APE2 IS A CRITICAL REGULATOR OF THE DNA DAMAGE RESPONSE TO MAINTAIN GENOME INTEGRITY IN MAMMALIAN CELLS

by

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A dissertation submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Biology

Charlotte

2021

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ABSTRACT

MD AKRAM HOSSAIN. APE2 is a critical regulator of the DNA damage response to maintain genome integrity in mammalian cells. (Under the direction of DR. SHAN YAN)

The maintenance of genome integrity and fidelity is essential for the proper function and survival of all organisms. Recent studies have revealed that APE2 is required for the activation of an ATR-Chk1 DNA damage response (DDR) pathway in response to oxidative stress and a defined DNA single-strand break (SSB) in *Xenopus laevis* egg extracts. However, it remains unclear whether APE2 is a general regulator of DDR pathway and what the biological significance of APE2 is in mammalian cells. Here, I provide evidence using mammalian cultured cell lines including human pancreatic cancer cells that APE2 is important for ATR DDR pathway activation in response to different stressful conditions including oxidative stress, DNA replication stress, and DNA double-strand breaks. Fluorescence microscopy analysis shows that APE2-knock-down (KD) leads to enhanced γH2AX foci and increased micronuclei formation. In addition, a small molecule compound is identified as APE2 inhibitor that specifically compromises the binding of APE2 to ssDNA, its 3′-5′ exonuclease activity, and the defined SSB-induced ATR Chk1 DDR pathway in *Xenopus* egg extracts. Notably, cell viability assays demonstrate that APE2-KD or APE2 inhibitor sensitizes pancreatic cancer cells to chemotherapy drugs. Overall, APE2 is proposed as a general regulator for DDR pathway in genome integrity maintenance in mammalian cells.

ACKNOWLEDGEMENTS

I thank Drs. Matthew Michael, Karlene Cimprich, Howard Lindsay, Scott Williams, Zhongsheng You and Doug Golenbock for reagents. I am grateful for the assistance by Dr. Didier Dreau for FACS analysis, Multi-plate Reader & Fluorescence Microscopic analysis & training. I also thank Dr. Christine Richardson, Dr. Donna Goodenow & David Gray for the assistance with Confocal Microscopy protocol, analysis & training respectively. I also appreciate the assistance of Dr. Paola Lopez-Duarte with the training, analysis and using of Leica Microscope. The Yan lab was supported, in part, by grants from the NIH/NCI (R01CA225637) and NIH/NIGMS (R15GM114713), and funds from UNC Charlotte. This study was made conceivable through the outstanding mentorship and supervision of Dr. Shan Yan. Thank you for the opportunity to work with you and your team in your lab, your guidance, advising, suggestions, personal remarks and especially the trust & belief that you had in me made my journey a wonderful one. I would like to thank my committee: Dr. Pinku Mukherjee, Dr. Christine Richardson, Dr. Kaushik Chakrabarti, and Dr. Jun Tao Guo for their suggestions and encouraging remarks. Thank you to the members, past and present, of the Yan lab, Dr. Yunfeng Lin, Dr. Jia Li, Dr. Haichao Zhao, Melissa McLeod, Mary Overton, Anh Ha, Katherine Jensen, Garrett Driscoll, Joshua Matos, Anne McMahon and all of the undergraduates who worked with us. I am ever grateful to the University of North Carolina at Charlotte Graduate School for providing me Graduate Assistant Support Award (GASP) for whole 5 years of my doctorate program and The Graduate School Summer Fellowship Program for supporting my graduate education in Summer semesters. And most importantly, I'd like to thank my beloved wife Zobaida Zaman who had always been there

throughout this whole journey, keeping me away from stress and monotony of relentless work-oriented life and also want to thank my friends and family who have kept me calm, composure with huge amount of patience & perseverance throughout this PhD journey. I want to dedicate my doctorate degree to my parents who always believed in me that I would be good scientist one day and they really are the happiest person in this world to see the initial title of “Dr” before my name. I assume this is the most divine feelings if you make your parents proud of you that much.

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LIST OF ABBREVIATIONS

9-1-1 Complex: Rad9, Hus1, and Rad1. DNA damage checkpoint sliding clamp
AKI: Acute Kidney Injury
AP site: Apurinic/Apyrimidinic site
APE1/Ref-1/Apn1: Apurinic/Apyrimidinic Endonuclease
APE2/APEX2/Apn2: Apurinic/Apyrimidinic Endonuclease 2
APTX: Aprataxin
AMP: 5'-Adenylate / Adenosine monophosphate
ATM: Ataxia telangiectasia mutated
ATR: Ataxia telangiectasia and Rad3- related protein
ATRIP: Ataxia telangiectasia and Rad3- related- interacting protein
AZD6738: Ceralasertib, ATR inhibitor
AZD7762: Chk1 & Chk2 inhibitor
BER: Base Excision Repair
BLASTP: Basic Local Alignment Search Tool: Protein
bp: base pair
BRCA1: Breast Cancer Type 1 Susceptibility Protein
BRCT: BRCA1 C terminus
Cdc7: Cell division cycle 7
CDK: Cyclin Dependent Kinase
cGAS: Cyclic GMP-AMP Synthase
Chk1: Checkpoint Kinase 1
Chk2: Checkpoint Kinase 2
cisPt: Cisplatin, anti-cancer ("antineoplastic" or "cytotoxic") chemotherapy drug
CIP: Calf Intestine Phosphotase
CPT: Camptothecin (Topoisomerase I inhibitor)
COSMIC: Catalogue of Somatic Mutations in Cancer
CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9
CSR: Class switch recombination
CtIP/Sae2: C-terminal binding protein- interacting protein
DAPI: 4′,6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA.
DDR: DNA Damage Response
DE3: E. Coli cell line used for plasmid protein expression
DMSO: Dimethylsulfoxide
DNA: Deoxyribