **(D & E)** Schematic overview of the experiment. APE1 was down regulated in HEK293 cells using APE1 specific siRNA, and after 48 hours cells were transfected with expression plasmids containing WT or K27Q or H309N APE1. Immunofluorescence (IF) assay was performed using α-FLAG and α-AcAPE1 antibody to check co-localization. At least 50 FLAG positive cells were counted for co-localization. **(E)** Proximity Ligation assay (PLA) was performed using α-FLAG and α-AcAPE1 to examine the acetylation of WT, K27Q and H309N mutant APE1 in cells. At least 50 cells were counted and percentage of PLA signal is plotted for each APE1 mutant.

APE1 is acetylated after binding to AP site damages in the chromatin.

Our confocal and biochemical data clearly indicate that the positive charges of acetylable Lys residues in APE1, but not their acetylation, are essential for chromatin association (Fig. 2A & 2C). Still, we consistently observed that AcAPE1 is exclusively associated with chromatin in both interphase and mitotic cells (Fig.1B & 1D). This led us to test whether APE1 is acetylated after binding to AP site lesions in chromatin**.** If chromatin-binding is necessary for APE1 to be acetylated, then loss of chromatin-binding of K27Q or K31&32Q APE1 mutants (Fig. 2B) is expected to prevent APE1 acetylation at Lys6 residue in cells, and therefore should not be detected by AcAPE1 Ab. To test this, we downregulated endogenous APE1 levels in HEK293 cells using siRNA. After downregulating endogenous APE1, we ectopically expressed FLAG-WT or -K27Q or, -H309N mutants and immunostain with AcAPE1 Ab to compare APE1 acetylation at Lys6. We reported earlier that mutation of Lys27 residues in recombinant APE1 proteins does not affect acetylation by p300 at Lys6 in vitro and can be detected by AcAPE1 Ab in WB analysis (62). Our immunofluorescence (**Fig. 2D**) and PLA (**Fig. 2E**) assays show co-localization of AcAPE1 and FLAG Abs only in FLAG-WT APE1 and H309N APE1, but not chromatin-binding defective K27Q mutant expressing cells; providing evidence that chromatin association is necessary for APE1 to be acetylated.

To directly test that acetylation of APE1 occurs after binding to AP site lesions in the genome in cells, we abrogated binding of APE1 to AP sites by Methoxyamine (MX). Several earlier studies have established that MX covalently binds to AP sites to form methoxyamine-bound AP (MX-AP) sites, and competitively inhibits binding of APE1 to AP sites (91-93). These MX-AP sites are resistant to recognition and repair by the APE1 (91, 92). Thus, we treated the cells with different doses of MX for different time periods, and found that treatment of MX showed a dose- and time- dependent inhibition of

chromatin association of endogenous APE1 (**Fig. 3A and 3B)**. This indicates that observed chromatin association of APE1 was primarily due to AP sites damage binding on the genome (**Fig. 3A and 3B**). Interestingly, we observed that MX treatment completely abrogated APE1 acetylation in a dose and time-dependent manner (**Fig. 3A and 3B**). However, MX treatment did not affect the chromatin association of OGG1 (**Fig. 3C**), a DNA glycosylase which recognizes 8-oxo guanine DNA base damage (61). Thus, MX treatment does not inhibit chromatin association of other initial enzyme involved in the BER pathway. To further support the observation that APE1 acetylation occurs after binding to the AP sites, we induced generation of AP sites in the genome by treatment alkylating agent Methyl Methane sulphonate (MMS), glucose oxidase (GO), an oxidizing agent that induces oxidative base damages. These oxidative or alkylated base damages subsequently generate AP sites after their removal by DNA glycosylases. (94). As shown in figure **Fig. 3D**, blocking AP site with MX treatment abrogated acetylation of APE1 even after induction of AP sites with MMS treatment (**Fig. 3D**). Moreover, our biochemical assay revealed that treatment with GO significantly enhanced the levels of chromatin–bound AcAPE1 levels (**Fig. 3E)**. We also examined the binding or association of APE1 on the endogenous p21 promoter region in cells via ChIP using AcAPE1 ab after induction of DNA damage with MMS treatment. A significant enrichment of an AcAPE1-bound p21 promoter region was observed in MMS treated cells compared to control (**Fig. 3F**). Similarly, MX treatment reduced the enrichment of the AcAPE1-bound promoter region (**Fig. 3F**). Together, these data suggest that APE1 is acetylated after binding to AP sites in chromatin in cells.

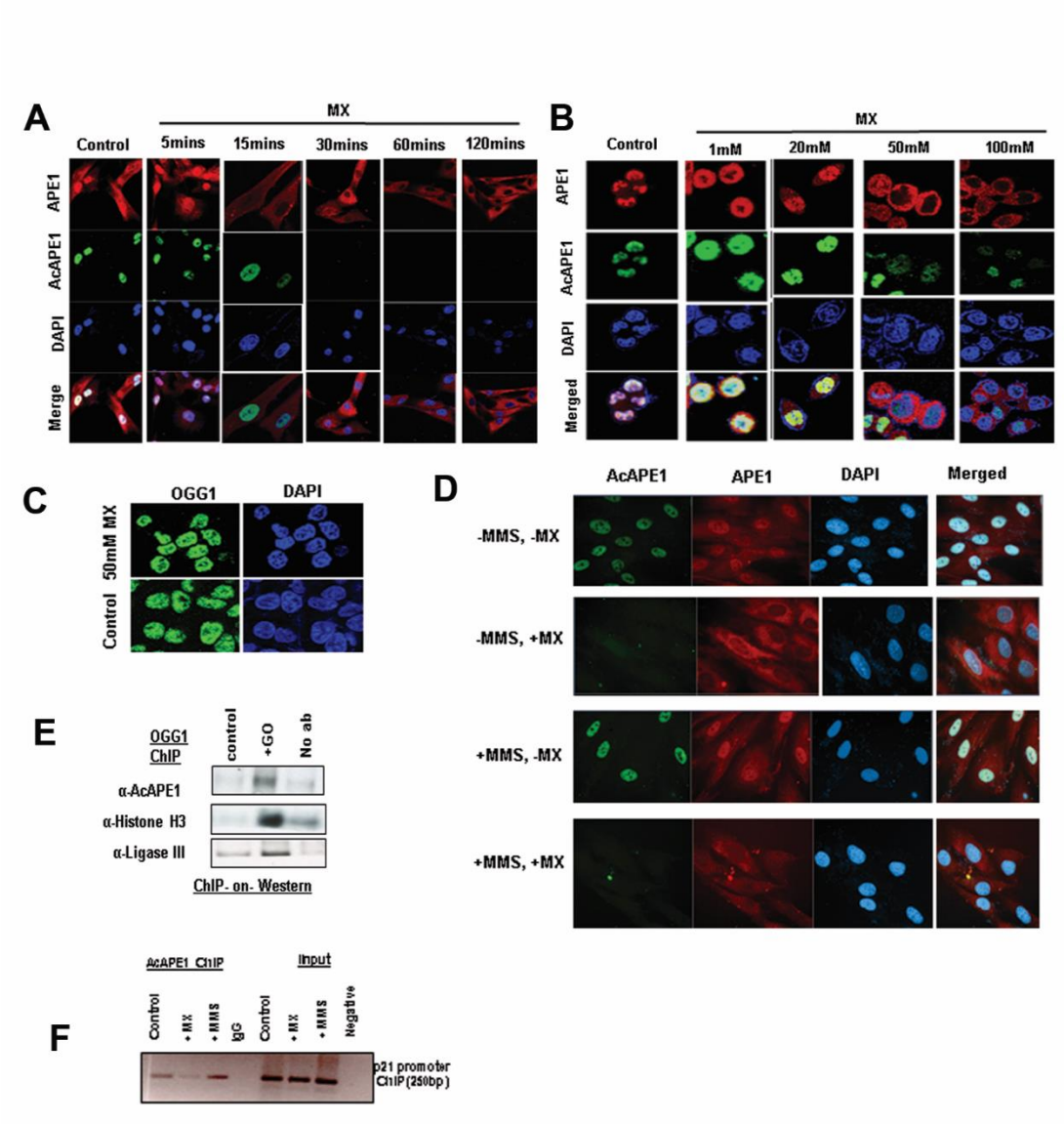


Figure 3: APE1 is acetylated after binding to AP sites in the chromatin. (A) BJ-hTERT cells were treated with Methoxyamine (MX) (50mM) for indicated time periods. IF was performed using α-APE1 and α-AcAPE1 and counterstained with DAPI. **(B)** HCT116 cells were treated with various doses of MX for 30 mins and IF was performed using α-APE1 and α-AcAPE1 and counterstained with DAPI. **(C)** HCT116 cells were treated with MX 50mM for 30 mins and IF performed using α-OGG1 and counterstained with DAPI. **(D)** BJ-hTERT cells pre-treated with or without MX 50mM for 30 mins was exposed to Methyl Methane Sulfonate (MMS) (2mM) for 1 hour. IF was performed using

α-APE1 and α-AcAPE1 and counterstained with DAPI. Confocal microscopy was used to visualize the AcAPE1 level in control and MMS or MX or both treated cells. **(E)** Chromatin Immunoprecipitation (ChIP) with α-OGG1 antibody followed by Western blotting (ChIP-on-Western) was performed, to examine the association of AcAPE1 and Ligase III on chromatin after induction of DNA damage with glucose oxidase (GO). **(F)** Association of AcAPE1 on endogenous p21 promoter was examined by promoter directed ChIP using α-AcAPE1 in control or MMS or MX treated cells.

Acetylation of APE1 enhances its AP endonuclease activity or catalytic efficiency.

We investigated directly whether acetylation affects the AP-endonuclease activity of APE1 *in vitro*. Purified WT APE1 was incubated with p300 HAT domain either in the presence or absence of AcetylCoA and then acetylation of APE1 was confirmed by Western analysis using our AcAPE1 specific Ab (**Fig**. **4A**). We found that acetylation of APE1 increased its AP-endonuclease activity in a dose-dependent manner (**Fig. 4B**). We determined the steady-state parameters for both recombinant unmodified APE1 and AcAPE1 during the linear increase period (up to 3 min, of product formation **(Fig. 4C and 4D**). Enzyme Kinetic analysis showed that both APE1 and AcAPE1 have comparable binding affinity (K_m) for the substrate AP sites (**Fig. 4E)**. However, acetylation enhanced catalytic (K_{cat}) turnover of APE1 *in vitro* (**Fig. 4E**). Thus AcAPE1 has a higher (~-3fold) k_{cat}/k_m ratio, i.e increased catalytic efficiency.

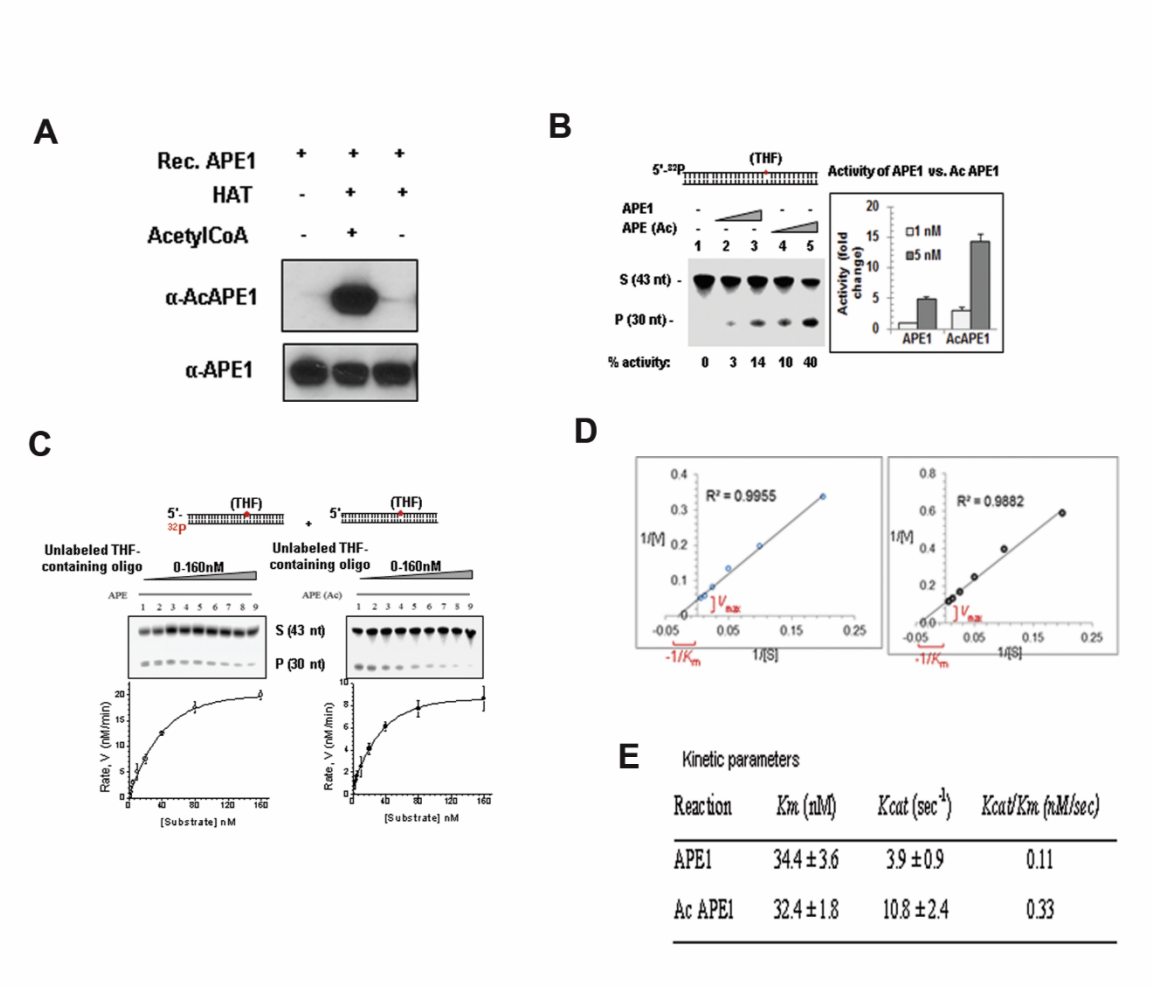


Figure 4: Acetylation of APE1 enhances its AP endonuclease activity. (A) Rec. APE1 was incubated with p300 HAT domain either in the presence or absence of AcetylCoA and Western blot analysis was performed with α-APE1 and α-AcAPE1 Abs to confirm acetylation of APE1. **(B)** Incision of the THF (reduced AP-site)-containing 43-mer duplex oligonucleotide (Substrate: S) by APE1 and *in vitro* acetylated APE1; the cleaved product (P). **(C, D)** The kinetic parameters Km and kcat were calculated by incubating 33 pM enzymes at 37°C for 3 min with substrates at various concentrations (0-160 nM). The enzyme kinetics data were fitted into nonlinear least-squares regression to obtain Vmax and Km with the Michaelis–Menten equation using Sigma plot software. **(E)** Comparison of kinetic parameter between APE1 and AcAPE1.

APE1 acetylation enhances its interaction with downstream BER proteins and stability on chromatin.

In cells, total (complete) repair of AP sites is not only dependent on AP-endonuclease activity of APE1 but also its interaction and coordinated recruitment of downstream BER proteins such as DNA polymerase beta (polß) and XRCC-1/DNA ligase III (95-97). APE1 has been shown to incise the AP-site and remains tightly bound to the cleaved AP site product and serve as a mediator for the next step in the BER pathway. Thus when bound to the cleaved AP site, APE1 physically interacts with polß and significantly stimulates its dRP-lyase activity (90, 97). Moreover, interaction of APE1 with XRCC1 was shown (98). Consistent with this idea, we observed that AcAPE1 co-localizes with Ligase III in chromatin (**Fig. 5A**). Furthermore, we found that treatment with TSA which enhances APE1 acetylation levels (57) increased association of XRCC1 with WT-APE1 but not with non-acetylable K6R/K7R mutant (**Fig. 5B**). This indicates that acetylation of APE1 enhances its interaction with XRCC1((99). Interestingly, we found that pre-extraction of loosely associated proteins with 0.5% Triton-X-100 and salt prior to fixation significantly decreased staining for non-modified APE1 but did not have any effect on AcAPE1 staining in the nucleus (**Fig. 5C**). This suggests that APE1 is more stable on chromatin when it is acetylated.

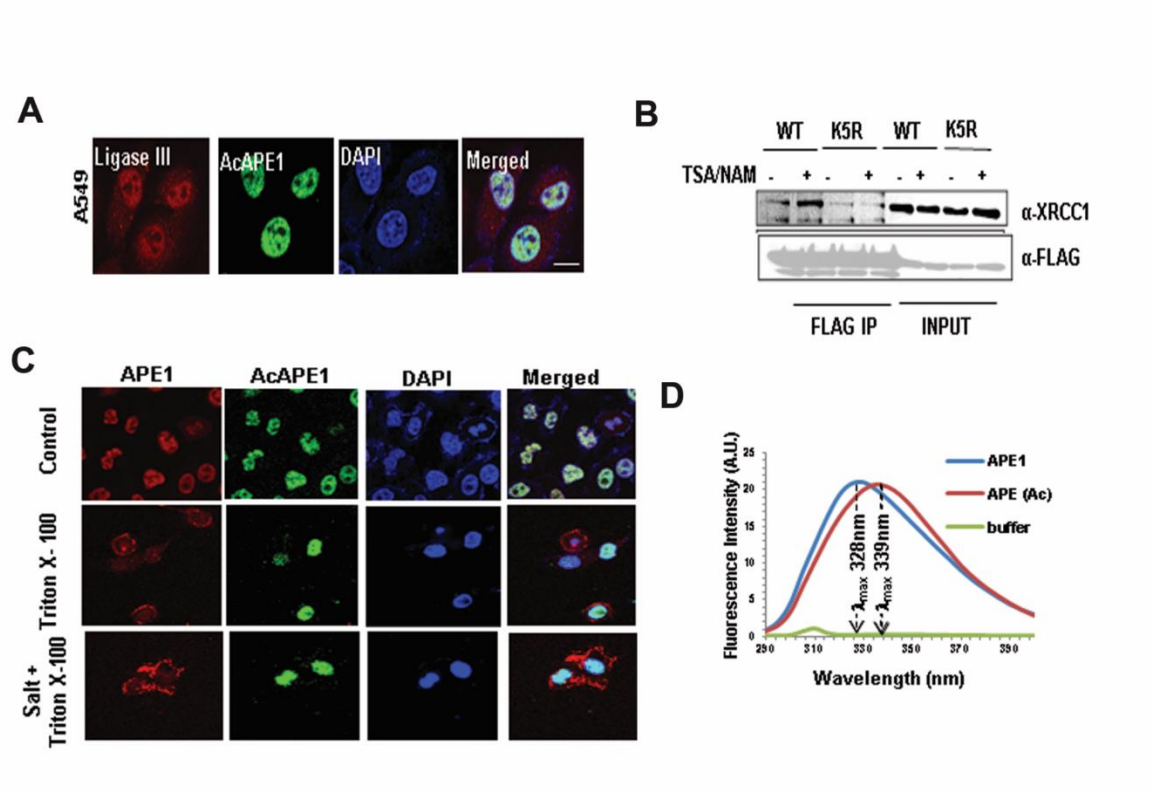


Figure 5: APE1 acetylation enhances its stability on chromatin and interaction with downstream BER proteins. (A) Co-localization of Ligase III and AcAPE1 in A549 cells. Cells were immunostained with α-ligase III and α-AcAPE1 Abs. **(B)** WT or K5R APE1 overexpressing HEK293 cells were treated with or without TSA/NAM for 6 hrs and nuclear extract was immonoprecipitated using α-FLAG Ab and immunoblotted with α-XRCC1 and α-FLAG Abs. **(C)** A549 cells fixed with paraformaldehyde before (upper panel) or after treatment with Triton X-100 (0.5%) (middle panel) or Triton X-100 plus salt (100 mM KCl, lower 203 panel) and immunostained with α- APE1, α-AcAPE1 Abs and counterstained with DAPI. **(D)** Acetylation of APE1 induces a conformational change in APE1. Distinct intrinsic fluorescence emission spectra of APE1 and AcAPE1 at 280 nm.

Acetylation induces a conformational change in APE1.

So far, our data suggest that upon binding to the AP site in the chromatin, APE1 is acetylated and AcAPE1 has higher catalytic efficiency. We tested the possibility that acetylation-mediated neutralization of positive charges of Lys residues in the N-terminal could induce a conformational change in APE1, and that this may increase its catalytic efficiency. We compared the UV fluorescence of both unmodified and AcAPE1. WT APE1 contains seven tryptophan (Trp) and eleven Tyrosine (Tyr) residues, all of which are located in its globular core domain (aa 42-318) (100). Trp residues that are exposed to water have maximal fluorescence at a wavelength of about 340-350 nm, whereas totally buried residues fluoresce at about 330 nm (101). Excitation at 280 nm (for both Tyr and Trp) showed a typical Trp emission spectrum of WT APE1 with λ_{max} 329 nm. However, AcAPE1 showed a red shift (λ_{max} 339 nm), indicating a more solvent exposed environment for the Trp residues when APE1 is acetylated. (**Fig. 5D**). These data together suggest that acetylation of APE1 induces a conformation change in APE1.

Absence of acetylation in APE1 cells accumulates AP-sites in the genome.

The observation that APE1 acetylation enhances its endonuclease activity raises the possibility that in the absence of acetylable Lys residues, cells will accumulate AP sites in the genome. We quantitated AP sites in the genome of cells expressing WTAPE1 and non-acetylable APE1 mutants by using an aldehyde-reacting probe (ARP). We used HEK293T^{APE1siRNA} cells stably expressing APE1-siRNA under a Dox-inducible promoter, as described earlier (57, 67). We treated the cells with Dox (1 ug/ml) to deplete endogenous APE1 and then ectopically expressed FLAG-tagged WT-APE1, or its mutants lacking the acetylation sites. AP sites in the genome were quantitated using aldehyde reactive probe (ARP) (94, 102). As expected, we observed that depleting

endogenous APE1 with Dox significantly increased AP sites in the genome compared to control (**Fig. 6A**). This effect can be partially rescued by ectopic expression of WT-APE1, but not with its non-acetylable K5R or chromatin-binding defective K5Q or NΔ33 mutants (**Fig 6A**). We also treated these cells with glucose oxidase to induce oxidative damages, which in turn produced AP sites in the genome. As shown in **Fig. 6B**, treatment with glucose oxidase significantly enhanced AP sites in the genome of APE1 downregulated cells. These can be reduced by ectopic expression of WT APE1, but not with either non-acetylable K5R or K5Q mutant. Together, these data indicate that the presence of acetylable Lys residues and acetylation of these residues in APE1 play a crucial role in endogenous DNA damage or AP site repair in cells.

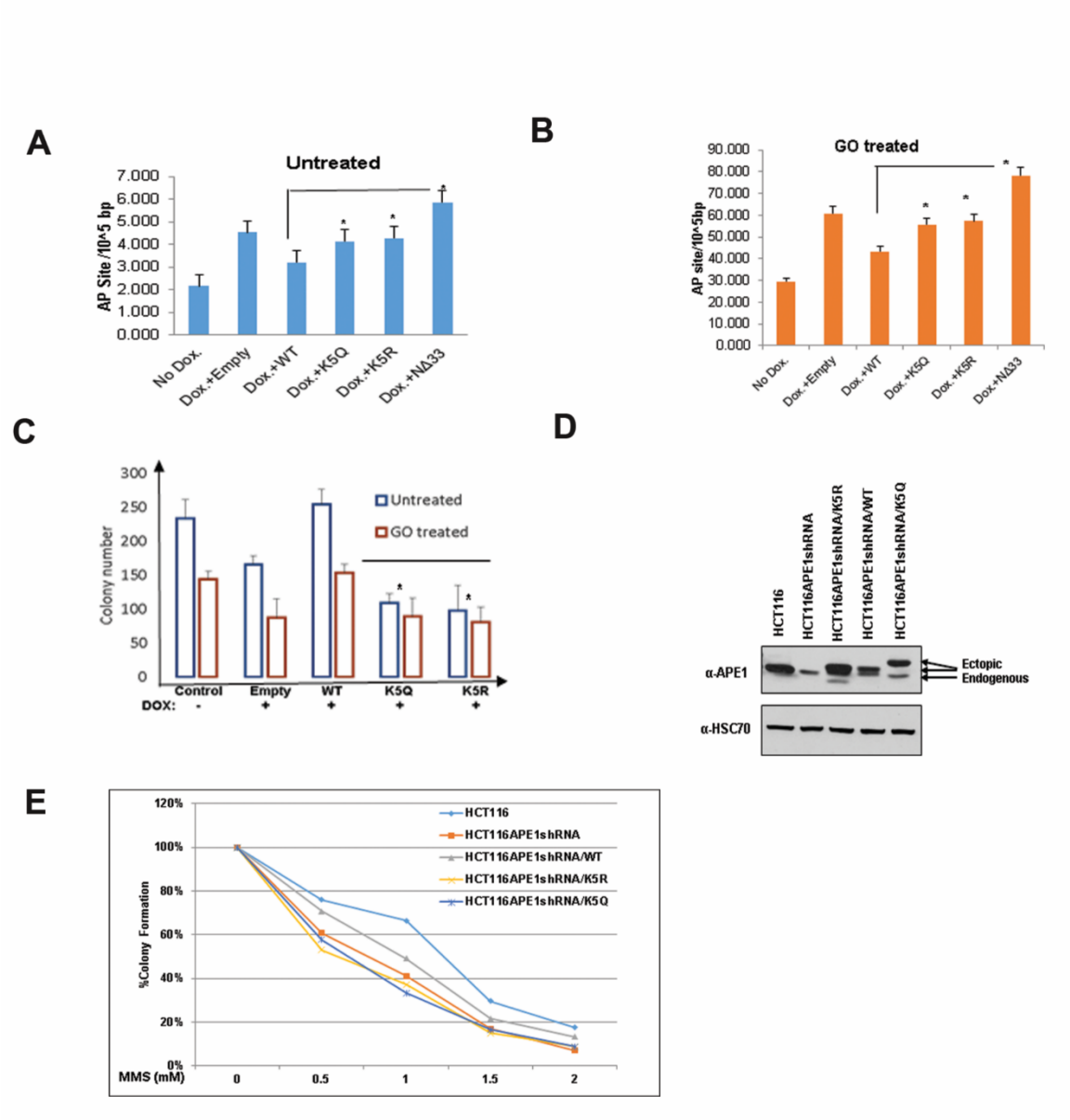


Figure 6: APE1 acetylation is essential for cell survival and/or cell proliferation and absence of APE1 acetylation sensitizes cells to DNA damaging agent. (A, B) EndogenousAPE1 was downregulated in HEK293T^{APE1siRNA} cells using doxycycline (Dox) treatment. FLAG tagged WT APE1 or acetylation defective (mutations of Lys6,7,27,31&32 to non-acetylable arginine (K5R) or glutamine (K5Q)) or N-terminal deletion (NΔ33) mutants were further overexpressed in these cells. Cells were treated with or without Glucose oxidase (100ng/ml for 30 mins) and AP sites were measured using ARP kit. Bar diagram representing number of AP site/10^5 bp in presence of

different APE1 mutants compared to vector control. Error bars indicate mean ±SD (n=3). **(C)** HEK293T^{APE1siRNA} cells were treated with/without DOX and WT, K5R and K5Q mutant APE1 were ectopically expressed. Cells were treated with or without glucose oxidase (100 ng/ml for 30 min) and colony formation assay was performed. Bar diagram representing number of colony formed in presence of different APE1 mutants compared to vector control. Error bars indicate mean ±SD (n=3). **(D, E)** HCT116 cells constitutively expressing APE1 shRNA; HCT116APE1shRNA, were transfected with FLAG tagged WT APE1 or acetylation defective (mutations of Lys6,7,27,31&32 to non-acetylable arginine (K5R) or glutamine (K5Q)) APE1 mutants. Western blot analysis was performed to examine APE1 levels using α-APE1 Ab. α-HSC70 was used as loading control. **(E)**Damage sensitivity was measured in HCT116APE1shRNA cells ectopically expressing different APE1 mutants. Cells were treated with increasing dose of MMS for 1hrs and colony formation assay was performed.

APE1 acetylation plays a critical role in cell survival and/or proliferation in response to genotoxic stress

We examined the role of APE1 acetylation in cell survival and/or proliferation, measuring cell survival by clonogenic survival assay. As expected, we observed that depleting endogenous APE1 with Dox significantly decreased the number of viable colonies compared to control (**Fig. 6C**). This effect can be rescued by ectopic expression of WT APE1, but not with non-acetylable K5R or K5Q mutants (**Fig. 6C**). We further examined the role of APE1 acetylation in cell survival or proliferation after induction of DNA damage. Treatment with GO decreased the number of viable colonies which can be rescued by ectopic expression of WT APE1, but not with its non-acetylable mutants (**Fig. 6C**). Furthermore, we transfected HCT116 cells that stably expressing APE1 shRNA (HCT116APE1shRNA) with FLAG-tagged WT, and noncaetylable K5R or K5Q mutants. Western blot analysis showed that the expression levels of WT APE1 was significantly low compared to non-acetyalable K5R , K5Q and endogenous APE1 level in control HCT116 cells (**Fig. 6D**). We found that treatment of alkylating agent MMS sensitized the HCT116APEshRNA cells in –a dose-dependent manner **(Fig. 6E)**. This effect can be partially rescued by ectopic expression of WT APE1, but not with non-acetylable K5R or K5Q mutant protein which is present in higher levels in cells (**Fig. 6E and 6D**). These data indicate that APE1 acetylation plays a role in cell survival and/or proliferation and that the absence of APE1 acetylation in cells sensitizes them to DNA damaging agents.

2.4 **Discussion**

Unrepaired AP sites in the genome can inhibit transcription, and block DNA replication (9, 75, 103). Thus, the presence of an efficient repair mechanism is essential for cell

survival and/or proliferation. We reported earlier that multiple Lys residues in the intrinsically disordered N-terminal domain of APE1 are modified by acetylation in cells (56, 62). Further, using conditional APE1-nullizygous mouse embryo fibroblasts (MEF), we showed that both the DNA repair function and acetylable Lys6 and Lys7 residues of APE1 are essential for cell survival (72). Moreover, a recent study showed that the absence of acetylation at Lys 6 and Lys7 sites in APE1 or its DNA repair function results in telomere fusion and mitotic defects (73). Given the key role of APE1 in the repair of AP sites which inhibits transcription and replication (5, 75, 103), the essential role of DNA repair function of APE1 in cell survival is not surprising (6, 24, 72, 78). However, what was unexpected was the critical importance of APE1's acetylation for cell survival and proliferation (72). In this study, we demonstrated that acetylation of APE1 plays a crucial role during the repair process of AP sites in the genome in cells via the BER pathway. Our study documents that APE1 is acetylated after binding to AP sites in the chromatin and acetylation not only improves the catalytic efficiency of APE1 but also may facilitate coordination and recruitment of the downstream enzyme in the BER pathway. Thus, acetylation of APE1 is likely to be an integral part of the APE1-dependent BER pathway for maintaining DNA integrity.

Although the N-terminal domain (1-61 aa) of APE1 is dispensable for its *in vitro* DNA repair activity (104, 105), this study unraveled the novel regulatory role of acetylation of multiple Lys residues in this domain, in AP site repair both *in vitro* and in cells. Our study provides direct evidence that APE1 after acetylation enhances its AP-endonuclease activity *in vitro*. We also have provided evidence that the absence of acetylation in APE1 cells accumulates AP sites in the genome and becomes sensitive to DNA damaging agents. Several lines of evidence support that APE1 is acetylated at Lys6 residues after binding to AP sites in the chromatin. First, AcAPE1 is exclusively present on chromatin

throughout cell cycles in all types of cells including primary, transformed, and tumor. Second, the inhibition of binding of APE1 to AP sites by MX treatment abrogates APE1 acetylation in a dose and time-dependent manner. Third, APE1 mutants that are proficient in acetylation but cannot bind to chromatin cannot be acetylated in cells. Finally, induction of AP sites in the genome by MMS treatment enhanced APE1 acetylation and occupancy of AcAPE1 on chromatin.

Stimulation of the AP-endonuclease activity of APE1 due to acetylation suggests multiple possible mechanisms. An acetylation-induced conformational change in APE1 could either increase its affinity for the substrate AP site in DNA; or, it could facilitate AP site cleavage or decrease APE1's affinity for the product (cleaved AP-site), thus increasing its turnover. Our data, showing that both WT and AcAPE1 have comparable affinity K_m for the substrate AP sites but different K_{cat}, suggest that an acetylation-induced conformational change in APE1 could either facilitates AP site cleavage or increase the dissociation of APE1 from the product (cleaved AP-site) thus increasing its turnover. However, APE1 has been shown to incise the AP-site, remain tightly bound to the cleaved AP site product, and serve as a mediator for the next step in the BER pathway (97). Efficient complete (total) repair of the AP site is not only dependent on APE1 endonuclease activity, but also on the coordination and interaction of APE1 with downstream BER proteins (32, 95, 97, 98). Thus, when bound to the cleaved AP site, APE1 physically interacts with DNA polß and significantly stimulates its dRP-lyase activity (90, 97). Interaction of APE1 with XRCC1 was also shown (98). Consistent with this idea, we observed that AcAPE1 interacts with Ligase III in chromatin and that acetylation of APE1 enhances its interaction with XRCC1. Thus APE1 after acetylation not only facilitates AP sites cleavage in the chromatin but may also enhance coordination of total AP site repair in cells.

In the absence of any structural information about full-length APE1 or its binary complex with AP site containing oligo, it is not easy to establish if these acetylable Lys residues directly interact with AP sites containing DNA or C-terminal active site domain. Our data shows that the positive charges of the acetylable Lys residues are important for its nuclear retention and/or chromatin binding and neutralization of positive charges of acetylable Lys residues reduced its chromatin association. It is likely that basic Lys residues in the N-terminal domain of APE1 may dynamically interact with DNA or with acidic residues in interacting partner proteins including nuclear importin complexes for nuclear localization or nucleosome remodeling complexes that facilitate binding access of APE1 to AP sites in nucleosomes in the context of chromatin in cells. Consistent with this, earlier studies by our group and others have shown that the APE1 N-terminal 33 aa is required for interaction with many interacting partners, and neutralization of positive charges in APE1 affects their interaction (67, 68). Intrinsically unstructured regions and electrostatic forces are known to be generally important for intramolecular interactions. Similar to our observation, a study has shown that NEIL1, a DNA glycosylase involved in oxidative damage repair, has an intrinsically disordered C-terminal domain (311-389 aa) that is involved in intramolecular interaction with a negative charge core domain (106). This domain was also shown to be essential for interaction of NEIL1 with many binding partners (107).

We propose a model (**Fig. 7**) by which APE1 acetylation regulate the AP site repair in cells. When APE1 locates AP sites in the genome, the first stable APE1-AP DNA complex is formed. Subsequent recruitment of p300 to the damage sites acetylates APE1. Acetylation-mediated neutralization of multiple positive charges at multiple Lys residues in the N-terminal may disrupt the intramolecular interaction, which induce a conformational change in the catalytically active domain in APE1 which facilitates AP

sites cleavage in DNA. Consistent with this, one study has shown that neutralization of the positive charges of the Lys residues in the N-terminal domain or deletion of the N-terminal domain of APE1 significantly enhanced the AP-endonuclease activity of APE1 in vitro (68). Moreover, our current data show a similar change (red shift) in Trp emission in APE1 after its acetylation. Since, acetylation of APE1 improves the turnover rate of APE1 and also enhances the interaction with XRCC1, it appears that the acetylation of APE1 has evolved not only to improve the catalytic efficiency but also to facilitate coordination and recruitment of the downstream enzyme in the BER pathway. We hypothesize that, after AP site cleavage, AcAPE1 remains bound to the cleaved AP-sites and subsequent recruitment of polß displace APE1 from the 5`-deoxyribose phosphate of cleaved AP site (but no from DNA) which is enhanced when APE1 is acetylated. Thus acetylation-induced conformation change in APE1 not only enhances its cleavage activity, and at the same time may facilitate co-ordination with the next BER proteins. Such a mechanism might also help to explain why AcAPE1 is associated with ligase III and the XRCC1 complex, and why acetylation stimulates their interaction in cells. Finally, recruitment of the histone deacetylase SIRT1 (108, 109), deacetylates AcAPE1, and that displaces APE1 from the DNA. Consistent with this finding, an earlier study showed SIRT1 can deacetylate APE1 in vitro and in cells and that it regulates cellular BER (109).

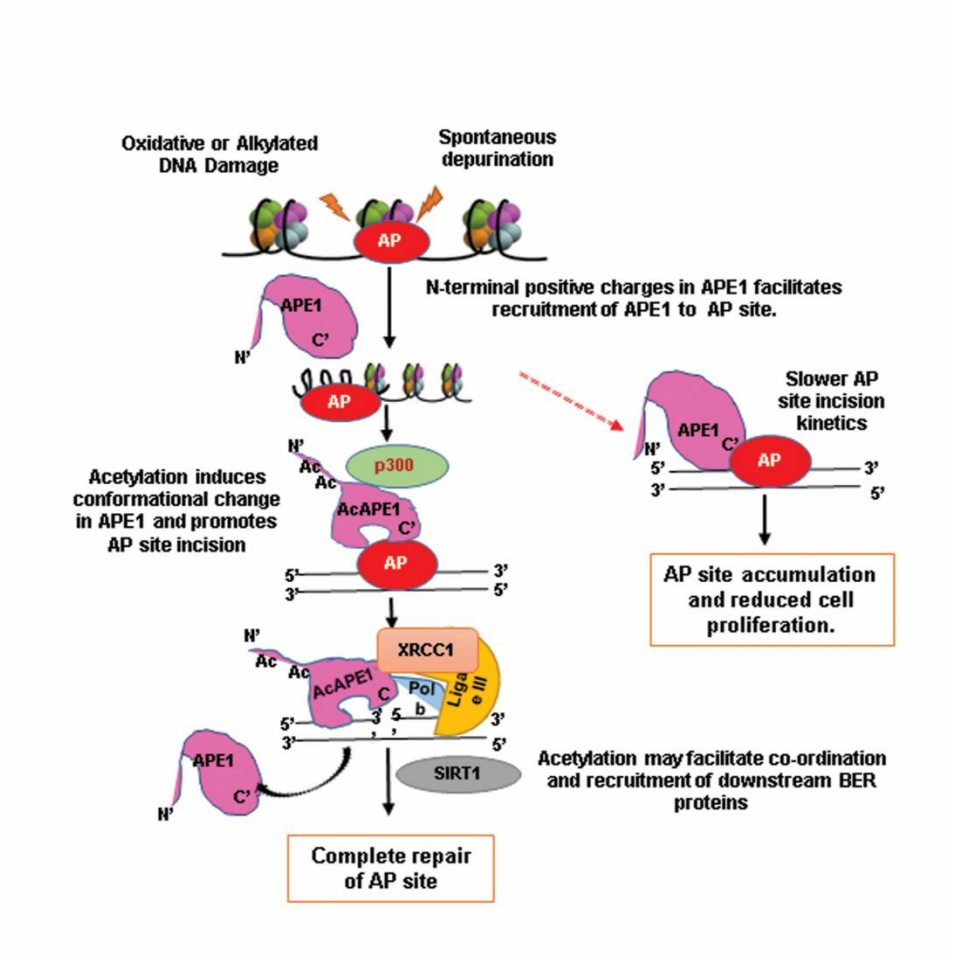


Figure 7: Schematic model for regulation of AP sites repair in cells by acetylation of APE1 via BER pathway.

Our novel discovery highlights that the conserved acetylable Lys residues in unique N-terminal domain (present only in mammalian APE) of APE1, and that their modifications are evolved to finely regulate and co-ordinate the efficient repair of the AP site in mammalian cells. Thus mutation of either catalytically active sites or acetylation sites in the N-terminal would have profound cellular consequences such as growth arrest and/or cell death, Consistent with this hypothesis, we showed earlier that both the DNA repair function and acetylable Lys6 and Lys7 residues of APE1 are essential for cell survival (72). Moreover, a recent study by Madlener and colleagues showed that APE1 is essential in maintaining telomere length, presumably through maintenance of DNA integrity in that region (73). They found that both the DNA repair function active H309 or N212 sites and acetylable Lys 6/7 sites in APE1 are essential for maintaining telomere length (73). We believe that mitotic telomeres might be prone to oxidative damages and generate AP sites and the absence of AcAPE1-mediated AP site repair leads to accumulate DNA breaks in this region, this will lead to telomere fusion and mitotic defects. Indeed, they found that absence of acetylation of APE1 or its DNA repair function leads to mitotic defects. Of note, our confocal IF data demonstrate the presence of AcAPE1 on condensed chromatin during all stages of mitosis. This indicates that AP sites are also generated in the genome during mitosis; AcAPE1-mediated BER may be also operative. Further studies are necessary to establish this.

Nonetheless, our findings demonstrate that APE1 acetylation is an integral part of the BER pathway in cells for maintaining genomic stability and also provide the mechanism by which APE1 acetylation plays a key role in the repair of AP sites or oxidative DNA damages. Our study also implicates that dysregulation of APE1 acetylation/deacetylation cycle may lead to genomic instability and cause many human diseases, including cancer, premature aging.

CHAPTER 3

Endogenous oxidative base damage and base excision repair regulate the formation of G-quadruplex structures in the genome

The materials presented in this chapter is submitted to a peer-reviewed journal as of May 2019.

3.1 **Introduction**

G-quadruplexes (G4) are non-canonical tetrahelical nucleic acid structures that arise from the self-stacking of two or more guanine quartets, a planar array of four guanine residues coordinated through Hoogsteen hydrogen bonding (110, 111). Numerous in vitro biochemical and structural analyses have established that both DNA and RNA sequences having a specific consensus motif ($G_{\geq 3}\ N_{1\text{-}7}G_{\geq 3}\ N_{1\text{-}7}G_{\geq 3}\ N_{1\text{-}7}G_{\geq 3}$) can form G4 structures (112). Formation of G4 DNA structures in the genome have emerged as an epigenetic mechanism for regulating transcription, replication, translation and telomere maintenance (113). Deregulation of formation (folding) or unfolding of G4 has been implicated in transcriptional dysregulation, telomere defects, replication stress, genomic instability and many human diseases including cancer and neurodegeneration (114-118). A breakthrough in establishing the regulatory role of G4 in vivo came from the recent genome-wide mapping of G4s in human cells using high-throughput chromatin immunoprecipitation-sequencing (ChIP-Seq) with a specific antibody directed against G4 structure (113, 119, 120). Mapping revealed that G4 structures are non-randomly located and overrepresented in areas of key regulatory regions like gene promoters, 5’ and 3’ untranslated regions and telomeric regions, suggesting a positive selective pressure for retention of these motifs at specific sites in the genome for regulating multiple biological processes. In vitro, many G4 DNA structures once formed are thermodynamically more stable than double-stranded DNA (121). However, for biological functions, their formation (folding), stabilization, and unfolding must be regulated. While several proteins (DNA or RNA helicases) that bind to and resolve G4 structures have been characterized (122), the mechanism(s) underlying spatiotemporal formation and stabilization of G4 structures in the genome are largely unknown.

Guanine (G) residues in potential G-quadruplex-forming sequences (PQS) have the lowest oxidation potential and are likely to be susceptible to the formation of 8-oxoguanine (8-oxoG), the most prevalent endogenous oxidative base damage in the genome (123, 124). 8-oxoguanine DNA glycosylase (OGG1) initiates the repair of 8-oxoG via evolutionary conserved DNA Base Excision Repair (BER) pathway by removing the oxidized base and generating an Apurinic/apyrimidinic (AP/abasic) site (125, 126). Human AP-endonuclease 1(APE1) is then recruited to the AP sites for repair through the BER pathway (77). APE1, a key enzyme in the BER pathway, is a multi-faceted protein involved in telomere maintenance, transcription regulation and antibody genesis, highlighting role of BER beyond its genome maintenance function (34, 72, 73, 76, 127). However, the molecular and functional connection of the endogenous DNA damage and APE1 with the G4 structures remains largely unclear. Here, we conduct an unbiased genome-wide mapping of G4 structures, along with oxidative base damage, AP sites, and binding of OGG1 and APE1 proteins, and provide the first direct evidence that occurrence of endogenous base damage is non-random and is predominant in PQS sequences. Visualizing G4 dynamics in cells using high-resolution microscopy, we demonstrate that oxidative base or AP site damages and the associated repair complexes play a critical role in the spatiotemporal regulation of G4 structures. Loss of either repair function or acetylation of APE1 results in the abrogation of G4 structures and deregulation of gene expression. Using in vitro biophysical and cell biological assays, we provide evidence that AP site damage and binding of APE1 to PQS promotes G4 formation and facilitates transcription factors loading to regulate gene expression. Overall, our study comprehensively elucidates the role of endogenous base damage and the BER pathway in controlling the formation of G4 structures in the genome to regulate transcription and other biological processes.

3.2 **Materials and methods**

Cell culture, plasmids, reagents: The human lung epithelial adenocarcinoma cell line A549 (ATCC # CCL-185), human lung fibroblast cells IMR-90 (ATCC #CCL-186), human embryonic kidney HEK-293T (ATCC # CRL-3216), human pancreatic ductal adenocarcinoma PANC-1 (ATCC #CRL-1469) and wild type and Ogg1-null mouse embryonic fibroblast (MEF) cells (kindly provided by Dr. Istvan Boldogh, University of Texas Medical Branch, Galveston), were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Thermo Fisher Scientific). Human Colon cancer HCT116 (ATCC #CCL-247) was grown in McCoy's 5A medium (Thermo Fisher Scientific). All media were supplemented with 10% fetal calf serum (FCS; Sigma) and an antibiotic mixture of 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco-BRL). All cell lines were authenticated by STR DNA profiling by Genetica DNA laboratories, Burlington, NC. For APE1 knockdown studies, HCT116 cells stably expressing APE1-shRNA or control shRNA (83) were maintained in 10% FBS supplemented MaCoyx 5A medium with 1ug/ml puroMycin. Transient APE1 knockdown was achieved using APE1siRNA (sigma) transfected to indicated cell lines for 72 hours. To generate the Doxycycline-inducible APE1 shRNA or control shRNA expressing stable A549, HCT116, HEK-293T, PANC-1 cells, three different SMARTvector doxycycline inducible Human APE1-shRNA lentiviral constructs (shRNA #V3IHSHEG_5634292/6377584/7228555 named as 1/2/3 respectively; Dharmacon) or non-targeting control shRNA construct with GFP were transfected individually into HEK-293T with packaging plasmids using x-tremeGENE HP DNA transfection reagent (Sigma) to generate lentiviral supernatants and individual supernatants were transduced into above-mentioned cell lines and selected with 1 ug/ml puromycin. All doxycycline-inducible APE1-shRNA stable cell lines were maintained in respective media supplemented with tetracycline free 10%

Fetal Bovine Serum (Atlanta biologicals). For rescue experiments, HEK-293T ^{APE1shRNA} / HCTT16^{APE1shRNA} cells were treated with Doxycycline (2 ug/ mL) for 3 days to knockdown the APE1 levels and then cells were transfected with FLAG-tagged WT APE1, or acetylation defective K5R (Lysine 6,7,27,31,32 to arginine) or repair defective H309A or redox defective C65,99S mutants of APE1 using Lipofectamine 3000 (Invitrogen) in serum-free Opti-MEM media (Thermo Fisher Scientific) and cell were fixed after 24 hrs of transfection. Expression plasmid Adenovirus EIA12S (86) were transfected using Lipofectamine 3000 (Invitrogen) and harvested after 48 hrs. Methoxyamine, Glucose oxidase, hydrogen peroxide, Methyl Methanesulfonate, ActinoMycin D, Aldehyde Reactive Probe (ARP) were obtained from Sigma.

Immunofluorescence analysis: Cells were cultured on coverslips, fixed for 30 min in 4% formaldehyde (Sigma) and then permeabilized and blocked in PBS with 0.5% Triton X-100 (Sigma), 10 % goat serum (Thermo-Fisher #50062Z), glycine, sodium azide for 1 hour at room temperature. Cells were then incubated with primary antibodies in blocking buffer overnight at 4°C followed by 1 h further incubation with the corresponding secondary antibodies Alexa Fluor 488 anti-mouse IgG (Life Technologies, 1:500) or Alexa Fluor 594 anti-rabbit IgG (Life Technologies; 1:500) at room temperature (RT) in blocking buffer. Cells were then washed in PBS and mounted using mounting media with DAPI (Vector Laboratories –Item # VV- 93952-27) for confocal microscopy and super-resolution structured illumination microscopy (SIM). For G quadruplex staining, after formaldehyde fixation, cells were permeabilized in PBS containing 0.5% Tween 20 for 20 mins at 37° incubator, then treated with 20 µg/ 500 µL RNase A (Invitrogen) and subsequently blocked and processed as mentioned previously (128). For DNase experiments; cells were fixed in 4% PFA in PBS for 30 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 1 min and washed in PBS. They were

then incubated for 2 hrs at 37 °C in 40 mM Tris-Cl (pH 8), 5 mM $CaCl_2$, 2 mM $MgCl_2$, 100 ug/ml BSA alone or including 0.06 U/ul of DNase I (RQ1 DNase, Promega), washed in PBS and then stained with antibodies. Three- dimensional (3D) SIM images were collected with an ELYRA PS.1 illumination system (Carl Zeiss). All images were analyzed using the ImageJ software. The Pearson correlation coefficients were calculated using the JACoP co-localization analysis module of the ImageJ software. To quantify the level of co-localization, a threshold was first established using the inbuilt JACoP threshold optimizer followed by calculation of correlation coefficients. Primary antibodies used for immunofluorescence studies were mouse monoclonal anti-APE1 (1:100; Novus Biologicals; # NB100-116), anti-AcAPE1 (1:50; (55)), Anti-AcOGG1 (1:50; (61)),Anti-G4-1H6 (1:50; Millipore), Anti-ECD (1:100;(129)) Anti-H3K27 acetylated histone (1:100; Millipore; #05-1334), Anti- H3K4Me3 (1:100; Millipore) and anti-c-Jun (1:100;Abcam).

Chromatin Immunoprecipitation (ChIP) Analysis: Cells were plated in 150 mm culture dish for overnight. Next day, cells were subjected to 1% formaldehyde crosslinking (15 min incubation at room temperature) in PBS. The crosslinked cells were washed thrice in PBS and scraped off the cells in PBS (containing PI), pelleted at 1000 rpm (10 min, 4 ºC). The pelleted cells were lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8), incubated on ice for 10 min and subjected to sonication (Misonix sonicator 3000) on ice by setting the pulse at 4 min for 4 times with 15 seconds interval in between, followed by centrifugation (14000 rpm, 15 min, 4ºC) to collect the sonicated clear sheared chromatin lysate. IP was done in this lysate with corresponding antibody (5 µg; control IgG included in a separate IP) in a total volume of 2 ml (diluted 1:10 with ChIP dilution buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl) overnight (4ºC) with constant shaking. Next day, 25 ul

dynabeads were added to each reaction and incubated in 4°C shaker for 2 hours. The IPs were washed sequentially with low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl), high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 500 mM NaCl), LiCl immune complex Wash Buffer (0.25 M LiCl, 1% NP40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8) and TE Buffer (10 mM Tris-HCl, 1 mM EDTA pH 8). The protein-DNA complexes were eluted in ChIP elution buffer (1% SDS, 0.1 M $NaHCO_3$), de-crosslinked in 200 mM NaCl for overnight at 65ºC. ChIP DNA was purified by RNAse treatment, proteinase K digestion, phenol-chloroform extraction, and precipitation by 100% ethanol precipitation using a standard protocol. The ChIP-purified DNA was finally dissolved in ultrapure water. For damage AP-seq, 1 mM biotin-conjugated ARP was fed to cells for 3 hours, and direct streptavidin pull-down was performed on sonicated lysate followed by other steps as described above. The ChIP and 1% input DNA were subjected to SYBR GREEN-based Real-Time PCR (7500 Real-Time PCR System; Applied Biosystems) with primers (Table below). Data were represented as % input calculation ((2^(adjusted input-ChIP CT))*100).Immunoprecipitation (IP) was performed with antibodies: rabbit α-APE1, rabbit α-AcAPE1 (55), rabbit α-AcOGG1 (61), mouse α-BG4 (Absolute biology), mouse α -MAZ (Santa Cruz), or control IgG (Santa Cruz) and Dynabeads Protein A/G Magnetic beads (Millipore, # 16-661).

Primer name	Forward sequence	Reverse sequence
KRAS G4	GTACGCCCGTCTGAAGAAGA	GAGCACACCGATGAGTTCGG
Neg G4	CTCCGACTCTCAGGCTCAAG	CAGCACTTTGGGAGGCTTAG
MYC	CAGGCAGACACATCTCAGGG	CGTATACTTGGAGAGCGCGT

P21	CAGGCTGTGGCTCTGATTGG	TTCAGAGTAACAGGCTAAGG

ChIP-Sequencing analysis: The ChIP-purified DNA was provided to University of Nebraska Epigenomics Core. ChIP DNA was quantified by Qubit (Invitrogen), and then New England Biolabs NEB Next Ultra II DNA Library Prep Kit for Illumina was used to create library. High-throughput sequencing was conducted at the UNMC Sequencing Core, using an Illumina HiSeq 2500 Genome Analyzer. Adaptor sequences and low quality (Phred score < 20) ends were trimmed from sequences using Trim Galore software package (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Resulting fastq files were aligned to the human genome (GRCh37/hg19) using the sequence aligner Bowtie2 (version 2.2.3) (130). The software package Picard routine Mark Duplicates (http://broadinstitute.github.io/picard/) was used to remove sequence duplications. 20 million reads per sample were then analyzed using both MACS2 peak caller (131) software (version 2.1.1) and Homer. Enrichment of DNA sequence with specific Ab over IgG ($p<.001$; fold change >2; reads>15 contributing to each peaks) was considered significant. Both analysis programs scored read-density across genomic DNA and define peak regions where a bimodal enrichment of reads has occurred. All bigWig files were generated using the deeptools bamCoverage routine (https://deeptools.readthedocs.io/en/develop/) were uploaded and displayed on the UCSC Genome browser with a cut off at 5 reads. SeqMonk was used to analyze distribution with respect to TSS. Graph-pad Prism was used to calculate correlation coefficient between BG4 and APE1 or AcAPE1 promoter binding peaks.

Western blotting: Western blotting was performed using standard methods, as described previously ((56)). Primary antibodies used in the study includes mouse

monoclonal anti-APE1 (1:5000; Novus Biologicals; # NB100-116), mouse anti-HSC70 (1:10000; Santa cruz), Anti-ECD (1:1000;(129)) , beta-actin (1:1000).

RNA Seq and quantitative RT PCR: Doxycycline inducible A549^{APE1shRNA} cells were treated with Doxycycline (2 ug/ mL) in triplicate for 3 days to knockdown the APE1 levels. Total RNA was purified using a Qiagen kit (#74104). Total RNA was subjected to Illumina Hi-seq. For q RT-PCR, total RNA was isolated from cells using TRIZOL method. cDNA synthesis of 1 ug of total RNA was performed with the MulV RT kit (Invitrogen) using random hexamer primers. For quantitative Real-Time PCR (qRT-PCR) analyses, 1/50th of each reaction was used. RT-PCR analysis was performed using SYBR green (Applied Biosystems) for detection with an Applied Biosystems StepOne Plus system. Fold change was calculated by specific gene $2^{\wedge\text{-(target-reference)}}$/*GAPDH* $2^{\wedge\text{-(target-reference)}}$. The following gene-specific primers were used,

Primer name	Forward sequence	Reverse sequence
KRAS	TCTTGCCTCCCTACCTTCCACAT	CTGTCAGATTCTCTTGAGCCCTG
GAPDH	TGGGCTACACTGGAGCACCAG	GGGTGTCGCTGTTGAAGTCA

Site-directed mutagenesis and Luciferase assay: The Del4 luciferase reporter plasmid, harboring the 22-mer *c-MYC* G4 forming sequence in the P1 promoter upstream of the luciferase reporter (Addgene plasmid # 16604) was mutated using the QuickChange Site-directed Mutagenesis kit (Agilent Technologies, CA, USA) to generate a mutated G4-forming sequence.

- Del4: 5′-G4AG3TG4AG3TG4-3′

- G12A: 5′-G4AG3TGAG2AG3TG4-3′ (Kindly provided by Dr. Jyoti Dash; Indian Association for the cultivation of science; India,(132))
- G18A: 5′-G4AG3TG4AGAGTG4-3′

pRL -TK renilla luciferase (Addgene plasmid #E2241) control reporter vector was used for measuring transfection efficiency and as a non-G4 sequence control. 1 ug Del4 plasmid with 100 ng of pRL -TK plasmid was transfected to cells plated on a six-well plate using Lipofectamine 3000 as per manufacture's protocol. After 48 hours cells were lysed and luciferase assay was performed using Dual-luciferase reporter assay (Promega) according to manufacturer protocol in Glow-max (Promega) system.

Preparation of G4 DNA-containing templates: Synthetic single-stranded DNA templates and complimentary strands were purchased from Midland-certified reagents. The oligomer sequences are listed in Table. Synthetic double-stranded DNA templates were generated by incubating 10 uM of each strand in a final volume of 100 µL of annealing buffer for 5 min at 95°C, followed by slow cooling down from 95 °C to 37 °C. To induce G4 DNA formation, the annealing reaction was carried out in the presence of 100 mM KCl. After the annealing step, samples were stored at −20°C.

Oligo name	Sequence
MYC-AP 75 mer template strand	(Cy5)ATAAGCTTCCCGGGGTCGACCACGTCTGGGGAGGGTG(abasic) GGAGGGTGGGG AAGGTCTAGATCTGGTACCGAATTCT
Complimentary strand	TATTCGAAGGGCCCCAGCTGGTGCAGACCCCTCCCACTCCTCCCAC CCCTTCCAGATCTAG ACCATGGCTTAAGA

CD spectroscopy: Circular Dichroism (CD) spectra were obtained on a JASCO J-810 spectropolarimeter, equipped with a thermostated cell holder, with 1 μM oligonucleotides solutions in 50 mM HEPES pH 7.5, 10 mM NaCl, 10 mM MgCl2, 5% glycerol, 1 mM DTT, 0.1 mg/ml BSA. The spectra were recorded in 10 mm quartz cuvette at 20 °C. Scans were performed at 20 °C over a wavelength range of 210-330 nm with a response time of 0.5 s, 1 nm pitch and 1 nm bandwidth. Blank spectra of samples containing buffer were subtracted from DNA samples. The spectra are reported as ellipticity (mdeg) versus wavelength (nm). Each spectrum was recorded five times, smoothed and subtracted to the baseline.

EMSA: A 75-mer oligonucleotide containing the G4-forming sequence and an AP site analog, tetrahydrofuran at position 12 of the c-Myc NHEIII1 was labeled at the 5' end with cy5 and annealed to a complementary strand as described above. This substrate (30 nM) was mixed at with increasing dose of APE1 (6.4, 64 and 640 ng) at room temperature in electrophoretic mobility shift assay buffer containing 50 mM HEPES pH 7.5, 10 mM NaCl, 10 mM MgCl2, 5% glycerol, 1 mM DTT, 1 mM EDTA, 0.1 mg/ml BSA. One ug of poly-deoxy-inosinic-deoxy-cytidylic acid (Poly[dI-dC]) was used in each reaction as non-specific competitor. Aliquots of the binding mixtures were chilled in ice after incubation for 10 min at room temperature. The protein-bound DNA was separated from the free substrate by electrophoresis on a 10% non-denaturing polyacrylamide gel at 100 V/cm2 for 60 min.

Fluorescence Recovery After Photobleaching (FRAP): A small region in the nucleus of a cell was photobleached with three iterations at 100% laser intensity, and subsequently recovery of fluorescence was monitored 200 times every 20 ms at 1% laser intensity (25 mW Argon laser, 514 nm line) to allow the measured fluorescence to

reach a steady state level. The shown FRAP data were corrected for background noise and normalized to pre-bleach values.

Apurinic/apyrimidinic (AP) endonuclease activity assay: A 75-mer oligonucleotide containing AP site analog tetrahydrofuran (THF) at nucleotide 37 (Midland Corp) was 5′-end-labeled with Cy5. Labeled oligo was annealed to the complementary strand with G residue opposite THF. This THF-containing duplex oligomer was incubated with recombinant WT-APE1 or recombinant AcAPE1, in a 15 µL reaction mixture containing 50 mM Tris-HCl pH 8.5, 50 mM KCl, 1mM $MgCl_{2,}$ 1 mM DTT, 0.1 mM EDTA and 100 µg/ mL bovine serum albumin at 37 º C for increasing time points during which the reaction rate was linear as described earlier(104, 133). The reaction was stopped with 10 µL 80% formamide/40 mM NaOH containing 0.05% xylene cyanol, followed by heating at 95 °C for 5 min. The samples were run in a 20% polyacrylamide containing 8M urea denaturing gel electrophoresis to separate the substrate oligomer from the cleaved product. The gel was visualized in odyssey Li-cor machine.

3.3 **Results**

Genome-wide mapping of endogenous AP site damage and binding of repair proteins.

We set out to elucidate the relationship between endogenous base damage and formation of G4 structures in the genome. We first mapped the genome-wide occurrence of AP sites, the most prevalent type of endogenous DNA damage in cells, which is generated spontaneously or after cleavage of modified bases including oxidative or alkylated G in the BER pathway (134, 135). We developed a technique to map genome-wide AP site damage (AP-seq; **Fig. 1A**) at approximately 300 bp resolution in the genome.

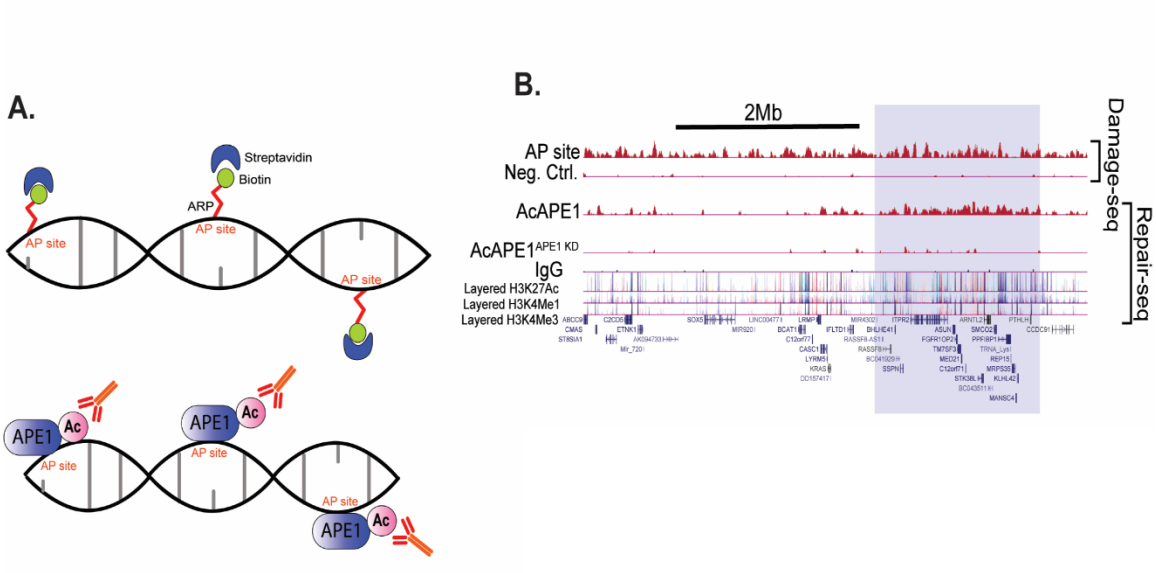
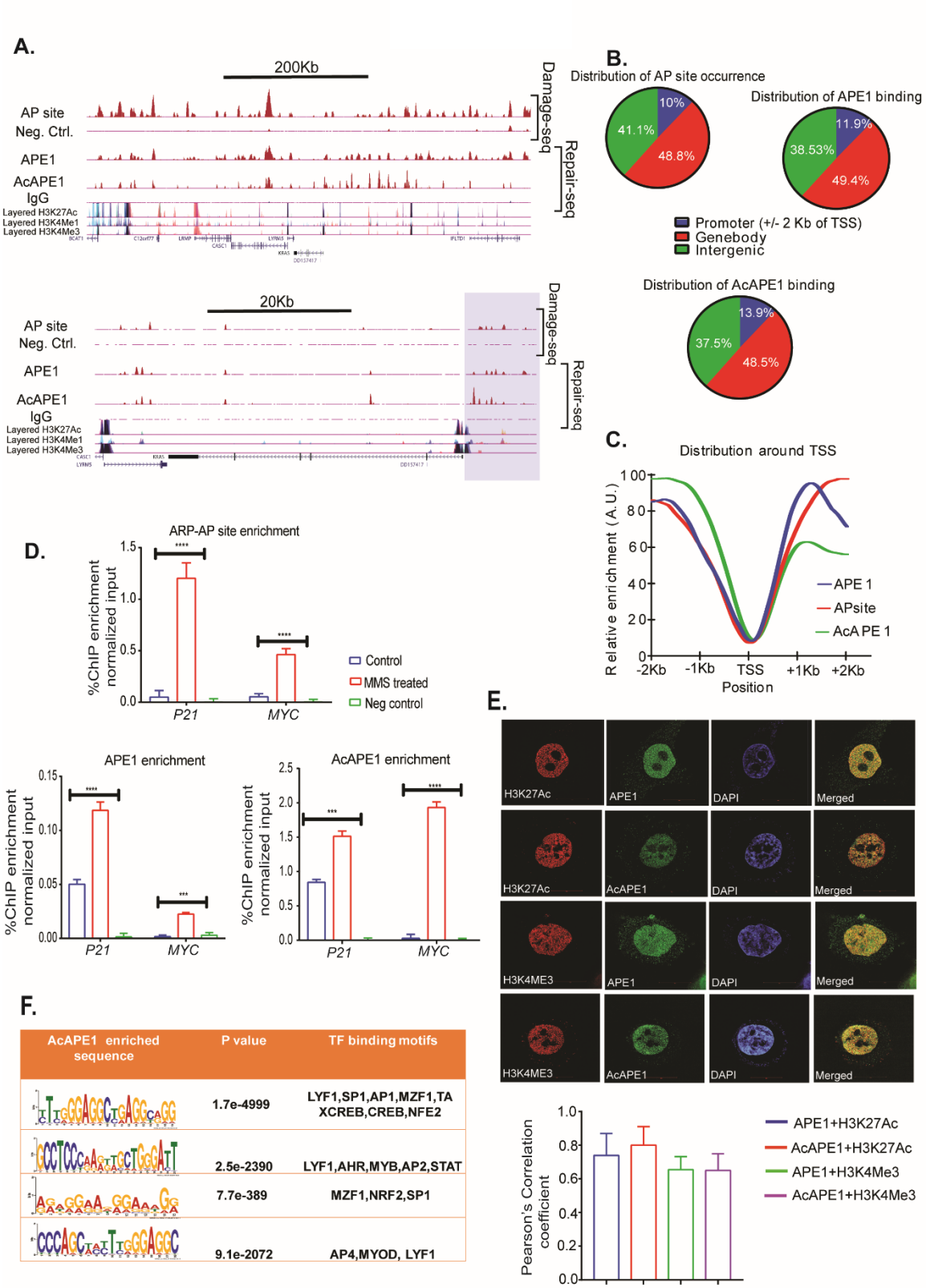


Figure 1: Genome-wide mapping of endogenous AP site damage by AP-Seq and binding of repair protein APE1 and AcAPE1. A) Top, schematic of AP-seq, designed to detect apurinic-sites (AP-sites) in the genome. AP-sites are captured using a biotin-tagged aldehyde reactive probe (ARP) which specifically recognizes AP-sites (100, 136) and pulled down with streptavidin. The enriched DNA is processed for sequencing and mapped to reference genome. The damage level across genome is quantified by analyzing the number of mapped reads. Bottom, schematic of Repair-seq, strategy to map genome-wide binding enrichment of APE1 and AcAPE1 by ChIP-seq. **B)** Representative region of chromosome 8 showing AP site occurrence and AcAPE1 binding profiles compared to IgG in control HCTT16 and isogenic HCT116 expressing APE1 shRNA (APE1-KD) cells. The purple box highlights the disappearance of AcAPE1 peaks in APE1 KD cells.

We found a reproducible occurrence of AP site damage in 21,228 and 22,314 regions ($p<0.0001$) in the genome of lung adenocarcinoma A549 and colon carcinoma HCT116 cells respectively (**Fig. 2A and 1B**). Furthermore, to examine whether APE1, the primary enzyme responsible for repairing the AP sites, binds to these regions, we also mapped genome-wide occupancy of APE1 and Acetylated APE1 (APE1 is acetylated (AcAPE1) at AP site damage in chromatin (137), by ChIP-Sequencing (repair-seq; **Fig. 1A**) analysis in A549 and HCT116 cells, using α-APE1, α-AcAPE1 antibodies (Abs). Multiple ChIP-Seq data analyses revealed ~ 24894 and 25904, ($p<0.0001$) APE1 and AcAPE1-enriched peaks respectively (**Fig. 2A and 1B**). The disappearance of AcAPE1 binding peaks in HCT116 cells expressing APE1-specific shRNA compared to isogenic wild type (WT) HCT116 cells (**Fig. 1B**), confirms the specificity of APE1 binding. A significant ($p<0.0001$) overlap between AcAPE1 binding peaks (repair-seq) and AP-seq in multiple independent biological replicates in several cell lines suggest that generation of these endogenous DNA damages are non-random, predominantly occurring in specific regions and are primarily repaired by APE1-mediated BER pathway. Analysis of endogenous AP site damage and AcAPE1 binding distribution relative to annotated genomic features revealed predominant occurrence (~ 60%) in the transcribed gene regions (exon and intron) and gene promoter regions (2000 bp upstream (-) and downstream (+) of the transcription start site (TSS)) (**Fig. 2B**). Interestingly, although the promoter regions represent only a tiny fraction of the human genome, we observed a significant fraction (~ 10%) of AP site damage and repair protein binding, suggesting predominant occurrence of AP site and APE1-binding in promoter regions relative to gene body. The occurrence of AP site damages and binding of APE1 were significantly higher in both upstream and downstream regions compared to TSS which have the lowest AP site damage-repair enrichment (**Fig. 2C**). We further validated the formation

of AP sites and binding of APE1 and AcAPE1 to *MYC* and *P21* gene promoters with or without induction of damage using Real-Time ChIP-PCR analysis (**Fig. 2D**). Consistent with our ChIP-seq data, super-resolution (110 nm) structured illumination microscopy (SIM) revealed that AcAPE1 localizes to specific regions in the genome that bear the active enhancer marker H3K27ac and active promoter marker H3K4me3 (**Fig. 2E**). Collectively, these results demonstrate that endogenous base damages are not randomly distributed in the genome but are predominant at defined gene regulatory (promoter/enhancer) regions.



AcAPE1 enriched sequence	P value	TF binding motifs
	1.7e-4999	LYF1,SP1,AP1,MZF1,TA XCREB,CREB,NFE2
	2.5e-2390	LYF1,AHR,MYB,AP2,STAT
	7.7e-389	MZF1,NRF2,SP1
	9.1e-2072	AP4,MYOD, LYF1

Figure 2: Genome-wide mapping of endogenous AP site damage and binding of repair proteins A) Representative region of chromosome 12 showing the distribution of AP site damages and binding profiles of APE1 and AcAPE1 in A549 cells with increasing resolution, zooming in on KRAS gene. ARP untreated cells (Neg. Ctrl.) or IgG were used as controls. Layered H3K27Ac, H3K4Me1 (active enhancer), and H3K4Me3 (active promoter) marks on seven cell lines from ENCODE shown in the bottom panel. The purple box highlights the promoter region with co-occurrence of AP site damage and repair protein APE1 and AcAPE1 binding. **B)** Gross genome-wide AP site occurrence, APE1 and AcAPE1 binding is shown in the context of the promoter, gene-body, and intergenic regions in A549 cell. **C)** Metprofiles of ~ 7500 protein coding genes showing relative enrichment of AP site occurrence, APE1 and AcAPE1 binding distribution within -/+ 2000 bp with respect to transcription start site (TSS). **D)** Validation of ChIP-seq data for binding of APE1 AcAPE1 and ARP on *P21* and *MYC* genes promoter regions in A549 cells, by Real Time ChIP-PCR analysis. Methyl methane sulfonate (MMS), an AP site damage-inducing agent, was used as a positive control. ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, $^{ns}p>0.05$, calculated using unpaired t test. Error bars denote ±SD**. E)** 3-Dimensional Structured Illumination Microscopy (SIM) images show co-localization of APE1 or AcAPE1 with H3K27ac (active enhancer) and H3K4Me3 (active promoter) in A549 cells; counterstained with DAPI. Pearson coefficient was calculated (n=10 cells) as an indicator of colocalization frequency. **F)** AcAPE1-bound promoter sequences were analyzed for finding de novo transcription factor (TF) binding motifs. Highly enriched motifs are shown with corresponding TF binding site.

Genome-wide mapping of occurrence of oxidative base damage and G4 structure formation.

Analysis of promoter sequences occupied by AcAPE1 showed enrichment of G-rich sequence motifs containing putative binding site of many transcription factors (TFs) (**Fig. 2F**). Since G residues in PQS are susceptible for oxidation by endogenous oxidants and 8-oxoG is primarily removed by OGG1(123, 138), we mapped genome-wide binding of OGG1 using our previously generated acetylated OGG1 (AcOGG1) antibody (61) (**Fig. 3A**). AcOGG1 peaks are predominantly localized to gene promoters and overlap with APE1 and AcAPE1 peaks (**Fig. 3A**). This raises the possibility that formation of 8-oxoG in these regions recruits OGG1 which cleaves 8-oxoG, generating an AP site, and recruits downstream BER enzyme APE1. With the help of QGRS (a web-based server for G quadruplex prediction) mapper(139), we found 67% of AcAPE1 bound promoter sequences to have a PQS score higher than 40 (PQS score >20 is considered as significant). To examine the relationship between AP site damage, binding of repair proteins and G4 formation, we mapped genome-wide occurrence of G4 structures using an antibody generated against G4 structure (BG4)(120, 140). We found that G4 structures are enriched in the promoter, 5' UTR and gene body of transcribed gene regions as observed earlier (113). Intriguingly, we found a significant ($p<0.0001$) genome-wide association between AP site damage, AcAPE1, AcOGG1 occupancy and G4 structures formation across the whole genome including many promoter regions that are known to form G4 (**Fig. 3B and 3C**). Overall, high Pearson correlation ($r=0.64$ and $r=0.69$) was observed between G4 formation and APE1 or AcAPE1 across the genome (**Fig. 3C**), particularly across +/- 2000 bp of TSS (**Fig. 3D**). Previously, QuadParser analysis showed that the frequency of PQS are not equal between chromosomes as some chromosomes are highly enriched with PQS irrespective of their sizes (141).

Consistent with this, our chromosome-wise analysis reveals a positive correlation between theoretically predicted PQS or experimentally observed G4s, and AcAPE1 binding peaks (**Fig. 3E**). High abundance of G4 structures in the promoter regions throughout the genome and a significant overlap between G4 structures, AcOGG1 and AcAPE1 in gene promoters (**Fig. 3F**) highlight a strong relationship between endogenous oxidative base damage, associated repair complex with the formation of G4 structures and transcription. Many have reasoned gene promoters experience negative super-helicity resulting from active transcription and that is sufficient to convert the duplex DNA to a G-quadruplex on the purine-rich strand (142). If formation of G4 structures and endogenous damage and repair are coupled to active transcriptional status, then inhibiting transcription would cause a concomitant shift in the binding of repair proteins and G4 profiles. Thus, we inhibited transcription by Actinomycin D (143) and performed APE1-seq, AcAPE1-seq, AcOGG1-seq, and G4 ChIP-seq. After inhibition of transcription we found no significant alteration of APE1-seq or G4 ChIP-seq profile, indicating that occurrence of AP sites or G4 is not dependent on active transcription and it is not an indirect consequence of transcription (**Fig. 3G**). However, we found a significant reduction of enrichment of sequences when we used acetylation-specific antibody against AcAPE1 or AcOGG1 indicating acetylation of these proteins, but not their promoter occupancy, is dependent on active transcription **(Fig. 3G)**. We further validated these observations by promoter-directed Real-Time ChIP-PCR analysis using *MYC* and *P21* sequence-specific primers. (**Fig. 3H**). Together, our genome-wide association data provide the first experimental evidence that endogenous oxidative base and/or AP site damage and BER initiating proteins (OGG1 and APE1) are predominantly localized to specific G-rich transcriptional regulatory regions that can form G4 structures,

suggesting a potential link between endogenous damage, activation of BER pathway and G4 formation *in vivo*.

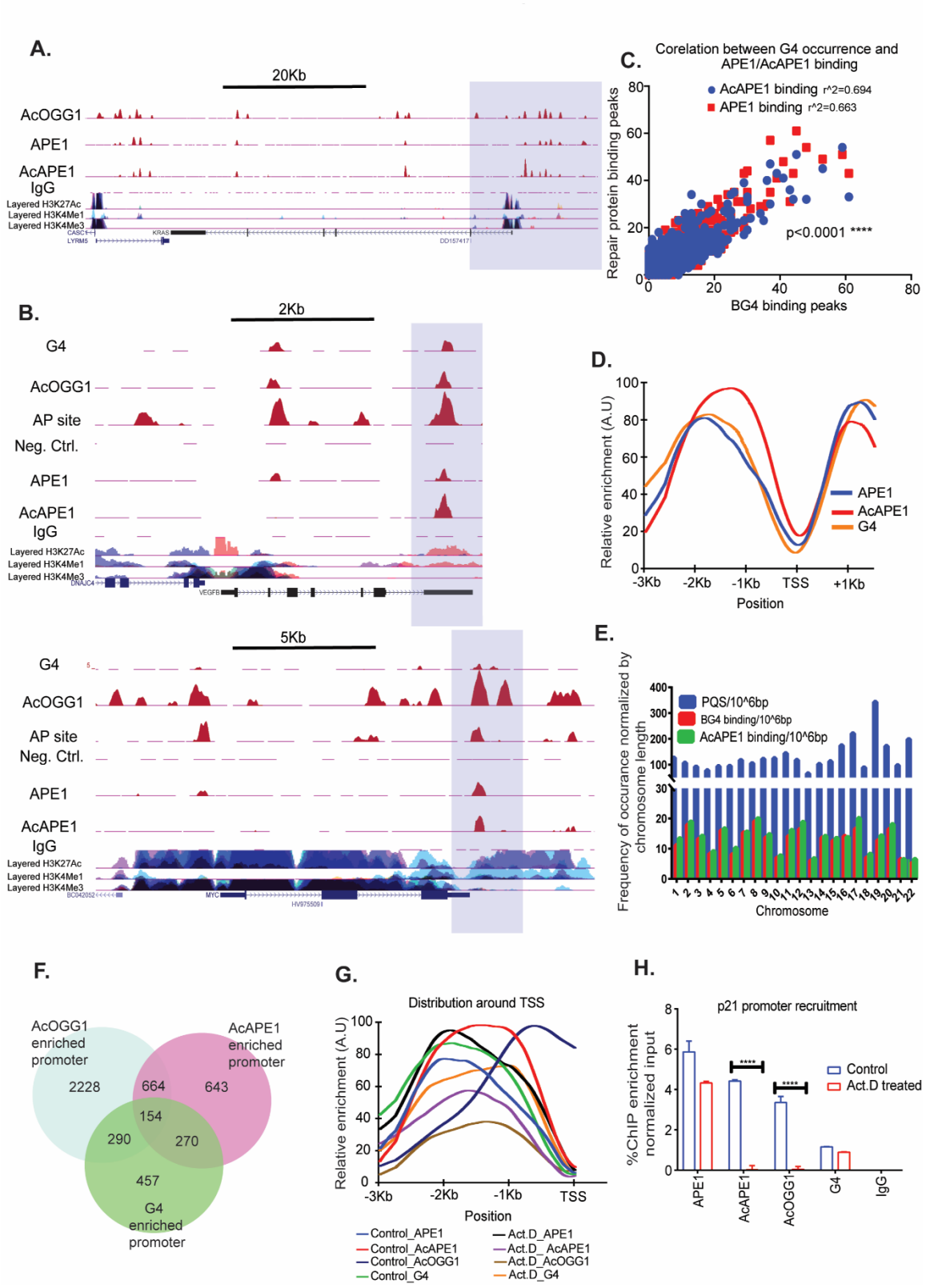

A.
20Kb
AcOGG1
APE1
AcAPE1
IgG
Layered H3K27Ac
Layered H3K4Me1
Layered H3K4Me3
B.
2Kb
G4
AcOGG1
AP site
Neg. Ctrl.
APE1
AcAPE1
IgG
Layered H3K27Ac
Layered H3K4Me1
Layered H3K4Me3
5Kb
G4
AcOGG1
AP site
Neg. Ctrl.
APE1
AcAPE1
IgG
Layered H3K27Ac
Layered H3K4Me1
Layered H3K4Me3
C.
Corelation between G4 occurrence and APE1/AcAPE1 binding
AcAPE1 binding r^2=0.694
APE1 binding r^2=0.663
Repair protein binding peaks
BG4 binding peaks
p<0.0001 ****
D.
Relative enrichment (A.U)
APE1
AcAPE1
G4
-3Kb
-2Kb
-1Kb
TSS
+1Kb
Position
E.
Frequency of occurance normalized by chromosome length
PQS/10^6bp
BG4 binding/10^6bp
AcAPE1 binding/10^6bp
Chromosome
F.
AcOGG1 enriched promoter
AcAPE1 enriched promoter
G4 enriched promoter
2228
664
643
154
290
270
457
G.
Distribution around TSS
Relative enrichment (A.U)
-3Kb
-2Kb
-1Kb
TSS
Position
Control_APE1
Control_AcAPE1
Control_AcOGG1
Control_G4
Act.D_APE1
Act.D_ AcAPE1
Act.D_AcOGG1
Act.D_G4
H.
p21 promoter recruitment
%ChIP enrichment normalized input
Control
Act.D treated
APE1
AcAPE1
AcOGG1
G4
IgG

Figure 3: Genome-wide mapping of occurrence of oxidative base damage and G4 structures. A) ChIP-Seq. analysis showing binding profiles of AcOGG1, APE1, and AcAPE1 in A549 cells. **B)** Overlap of G4 structure, AP site damages and binding of AcOGG1, APE1 and AcAPE1 in MYC, VEGF promoter regions (highlighted) that were previously shown to form G4. **C)** Co-relation analysis was performed for genome-wide enrichment of AcAPE1 and G4 structures. **D)** Metaprofiles of ~ 7500 protein coding genes within +/- 2000 bp of TSS showing relative enrichment of G4, APE1 and AcAPE1 binding sites in A549 cells. **E)** Blue histograms represent predicted numbers of PQS/ 10^6 bp mapped in the GRCH37/hg19 human genome by using QuadParser after normalization of chromosome-size. Red and green histograms represent the observed frequency of occurrence of G4 and AcAPE1 occupancy respectively, on each chromosome by ChIP-Seq analyses. **F)** Venn diagram is representing overlapping p romoters with AcAPE1, AcOGG1 and G4 enrichment ($p<0.001$). **G)** Metaprofiles of ~ 7500 protein coding genes, in upstream of TSS, showing relative enrichment of G4, AcOGG1, APE1 and AcAPE1 upon Actinomycin D (10 ug/ ml for 2 hours) treatment, in A549 cells. **H)** Binding of APE1, AcAPE1, AcOGG1 and occurrence of G4 were validated in *P21* gene promoter upon Actinomycin D treatment by promoter directed ChIP. ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, $^{ns}p>0.05$, calculated using unpaired t test. Error bars denote ±SD.

DNA repair function and acetylation of APE1 play a crucial role in the formation of G4 structures in cells.

Having established a genome-wide correlation between base damage and G4 formation, we asked the question whether APE1 plays any role in regulating the formation and/or stability of G4 structures. G4 structures were visualized in human cells by confocal and SIM microscopy by using G4 DNA-specific antibody 1H6 (128). We found the formation of G4 foci in the genomic DNA of a number of human cell lines including primary lung fibroblast IMR90, A549, HCT116, and Mouse Embryonic Fibroblast (MEF) cells (**Fig. 4A, 5A, and 5B**); formation of G4 foci was sensitive to DNase treatment, but did not change upon RNase A treatment (**Fig.4B**), confirming its specificity in recognizing G4 structures in genomic DNA.

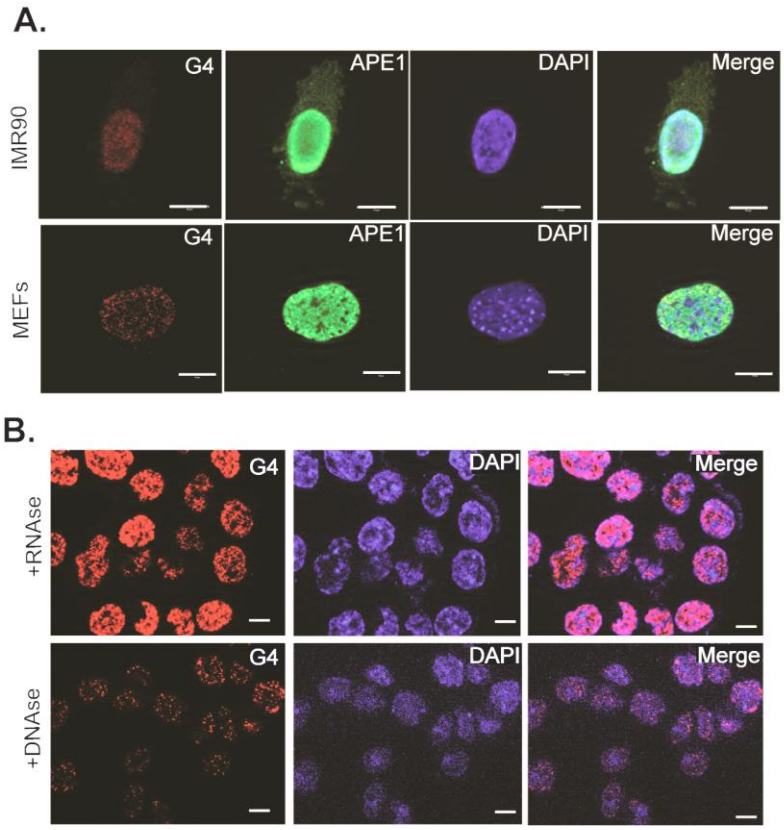
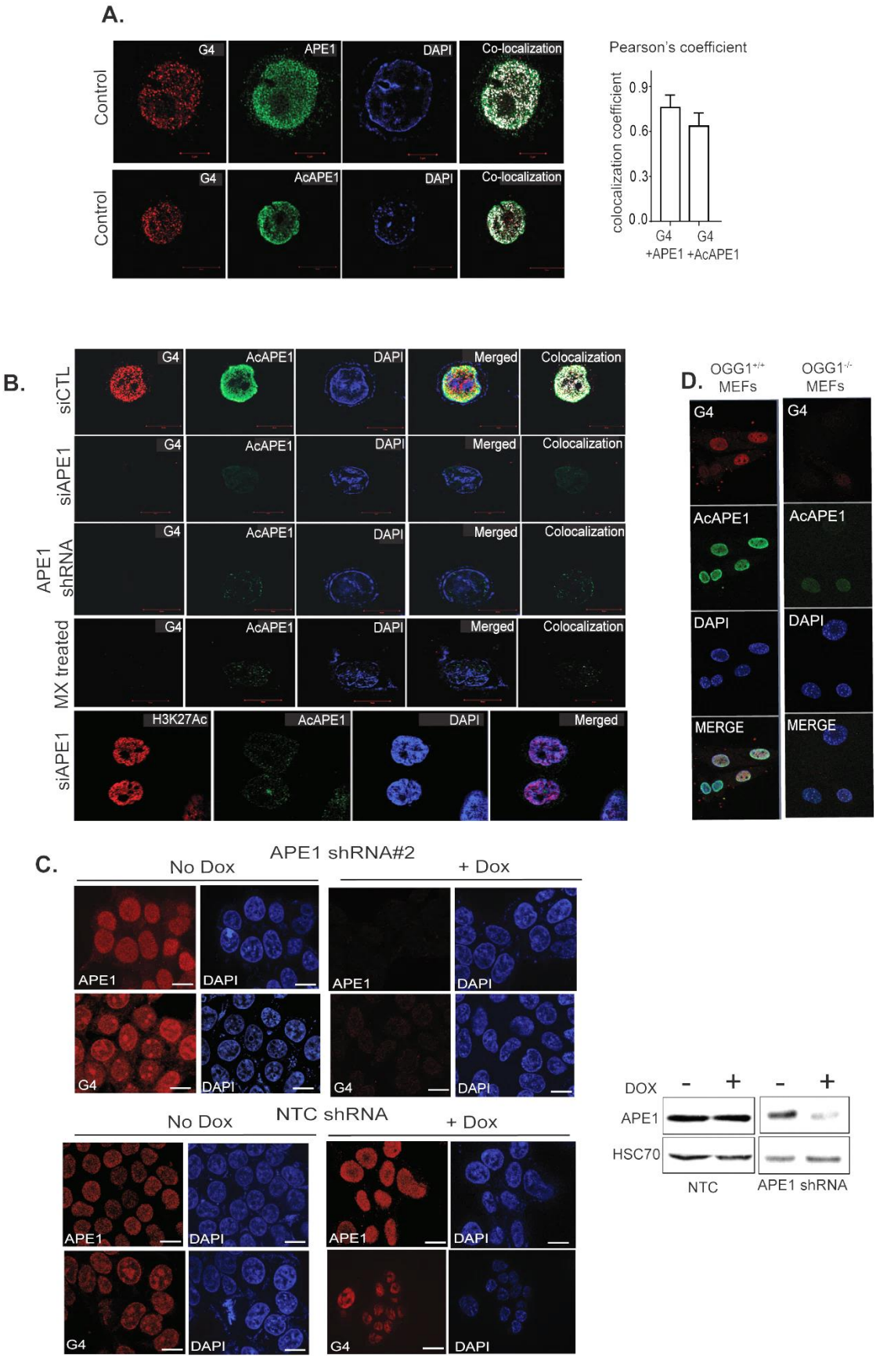


Figure 4: Visualization of G4 structures in cells and specificity of G4 antibody. A) Lung fibroblast cells IMR-90 and mouse embryonic fibroblast (MEF) cells were immunostained with G4 structure-specific antibody α-1H6, and α-APE1 Abs, counterstained with DAPI and visualized by 3D SIM. **B)** A549 cells treated with RNase A or DNase and immunofluorescence (IF) was performed using α-1H6, counterstained with DAPI.

We observed a high colocalization frequency (r=0.78) of G4 structures and APE1 or AcAPE1 staining (**Fig. 5A**). Downregulation of APE1 levels by stable expression of APE1shRNA in HCT116 cells abolished the formation of G4 foci compared to isogenic WT HCT116 cells, indicating the critical importance of APE1 in regulating the formation and/or stabilization of G4 structure in the genome (**Fig. 5B**). We further confirmed this finding by transient downregulation of APE1 level with siRNA (**Fig. 5B)**. We generated HEK293T, HCT116, and A549 stable cell lines expressing two independent APE1shRNA under a Dox-inducible promoter. Downregulation of APE1 levels in these cells with Dox treatment showed a significant reduction in G4 foci formation suggesting the essential role of APE1 in regulating the formation of G4 structures in the genome (**Fig. 5C**). No change in staining of other proteins such as histone H3K27Ac mark further supports that APE1 downregulation specifically reduced G4 staining (**Fig. 5B**). To further examine whether binding of APE1 to AP site damages is essential for the formation of G4 structures, we treated HCT116 cells with methoxyamine (MX), a small molecule which binds to AP sites and competitively inhibits binding of APE1 to AP sites both in vitro and in cells (91). We found that pretreatment of cells with MX significantly inhibited the formation of G4 structures suggesting that binding of APE1 to AP sites may be important for promoting the formation of G4 structures (**Fig. 5B**). To test that whether OGG1 DNA glycosylase which initiates the repair of 8-oxoG for generating an AP site also plays a role in the formation of G4, we compared the G4 staining between WT MEF and $OGG1^{-/-}$ MEF cells (144). Formation of G4 foci significantly decreased in $OGG1^{-/-}$ MEF (**Fig. 5D**). As a non-BER related protein control we used adeno-Cre expressing $ECD^{fl/fl}$ MEFs (145) and found deletion of *ECD* gene by Cre expression did not change G4 foci formation in $ECD^{fl/fl}$ MEFs (data not shown). These data together demonstrate that the absence of either OGG1 or APE1 abolished the formation of G4 in cells, suggesting their

critical role in the G4 formation. Furthermore, staining intensity of G4 foci increased in live cells after exposure to G4 ligand pyridostatin (PDS) (141) which traps G4 structures and stabilizes it in cells (**Fig. 5E**). However, G4-stabilizing ligand PDS had no effect on the staining of G4 foci in same cells when APE1 was downregulated by shRNA or siRNA, suggesting an essential role of APE1 in the formation of G4 structure in cells (**Fig. 5F**).

APE1 is a multifunctional protein with DNA repair function (AP-endonuclease activity) and redox-mediated transcription regulatory (Ref-1) function (40, 72). Furthermore, several of our previous studies had shown that APE1 is acetylated at multiple Lys (Lys6,7, 27, 31 and 32) residues at AP site damages in chromatin, and AcAPE1 modulates both DNA damage repair and expression of genes via functioning as a transcriptional activator or corepressor (55, 56, 62, 67, 83, 137, 146). To elucidate which function of APE1 (DNA repair, redox or acetylation) is important for regulating the G4 formation in cells, we ectopically expressed WT APE1, DNA damage repair defective H309A (147), redox function deficient Cys65/Cys99S (40) and acetylation-defective K5R (acetylabe Lys 6,7, 27,31 and 32 were mutated to arginine) (137) APE1 mutants in Dox-inducible APE1 downregulated cells. Our data show that while expression of WT APE1 or redox mutant Cys65/Cys99 APE1 was able to restore the formation of G4 structures in endogenous APE1-downregulated cells, both DNA repair-defective and acetylation-defective mutants failed to restore the formation of G4s in cells (**Fig. 5G**). Together, these results suggest that presence of DNA glycosylase OGG1 and both AP-endonuclease function and acetylation of APE1 play a crucial role in the formation of G4 structures in the genome in cells.


A.
G4
APE1
DAPI
Co-localization
Control
G4
AcAPE1
DAPI
Co-localization
Control
Pearson's coefficient
colocalization coefficient
0.9
0.6
0.3
0.0
G4
+APE1
G4
+AcAPE1
B.
siCTL
siAPE1
APE1 shRNA
MX treated
siAPE1
G4
AcAPE1
DAPI
Merged
Colocalization
H3K27Ac
D.
OGG1+/+ MEFs
OGG1-/- MEFs
G4
AcAPE1
DAPI
MERGE
C.
APE1 shRNA#2
No Dox
+ Dox
APE1
DAPI
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HSC70
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APE1 shRNA

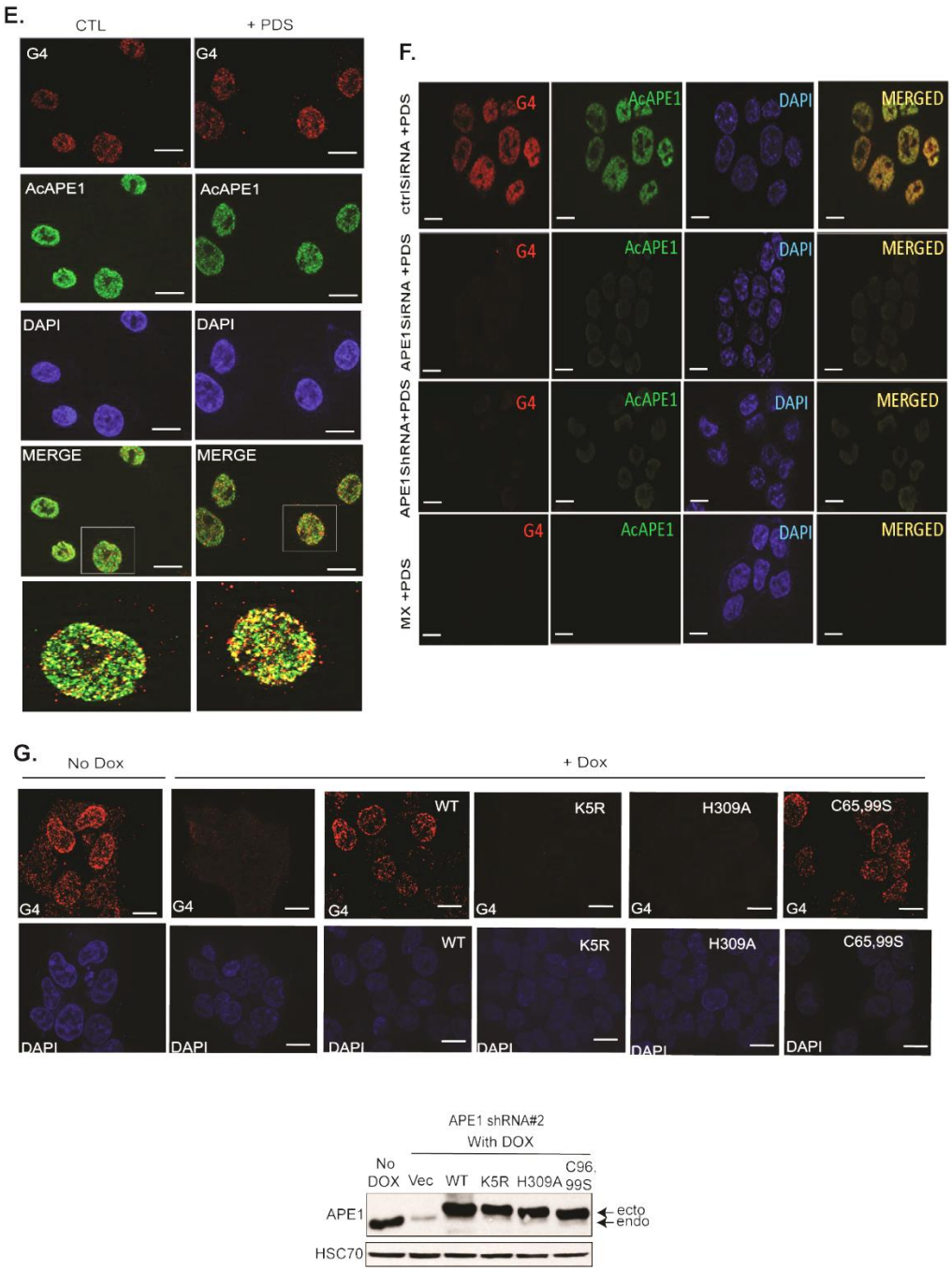

Figure 5: DNA repair function and acetylation of APE1 play a crucial in the formation of stable G4 structures in the genome. A) Lung adenocarcinoma A549 cells were immunostained with G4 structure-specific antibody α-1H6, and α-APE1 or α-AcAPE1 Abs, counterstained with DAPI and visualized by 3D SIM. Pearson coefficient

was calculated (n=10 cells) as an indicator of colocalization frequency. **B)** HCT116 cells transiently transfected with APE1siRNA or HCT116 cells constitutively expressing APE1 shRNA or cells treated with Methoxyamine (MX, 50 mM) for 30 mins were immunostained α-1H6 and α-AcAPE1. HCT116^{siAPE1} cells were immunostained with α-H3K27Ac and α-AcAPE1 as a control. **C)** HEK293T cells expressing a Doxycycline-inducible non-targeting shRNA control (NTCshRNA) and APE1-specific shRNA (APE1shRNA) were treated with Doxycycline (2 ug/ mL) for 2 days and immunostained with α-1H6 and α-AcAPE1. The level of APE1 in these cell extracts was examined by Western blot analysis with α-APE1 and α-HSC70 (as loading control). **D)** Mouse embryonic fibroblast cells established from OGG1 null-mice, OGG1$^{-/-}$ MEF and OGG1$^{+/+}$ MEF were immunostained with α-1H6 and α-AcAPE1 and visualized by confocal microscopy. **E)** HCT116 cells were pretreated with 1 uM of pyridostatin (PDS) for 1 hrs and immunostained with α-1H6 and α-AcAPE1 and visualized by confocal microscopy. **F)** HCT116 cells transfected with control siRNA (siCTL), APE1-specific RNA (siAPE1), HCT116 cells constitutively expressing APE1 ShRNA (APE1shAPE1) and HCT116 cells pretreated with MX (50 mM) 30 mins were subjected to PDS treatment for 1 hour and immunostained with α-1H6 and α-AcAPE1 and visualized by confocal microscopy. **G)** HEK-293T cells expressing APE1shRNA were treated with Doxycycline (2 ug/ mL) for 2 days to knockdown the APE1 levels and then cells were transfected with FLAG-tagged WT APE1, or acetylation defective K5R (Lysine 6,7,27,31,32 mutated to arginine) or repair defective H309A or redox defective C65,99S mutants of APE1 for 24 hrs and immunostained with α-1H6 and α-AcAPE1 and visualized by confocal microscopy. The level of APE1 in these cell extracts was examined by Western blot analysis with α-APE1 and α-HSC70 (as loading control).

APE1 modulates G4-mediated expression of genes

To compare gene expression profile between control and Dox-inducible APE1 knock-down (APE1KD) A549 cells, we performed RNA-seq and focused on differentially expressed (>2 -fold change) genes. We found that 31% of genes that are differentially expressed in the absence of APE1 have both AcAPE1 binding and G4 structure enrichment. Formation of G4 structure in the promoter region of many proto-oncogenes such as MYC, KRAS, BCL-2 and its role in modulating their expression had been well characterized in multiple studies (77, 148, 149). We found all these oncogene promoters have endogenous AP site damage and AcAPE1 occupancy in A549 and HCT116 cells (**Fig. 6A**). Thus, to understand the role of APE1 in G4-mediated gene transcription, we took two oncogenes KRAS and MYC as a model in our study. We found significant enrichment of APE1 or AcAPE1 and G4 in previously reported KRAS G4 promoter region (148), but not in control non-G4 sequence region, by ChIP-qPCR with APE1 or AcAPE1 and BG4 antibodies (**Fig. 6B**). Further, to establish whether APE1 is involved in G4 folding in KRAS promoter, we performed ChIP-qPCR using α-BG4 in WT and APE1KD cells. Quantitative PCR amplification revealed that loss of APE1 affects G4 enrichment on KRAS promoter regions compared to negative control regions (**Fig. 6C and 6D**). Moreover, inhibition of APE1 acetylation by adenovirus E1A 12S (E1A inhibits HAT function of p300, the acetyltransferase responsible for APE1 acetylation) (137) protein overexpression also abrogated G4 enrichment in KRAS promoter (data not shown) **,** indicating that APE1 and its acetylation is important for G4 folding. A recent study has shown that specific G oxidation in PQS of KRAS promoter regulates KRAS expression (148). Further, it was demonstrated that G4 sequence in KRAS promoter facilitates the loading of several transcription factors including MAZ to the promoter region (148). MAZ activates the transcription of KRAS through binding to (5′-

GGG(A/C)GG) sites at the 5′ and 3′ ends of KRAS-G4 sequences. We examined the role of AcAPE1 in the recruitment and binding of MAZ to the KRAS-G4 promoter. ChIP-qPCR analysis in control or APE1KD cells showed decreased MAZ occupancy on KRAS-G4 promoter in the absence of APE1 (**Fig. 6C and 6D**). Furthermore, oxidative damage induction by Glucose Oxidase (GO) increased enrichment of G4, APE1 and MAZ occupancy on KRAS-G4 promoter in WT cells but not in APE1KD HCTT16 cells **(Fig. 6E**). To understand the role of oxidative damage and active BER pathway in G4-mediated KRAS gene transcription, we did gene expression analysis by Real-time PCR under various conditions. We found induction of oxidative damage by glucose oxidase treatment increases KRAS gene expression in WT cells but not in APE1KD cells (**Fig. 6F**). Importantly, APE1KD attenuates both basal and oxidative stress-induced KRAS expression (**Fig. 6F**). Further, we found that only ectopic expression of WT APE1 was able to restore the KRAS gene expression, but neither the acetylation defective K5R mutant nor the repair defective H309A APE1mutant was able to do so (**Fig. 6G**), confirming again that both DNA repair function and acetylation of APE1 are necessary for modulating the expression of KRAS.

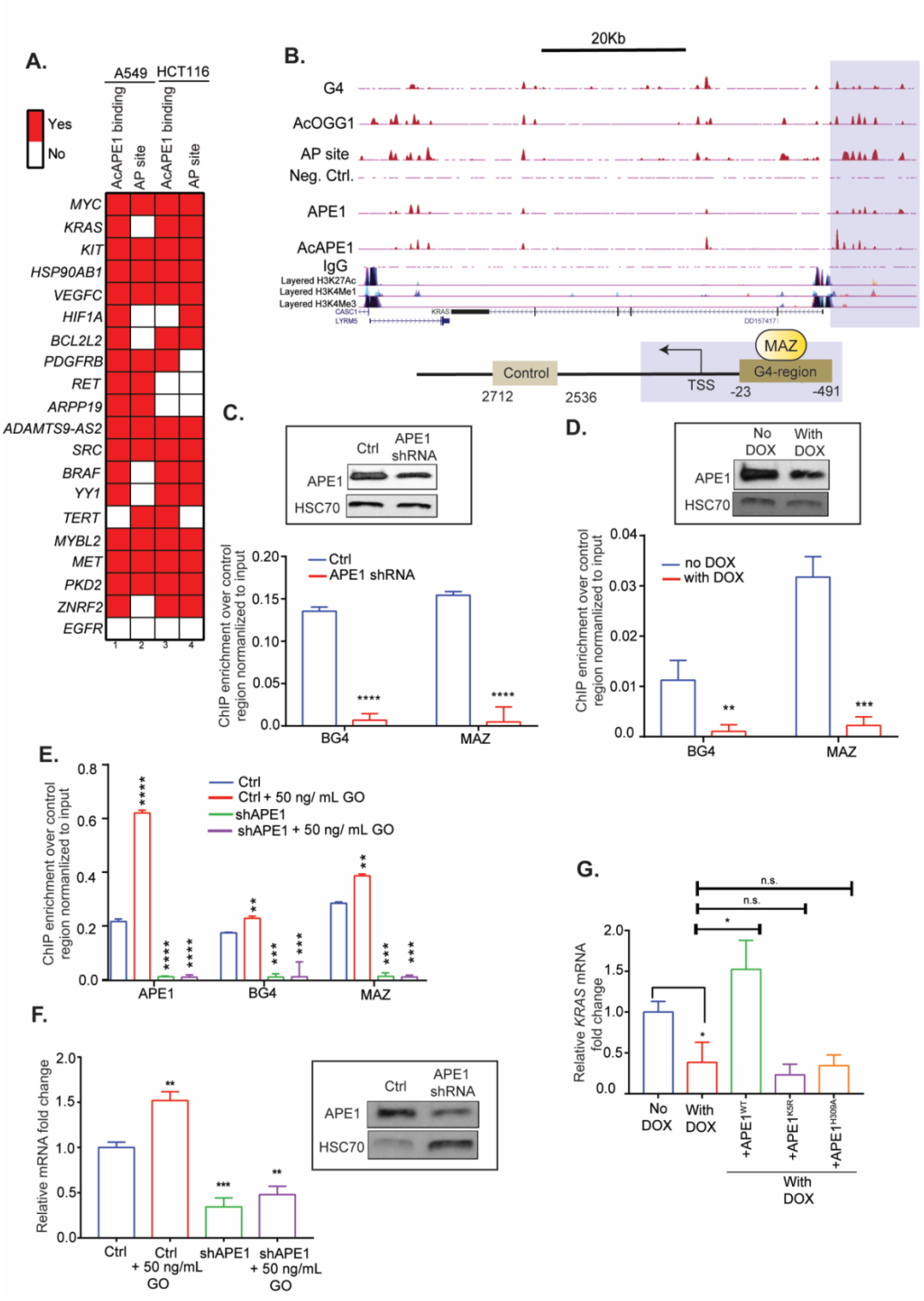

Figure 6: APE1 modulates G4-mediated expression of genes. A) Diagram shows occurrence of AP site and AcAPE1 binding in G4 containing oncogene promoters. **B)**

ChIP-Seq. analysis showing binding profiles and overlaps of occurrence of G4, AP site, and binding of AcOGG1, APE1, and AcAPE1 on KRAS gene in A549 cells. The purple box highlights AP site damage and repair protein AcOGG1, APE1, and AcAPE1 binding in the established G4 positive promoter region. Bottom schematic represents KRAS G4 promoter region, that harbors binding site for MAZ transcription factor, and the green box represents the G4 and non-G4 regions that were quantitated for examining the enrichment for G4 and occupancy of MAZ TF by real-time ChIP experiments in Fig. C and D. **C)** HCT116 cells and HCT116 constitutively expressing APE1 shRNA or **D)** HCT116 cells expressing doxycycline-inducible APE1shRNA cells showing the basal G4 enrichment, MAZ enrichment in specific G4 region over non-G4 control region in KRAS gene. The level of APE1 in these cell extracts was examined by Western blot analysis with α-APE1 and α-HSC70 (as loading control). **E)** HCT116 control and isogenic HCT116 shAPE1 cells were treated with glucose oxidase (GO) 50 ng/ml for 30 mins, ChIP qPCR was performed to examine APE1, BG4, and MAZ enrichment. **F)** Expression of KRAS gene by Real-time PCR, normalized to GAPDH. The level of APE1 in these cell extracts was examined by Western blot analysis with α-APE1 and α-HSC70 (as loading control). **G)** HCT116 cells expressing APE1shRNA were treated with Doxycycline (2 ug/ mL) for 3 days to knockdown the APE1 levels and then cells were transfected with FLAG-tagged WT APE1, or acetylation defective K5R (Lysine 6,7,27,31 and 32 to arginine) or repair defective H309A mutants of APE1 for 24 hrs, and RT-PCR performed to measure KRAS expression. ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, $^{ns}p>0.05$, calculated using unpaired t test. Error bars denote ±SD**.**

Our ChIP-seq data show enrichment of APE1 or AcAPE1 and G4 structures in previously reported MYC G4 promoter region (150) (**Fig. 3B**). To examine the role of APE1 in regulating G4-mediated gene expression, we utilized promoter-luciferase reporter with wild-type *c-MYC* (Myc- WT) G4 sequence in the upstream of promoter region of firefly luciferase coding gene. The expression of *c-MYC* firefly luciferase was normalized to relative expression of renilla luciferase gene from a non-G4 promoter sequence (pRL-TK). We found induction of oxidative damage by hydrogen peroxide activated Myc- WT luciferase expression in WT cells but not in APE1KD cells (**Fig. 7A**). Importantly, APE1KD attenuates both basal and oxidative stress-induced Myc-WT luciferase activity (**Fig. 7A**). *c-MYC* promoter PQS sequence has five G tracks and was shown to form two alternate G4 structures in vitro utilizing four G-tracks either 2345 or 1245 (**Fig. 7B**) (142, 150, 151). Next, to confirm whether the effect of APE1 on gene expression is mediated thorough the G4 sequence, we introduced two separate mutation in *c-MYC* G4 sequence of the promoter-luciferase reporter i) Myc- G12A (G to A mutation in 12th position of *c-MYC* G4; which can generate Myc 1245 G4 structure only), ii) Myc-G18A (G to A mutation in 18th position of *c-MYC* G4; which cannot generate any G4 structure) in the upstream region of firefly luciferase coding gene. Our results demonstrate that Myc-G12A (Myc 1245-G4) has increased luciferase activity whereas Myc-G18A (mutant G4) showed drastically reduced expression relative to Myc-WT indicating G4 structure (specifically Myc 1245) is involved in induction of promoter activity **(Fig. 7C)**. Consistent with these results, downregulation of APE1 level in cells reduced (~4-fold) Myc-WT and (~2-fold) Myc-G12A luciferase expression but did not significantly affect Myc-G18A mutant G4-luciferase expression (**Fig. 7C)**. . Overall, the results suggest that APE1 alters *MYC* or *KRAS* gene expression via promoting the formation of G4 structures and facilitating transcription factor loading.

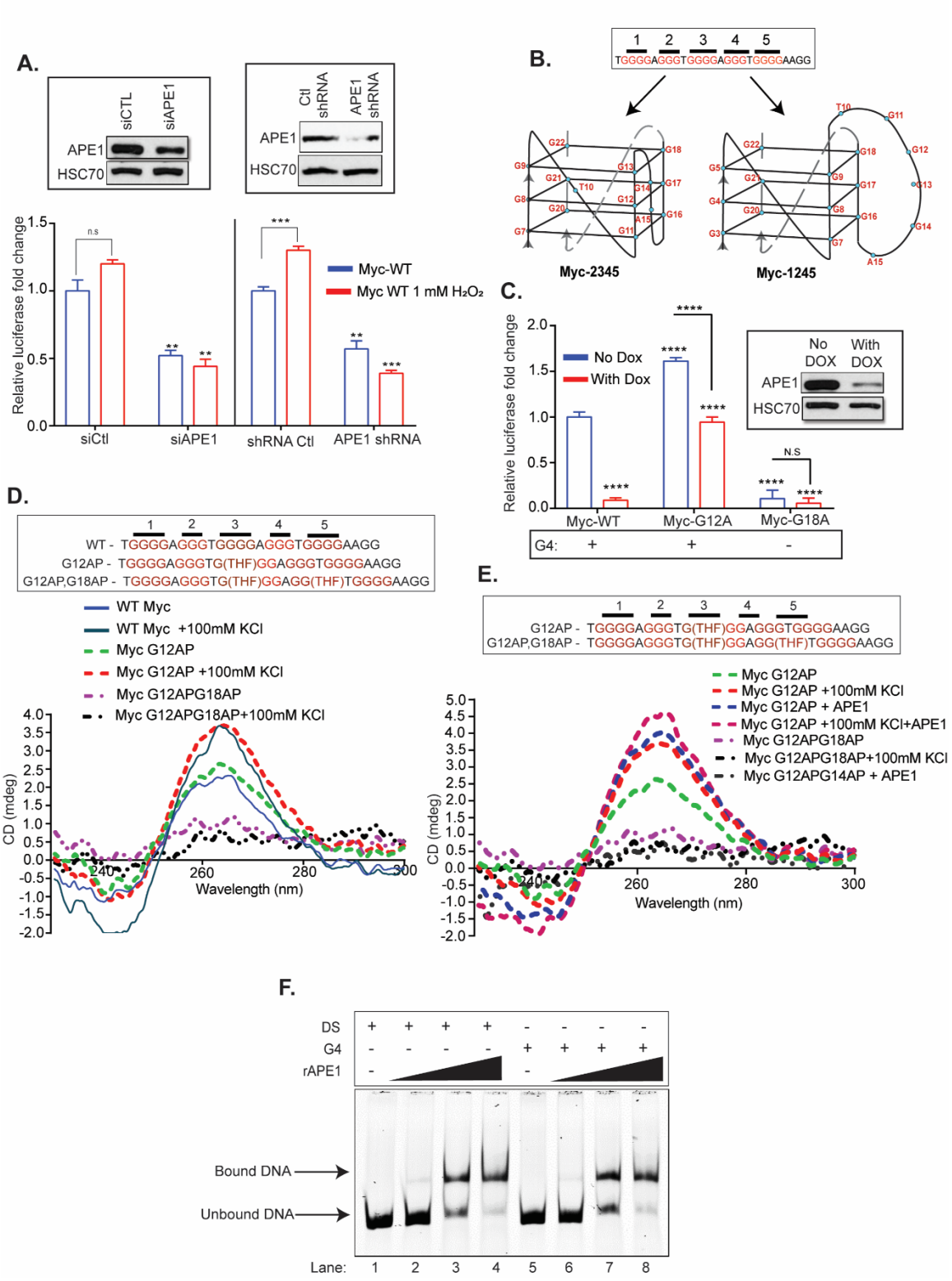
A.
siCTL
siAPE1
APE1
HSC70
Ctl shRNA
APE1 shRNA
Relative luciferase fold change
n.s
***
Myc-WT
Myc WT 1 mM H2O2
siCtl
siAPE1
shRNA Ctl
APE1 shRNA
B.
1 2 3 4 5
TGGGGAGGGTGGGGAGGGTGGGGAAGG
Myc-2345
Myc-1245
C.
No Dox
With Dox
No DOX
With DOX
N.S
Myc-WT
Myc-G12A
Myc-G18A
G4: + + -
D.
WT - TGGGGAGGGTGGGGAGGGTGGGGAAGG
G12AP - TGGGGAGGGTG(THF)GGAGGGTGGGGAAGG
G12AP,G18AP - TGGGGAGGGTG(THF)GGAGG(THF)TGGGGAAGG
WT Myc
WT Myc +100mM KCl
Myc G12AP
Myc G12AP +100mM KCl
Myc G12APG18AP
Myc G12APG18AP+100mM KCl
CD (mdeg)
Wavelength (nm)
E.
G12AP - TGGGGAGGGTG(THF)GGAGGGTGGGGAAGG
G12AP,G18AP - TGGGGAGGGTG(THF)GGAGG(THF)TGGGGAAGG
Myc G12AP
Myc G12AP +100mM KCl
Myc G12AP + APE1
Myc G12AP +100mM KCl+APE1
Myc G12APG18AP
Myc G12APG18AP+100mM KCl
Myc G12APG14AP + APE1
CD (mdeg)
Wavelength (nm)
F.
DS
G4
rAPE1
Bound DNA
Unbound DNA
Lane: 1 2 3 4 5 6 7 8

Figure 7: APE1 regulates G4-mediated *MYC* gene expression. A) *MYC* promoter-luciferase reporter containing wild-type G4 sequence; Myc-WT and pRL-TK-renila luciferase were co-transfected in control HCT116 cells or HCT116 cells transiently transfected with APE1 siRNA (siAPE1) or HCT116 cells constitutively expressing APE1 shRNA. 48 hrs after transfection, cells were treated with H_2O_2 1 mM for 1 hour and luciferase activity was measured and normalized with renila luciferase. The level of APE1 in these cell extracts was examined by Western blot analysis with α-APE1 and α-HSC70. **B)** Sequence of G4 forming Nuclease Hypersensitive element III1 (NHEIII1) element of c-Myc gene (150). Bases are numbered according to the sequence of NHEIII1. Two alternative G4 folding patterns (Myc 1245 and Myc 2345) of NHEIII1 are shown. **C)** Promoter luciferase constructs of Myc-WT, Myc promoter harboring G to A mutation in 12^{th} G position (Myc-G12A) and in 18^{th} position (Myc-G18A) in G4 sequence were transfected in control or doxycycline inducible $HCT116^{APE1shRNA}$ cells. Firefly luciferase activity was measured and normalized with renilla luciferase activity. The level of APE1 knockdown in these cell extracts was examined by Western blot analysis with α-APE1 and α-HSC70. ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, $^{ns}p>0.05$, calculated using unpaired t test. Error bars denote ±SD**. D)** 28-mer oligo sequences containing c-Myc G4 forming sequence with AP site analog (THF) are shown. Circular Dichroism (CD) spectra of WT-Myc , Myc G12AP, Myc G12AP, G18AP at 20 °C in presence or absence of 100 mM KCl. The ordinate indicates the ellipticity signal expressed in mdeg. **E)** CD spectra of Myc G12AP, Myc G12APG18AP at 20 °C in the presence of 100 mM KCl or 1 ug of APE1 alone or both APE1 and KCl together. **F)** Electrophoretic mobility shift assay was carried out to analyze binding of increasing amount (6.4, 64, 640 ng) of recombinant APE1 (rAPE1) to Cy5-labeled AP site containing Myc duplex (DS) or G4 oligonucleotide substrates.

Binding of APE1 to AP site damage in PQS promotes the G4-folding and acetylation of APE1 enhances residence time.

Finally, we investigated the mechanistic connection between AP site damage in PQS, binding of APE1 and formation of a stable G4 structures using *c-MYC* promoter G4 element. We performed in vitro studies using a 28-mer *c-MYC* promoter G4 (WT Myc) or containing a single AP site analog, tetrahydrofuran, in the 3rd G track (Myc G12AP) or two AP sites in the 3rd and 4th G track (Myc G12AP,G18AP) (**Fig. 7D**). We performed circular dichroism (CD) spectroscopy to determine the secondary structure of DNA (152, 153). The presence of a strong positive peak at ~265 nm with a weak negative signal at 240 nm is indicative of parallel G4 structure (154, 155). We found that KCl increased the folding of *c-MYC* oligo to a stable G4 structure in vitro when a single AP site (Myc G12AP) was present or absent (WT Myc) in the oligo (**Fig. 7D**). However, G4 oligo with AP sites in both the 3rd and 4th G track (Myc G12AP, G18AP) was unable to form G4 structure in the presence of KCl, which served as a negative control. To test whether binding of recombinant APE1 (rAPE1) at AP site in PQS can induce the formation of stable G4s, we incubated Myc G12AP and G12AP,G18AP oligos with increasing doses of recombinant APE1 in the absence of KCl. Addition of APE1 stimulated the folding of Myc G12AP oligo to a G4 structures even in the absence of KCl suggesting that APE1 binding promotes the formation of G4 folding in vitro **(Fig. 7E)**. Moreover, the presence of rAPE1 and KCl together further enhanced the formation of G4 structure in vitro **(Fig. 7E)**. In contrast, binding of APE1 to AP sites in Myc G12AP, G18AP oligo which cannot form G4 showed no effect on CD signals. We compared binding affinity of APE1 to AP site containing *c-MYC* duplex (DS) vs. pre-formed G4 oligo by EMSA assay. We found that recombinant APE1 has equal affinity for binding to the AP site when present in duplex or quadruplex forms (**Fig. 7F**).

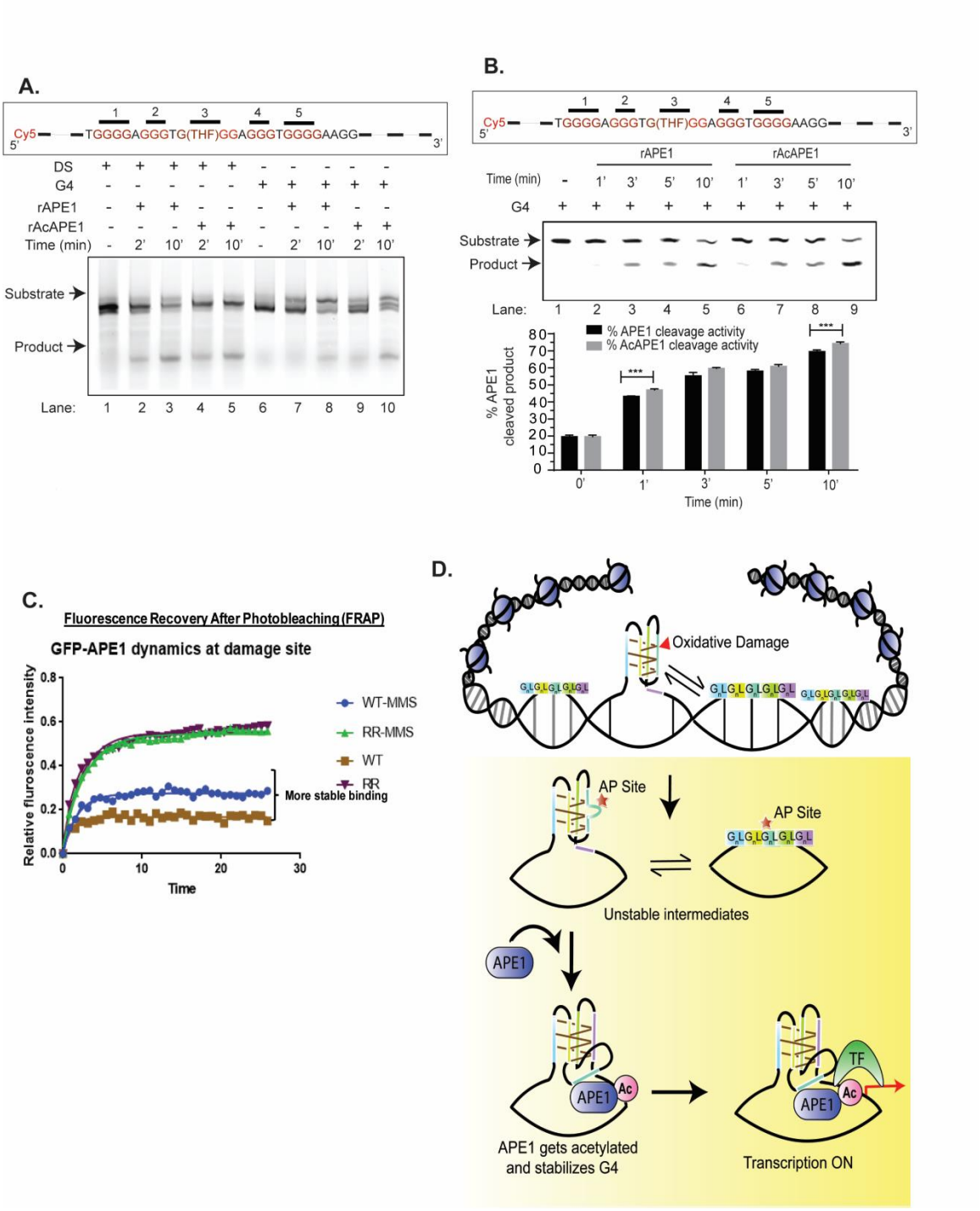

Figure 8: Binding of APE1 to AP site damage in PQS promotes the G4-folding and acetylation of APE1 enhances residence time. A) Comparison of endonuclease activity of recombinant APE1 or AcAPE1 on a Cy5-labeled 75-mer c-Myc duplex (DS) Vs quadruplex DNA (G4) (substrate, S). A 37-mer long cleaved product (P) was shown at 2

and 10 min. time points. **B)** Comparison of time-dependent cleavage activity of recombinant APE1 Vs. AcAPE1 on a Cy5-labeled 75-mer c-Myc quadruplex DNA (substrate, S). A 37-mer long product (P) was shown at different time points. Quantitation of the average cleaved product from three independent experiments are shown. **C)** FRAP analysis of GFP-APE1^{WT} and GFP-APE1^{RR} expressing cells was performed with or without 1 mM MMS treatment for 30 minutes. Each point represents the average of *n*=15 cells. Experiments were repeated at least three times and consistently showed similar mobility differences. **D)** Schematic representation of how binding of APE1 to AP site containing PQS sequence in the genome promotes folding of G4 and regulate transcription. G4 motif sequences (GnLGnLGnLGnLGn; where n≥3 and L is loop region containing any nucleotide) in PQS exits in a rapid equilibrium between duplex-quarduplex forms. Upon G oxidation, 8-oxoG base damage initiates the BER pathway and generates an AP site. Presence of AP site and subsequent binding of APE1 shifts the equilibrium to form a stable G4 structure. Subsequent acetylation of APE1 enhances its residence time on G4 and facilitates spatiotemporal stabilization of G4 structure, promotes transcription factor loading and gene expression.

The interaction between APE1 and AP site was previously studied in vitro in the G4 context, and it was found that APE1 binds AP sites in G4, but the cleavage rate was attenuated (151). Consistent with this, we also found that rAPE1 cleaves AP site less efficiently when it is present in G4 (**Fig. 8A**). We have recently shown that APE1 is acetylated after binding to AP site in chromatin (137) and our current study shows that both acetylation and DNA repair functions are essential for stable G4 (**Fig. 5H**) formation in cells**.** Therefore, we examined the effect of acetylation on endonuclease function of APE1 on G4 substrate. Recombinant AcAPE1 (rAcAPE1) had similar activity compared to unmodified rAPE1 when the AP site is present in folded G4 structure (**Fig. 8A and 8B**). However, its was shown earlier that APE1 remains bound to cleaved AP site and coordinate recruitment of downstream BER enzyme polymerase β (32, 95). To test whether acetylation of APE1 increases its residence time at AP site damages in chromatin in cells, we measured protein mobility at damage site by performing fluorescence recovery after photobleaching (FRAP) assay with WT APE1 and Lys 6 & 7 acetylation defective RR-APE1 GFP mutants with or without inducing AP site damages in cells. We found WT- APE1 GFP protein has higher residence time at damage site with less mobile fraction than non-acetylable RR-APE1 GFP in control or MMS treated cells (**Fig. 8C**). This suggests that acetylation may delay or slowdown the complete repair of AP site and increase the residence time of APE1 in G4 structure to coordinate transcriptional activation or repression via regulating loading of transcription factors to promoters. Consistent with this, our ChIP-seq data show several transcription factors binding site in AcAPE1 enriched promoter sequences (**Fig. 2F**), and AcAPE1 was shown to co-occupy with many transcription factors and coactivators in the promoter regions (31, 156, 157). Overall, the data suggest that binding of APE1 to AP site in PQS

promotes the folding of G4 structures and acetylation of APE1 enhances its residence time in chromatin which may spatiotemporally coordinate transcription and repair.

3.4 **Discussion**

Over the last few years, increasing direct evidence for the presence of G4 structures in vivo has started to provide insights in G4 occurrence and their functions in controlling multiple biological processes including transcription, replication, translation and telomere maintenance (117, 119, 158). Aberrant regulation of G4 structures are detrimental, causing transcriptional deregulation, replication stress, genome instability and human diseases (116). Understanding of how the formation (folding) and stabilization of G4s in the genome are regulated is fundamentally important. In this study, by conducting unbiased genome-wide mapping we demonstrate that the occurrence of endogenous base damage and APE1 binding is not random and are predominant in G4 sequences. Importantly, we demonstrate that endogenous oxidative base damage in G4 sequences and subsequent activation of BER drive the spatiotemporal formation of G4 structures which in turn regulate gene expression via facilitating transcription factors loading. Our study reveals a regulatory role of the BER machinery in coordinating the formation of higher order DNA secondary structures (G4s) in the genome.

Oxidatively induced endogenous DNA damage is conventionally viewed as detrimental to cellular processes. However, several recent studies have shown that oxidative DNA damages have a strong positive correlation with elevated oncogene expression (159). An interplay between oxidative-stress signaling, formation of 8-oxoG, binding of OGG1 and APE1 in promoters, and transcriptional activation or repression has been documented in mammalian genes (160-168). Moreover, recent studies have

demonstrated that 8-oxoG or AP sites when present in G4-forming promoter sequences of VEGF, BCL-2, and KRAS, directly upregulates transcription of the downstream genes (77, 148, 157, 164). Of note, these G4-promoter containing genes are known to be regulated by oxidative stress (169). Our study provides a mechanistic framework by highlighting a connection between oxidative base damage and the ability of certain DNA sequences to fold into G4 to regulate transcription.

We propose that PQS in the genome exists in a dynamic equilibrium between duplex and quadruplex forms and that guanine residues in PQS are oxidized to form 8-oxoG by cellular oxidants through intrinsic or extrinsic signal-dependent manner. Formation of 8-oxoG base damage in PQS recruits OGG1 to initiate the BER pathway (**Fig. 8D**). Our genome-wide overlap of OGG1 binding peaks with G4 support this (**Fig. 3B**). It was shown that unlike 8-oxoG paired with C, AP site in duplex DNA significantly impact the thermal stability of duplex DNA (164, 170, 171). We propose that cleavage of 8-oxoG and generation of AP site by OGG1 in PQS destabilizes it and opens up the duplex (172, 173), allowing the PQS to adopt an G-quadruplex fold where AP site containing G patch is looped out. This new conformation facilitates the binding of APE1 to AP sites for stabilizing G4 structure. Consistent with this, our in vitro CD-spectra data shows that the presence of an AP site and binding of APE1 in *MYC* PQS oligo promotes the formation of G4 structures in vitro (**Figure 7E**). These observations support a mechanism by which the AP site facilitates G4 formation by shifting the duplex-quadruplex equilibrium, and subsequent binding of APE1 to the AP sites promotes the formation of a stable G4 folding. However, we do not know the exact molecular basis of how APE1 binding promotes the stability of G4 folding. It is likely that APE1 binding increases the thermal stability of G4 structure. Alternatively, APE1 binding to G4 prevents the binding of G4-resolving helicases and thus spatiotemporally stabilize the G4 structures to regulate

transcription. Further, in vitro thermodynamics, DMS foot printing, and helicase activity studies are necessary to delineate the mechanistic details. We propose, oxidative DNA lesions at G4 sequences act as a sensor and initiation of BER pathway is intermediate in a signal transduction cascade that regulate G4 structure to ultimately modulate transcription. In a previous study, Fleming et al. (164) demonstrated that the fifth G-track of the VEGF PQS helped to reconstitute a G4 structure when one of the four upstream G-track contained a damaged base or AP site which induced VEGF transcription. Of note, most of the oncogenes that are regulated by G4 structure have promoter PQS with five or more G patch (149). Promoter G4s are more conserved than other regions according to the mining of human genome sequence analysis(174, 175). Evolutionary selection of such oxidative damage prone G rich sequences (G4) in the genome and their role in gene regulation suggest a novel oxidative damage signaling mechanism where BER co-ordinates the process through modulating DNA secondary structures (G4s) and maintaining the integrity of the regulatory sequences.

Consistent with our model, cell-based assays show that initiation of repair of 8-oxoG via BER plays a crucial role in the formation of stable G4s. Our data show that the loss of either the initial glycosylase, OGG1 or the downstream repair protein APE1, abolished the formation of G4 structures in cells (**Fig. 5B, 5C, and 5D**). The observations that loss of either the endonuclease activity or acetylation of APE1 abrogates G4 formation raises the questions about the biological importance of these functions of APE1 in the formation of G4. We propose that APE1 cleaves the AP site and remains bound to G4 structures. APE1 is then acetylated by histone acetyl transferase p300, commonly found in promoters and enhancers of the gene via its association with TFs or RNA Pol II (56). This spatiotemporal acetylation of APE1 delays its dissociation from AP sites and stabilizes G4 structures; APE1 is then well positioned to stimulate binding of TFs, which

activates transcription. Consistent with this, our data show that knockdown of APE1 or inhibition of its acetylation abrogates G4 formation and reduced MAZ1 TFs loading on the KRAS promoter. Such a mechanism explains why AcAPE1 has increased residence time in chromatin (**Fig. 8C**), interacts with many TFs in the promoter region (31, 55, 56, 67, 68, 83), and AcAPE1- bound regions significantly overlap with G4 structure and bear active enhancer and promoter histone marks. Many reports have found co-occupancy and interaction of APE1 with activating or repressor factors such as STAT3, HIF-1α, AP-1, NF-κB, and HDAC1at promoter regions (31, 39, 156, 157). The present data, in tandem with our previous studies, support AcAPE1 coordinates both DNA damage repair and transcription.

Although this study shows that G4 structure formation is coupled with endogenous oxidative DNA damage followed by activation of BER pathway, the source of an essentially site-specific G oxidation or base damage in PQS promoter sequence in the genome remains a question. Random oxidation of G is too erratic to constitute the mechanism. However, we have observed reproducible occurrence of AP sites or enrichment of OGG1 and AcAPE1 binding to specific regions of active genes in multiple independent experiments in multiple cell lines. This discovery raises one intriguing question about whether endogenous oxidative or AP site damages occur in a targeted or site-specific manner (167). Although few recent studies have shown region-specific distribution of DNA damages (176, 177), further high-resolution (single-base) mapping of base damage in the genome is warranted. Eliciting a targeted DNA base damage appears to be a common first step in hormone-induced activation of many genes. For example, Perillo et al.(161) have shown that estrogen-induced activation of BCL-2 gene occurs by flavin-dependent demethylation of H3K9me2 by LSD-1 in the promoter region of BCL-2 gene which generates H_2O_2. This local oxidation was shown to produce G

oxidation in the promoter of BCL-2 gene in a targeted manner. Interestingly, BCL-2 promoter regions contain PQS (77). They found LSD1-mediated oxidation in PQS and noted an essential role of OGG1 of BER in the activation process. Pan et al.(162) have also shown that oxidation of G in NF-κB binding site promotes NF-κB binding and stimulates transcription. Similarly, active demethylation of 5-methylcytosine by TET enzymes also produce H_2O_2 and excision of deaminated 5-meC (Thymine) by the BER glycosylases TDG1 are thought to play a key role in the transcriptional control and resetting of epigenetic memory during embryonic development and cellular programing (178). However, further studies are necessary to address how an indiscriminate oxidant like H_2O_2 liberated by LSD1 generates specific G oxidation in PQS sequences to induce G4 formation.

G4 structures are often formed in the 3' overhang regions of telomere sequences(112, 179-181) and can serve regulatory roles by protecting telomere cap structures (113, 117). A corollary to our study, Mandler et al.(73) has shown that both DNA repair and acetylation of APE1 are essential for maintaining telomere length and absence of either function of APE1 leads to telomere shortening, telomere fusion and formation of micro-nuclei in cell lines, perhaps de-stabilizing the G4 structure in telomere. G4-induced replication stress, DNA damage and genomic instability have been linked with many cancers (115, 116, 118). Mutations or loss of many G4 resolving helicases, such as WRN, ATRX and DDX21 are indicated to cause genome instability and disease pathology (122). Studies have found that G4 forming sequences are enriched at translocation breakpoints (182). Moreover, an expandable GGGGCC motif that can adopt a G4 structure, located in many gene locus, have been found to contribute to two well recognized neurodegenerative diseases, amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (116, 183). We propose that endogenous oxidative

damages and the activation of BER not only repairs damaged bases but also regulates the formation and stability of G4 structures in the genome to regulate multiple biological processes. It is noteworthy to mention that like G4 structures, crucial roles of the BER or APE1 in regulating multiple biological processes, ranging from transcription, maintaining telomere length, class-switch recombination, somatic hypermutation, and mRNA biogenesis has been demonstrated (34, 76, 127, 184-186). Our study introduces a new perspective in understanding that region-specific endogenous damage and activation of BER serve specific functions in regulation of G4 for controlling multiple cellular processes. This function of endogenous damage and the BER machinery defines a new role, beyond its well characterized role as a safeguard for maintaining genomic integrity and preventing mutations, dysregulation of which can lead to many human diseases.

CHAPTER 4

Overall conclusions and future directions

5.1: **Overall conclusions**

My dissertation was aimed at improving the understanding of the functional role of APE1 acetylation in DNA repair and transcription regulation. Characterization of AcAPE1 is important to understand the reason behind its upregulation in different cancers and necessity for cell survival and proliferation. It is well established that the C-terminal domain of APE1 is important for DNA repair function (7). However, the direct functional significance of its N-terminus and acetylation is still under investigation. Although acetylation was associated with APE1's transcription regulatory function, the underlying mechanism was not very clear (31). Thus, elucidating the underlying functions of AcAPE1 may provide sight for generating future cancer drug targets.

Towards that end, the work presented in *chapter 2* of this dissertation has demonstrated that AcAPE1 is exclusively localized to chromatin in all phases of the cell cycle, including mitosis. Genotoxic stress induces acetylation and APE1 is acetylated at AP site in chromatin (63). Although the N-terminal domain (aa 1 to 40) of APE1 is known to be dispensable for its *in vitro* DNA repair activity, our study demonstrated the novel regulatory role of acetylation of multiple lysine residues in this domain in AP site repair both *in vitro* and in cells. Using Acetylation defective mutants, we found the absence of acetylation in cells expressing APE1 results in the accumulation of AP sites in the genome, and the genome becomes sensitive to DNA-damaging agents. Our data suggested acetylation not only improves the catalytic efficiency of APE1 but also may facilitate the coordination and recruitment of the downstream enzyme in the BER pathway. Collectively, the acetylation of APE1 is likely an integral part of the APE1-dependent BER pathway for maintaining DNA integrity.

In *chapter 3* of this dissertation, to know the genome-wide distribution of APE1, AcAPE1, we conducted an unbiased mapping of AP sites, APE1, and AcAPE1 binding across the genome, we provide the first direct evidence that the occurrence of endogenous base damage and APE1 binding is not random. There are distinct patterns of AP sites, APE1, and AcAPE1 binding predominantly in the transcription regulatory regions and specifically in G-quadruplex (G4) sequences in the genome. Formation of G4 DNA structures in key regulatory regions (gene promoters, 5' and 3' untranslated regions and telomeric regions) in the genome has emerged as a secondary structure-based epigenetic mechanism for regulating multiple biological processes including transcription, replication, translation, and telomere maintenance (110, 113). Aberrant regulation of G-quadruplex structures are detrimental, causing transcriptional deregulation, genome instability, and human diseases (116). For biological functions, formation, stabilization, and unfolding of G4 must be spatiotemporally regulated. While several proteins that bind to and resolve G4 structures have been characterized, the mechanisms underlying spatiotemporal formation and stabilization of G4 structures in the genome are largely unknown. We comprehensively demonstrated that APE1, a key player in BER, is directly involved in the spatiotemporal regulation of G4 structures in the genome. This study shows that oxidation of G and the resulting AP site recruits APE1 which shift the duplex-quadruplex equilibrium to the G4 fold. This, in turn, promotes the formation of stable G4 structures and regulate gene expression via facilitating transcription factor loading to the promoter. Loss of APE1 or its acetylation abrogates the formation of G4 structures in cells. APE1 binding promotes G4-folding in-vitro, and acetylation of APE1 which enhances its residence time stabilizes G4 structures and facilitates transcription factor loading on promoters, providing mechanistic insight into the role of APE1 in G4-mediated gene expression. Our study unravels an acquired function

of endogenous base damage and BER in controlling the formation of higher order DNA secondary structures beyond its role in safeguarding the genomic integrity.

5.2 **Future directions**

5.2.1 **Role of acetylated APE1 in mitotic DNA damage bookmarking**

According to the classical concept, mitotic chromosome condensation is coupled with the cessation of transcription with dissociation of most sequence-specific transcription factors (187-189). However, recent studies have revealed the presence of open chromatin structures associated with active gene histone mark and presence of some regulatory proteins during mitosis in those regions (190). This novel epigenetic mechanism is thought to control the post-mitotic gene expression pattern. Although repair is not active during mitosis, but damage can occur to these open chromatin regions specifically in active gene enhancer-promoter regions (191). Recently, selective mitotic retention of regulatory proteins in the promoter-enhancer region, a phenomenon called mitotic gene bookmarking, has changed the dogma about mitosis (192). Thus, subsequent marking of these DNA damages and preferential repair of these regions is a prerequisite for stable TF-binding or RNA Pol II-loading to restore rapid post-mitotic activation of the genes. High proliferating cells, specifically tumor cells, will be benefitted by this kind of epigenetic mechanism that would facilitate rapid gene activation following mitotic exit. However, the major gap in our understanding is that how cells bookmark DNA damages in mitosis and preferentially repair these regions for post-mitotic reactivation of gene.

We discovered that AcAPE1 is exclusively associated with chromatin and remains bound to the condensed chromatin at all stages of mitosis. Our genome-wide binding analysis showed binding of AcAPE1 to selective regions (promoters and enhancers) of several thousands of active genes and persist throughout mitosis. Madlener et al. recently showed loss of either repair activity or Lys 6,7 acetylation leads

to telomere fusion and mitotic defects (73). It is important to elucidate the role of APE1 acetylation, BER status in mitosis, and the functional importance of sequence selective AcAPE1 retention in the mitotic chromosome. Understanding the role of pre-exiting AcAPE1 in selective regions in the chromosome in mitosis may reveal a novel function of AcAPE1 in mitosis and the pathophysiological importance of elevated AcAPE1 in cancer cells that are continuously undergoing through mitosis.

5.2.2 **Mechanistic insight into the role of AcAPE1 in G-quadruplex formation and stabilization**

The multitude of G4 regulated cellular processes extending in both DNA and RNA level highlights the presence of a tightly coordinated epigenetic mechanism, just like CpG methylation and histone modification (113). Instead of writer, reader, and eraser proteins, there must be signal dependent formation (folding), stabilization, and unfolding to regulate a biological function. Many helicases have been documented to resolve G4 structure to coordinate G4 mediated cellular function such as WRN, BLM and ATRX in telomere maintenance, Pfh1 and FANCJ in replication fork progression, XPD/XPB and WRN in transcription and DDX21 and DHS36 in translation and loss of these proteins are indicated to cause genome instability and disease pathology (122). However, the signals or the proteins regulating the structural shift of a double helix towards a G4 and maintaining their spatiotemporal stability in cells are largely unknown.

Our genome-wide correlation of endogenous AP site mapping, APE1 binding and G4 occurrence together with a causal effect of loss of APE1 in G4 formation and stabilization, first time illustrates a mechanism behind G4 folding in cells. However, the molecular necessity of base damage and BER in G4 formation is not clear. Further, using in vitro biophysical (thermal stability, FRET, and CD) biochemical assays (binding

kinetics, DMS and bromide foot printing), and G4 containing promoter reporter approaches, future studies will elucidate the molecular basis of how APE1 promotes G4 folding. This information is important to understand the G4 folding/ stabilization dynamics inside the cell to design targeted strategies.

As we found that acetylation of APE1 is essential for spatiotemporal G4 formation and G4-mediated gene expression. We have developed an APE1 Lys 6, 7 acetylation defective mouse model. This mouse model will be utilized to elucidate the impact of compromised BER on G4 formation, gene expression, telomere maintenance and mutation accumulation at G4-forming sequences in the genome. Our acetylation-defective mouse models have excellent potential for dissecting the relative importance of acetylation of APE1 and efficient BER in regulating the formation of G4 structures and perhaps illustrating the pathophysiological consequences of alteration of G4 mediated gene expression and mutation accumulation in G4 sequence in vivo.

5.2.3 **Intertwined role of DNA base damage repair and gene transcription in mammalian cells**

The mechanism of BER has been reconstituted in vitro and is well characterized (7). However, a full understanding of AP site repair must take into account how it occurs in live cells, where DNA is packed into chromatin and serves as a template for transcription. Genome-Wide comparison of open chromatin status, histone modifications, RNA expression, and DNA damage and repair would allow determining of how these processes are coordinated. Transcription, a DNA templated basic process occurring in every cell, is mutagenic in nature (193). Nevertheless, considering the inherent mutagenic nature of transcription and frequency at which DNA damage occurs (~10^4 events per day), it is remarkable that the overwhelming majority of these

deleterious DNA lesions are repaired with impressive accuracy and efficiency in transcribed gene regions to avoid mutations. There is a reason to believe that interdependency between transcription and DNA repair is finely regulated to orchestrate this choreographed process. However, a clear mechanistic link between DNA damage, repair, and transcription have not yet been established.

We have developed a genome-wide assay for mapping AP site damage (damage-seq) and repair by APE1 and AcAPE1 (repair-seq) in cells. We are using damage and repair-seq strategy to measure repair kinetics after Methylmethane sulfonate (MMS); an alkylating agent known to produce AP sites, induced damage and then related the kinetics of repair to chromatin state and transcription. Initial study shows AP sites damage predominantly occurs to transcriptionally active regions. Of note, not all APE1 binding sites overlapped with AcAPE1 sites indicating there are two pools of APE1. Interestingly, inhibition of transcription inhibits APE1's acetylation and alters repair kinetics of the active gene regions. Further, analyzing the damage-repair occurrence in context to different epigenetic features, transcription status will identify fundamental interdependency between endogenous AP site damage-repair with transcription.

Together, this dissertation research study have provided valuable molecular insights into the role of acetylated APE1 in mammalian cells and warrant further studies to delineate molecular networks and pathophysiological consequences of aberrant regulation of APE1 N-terminal acetylation.

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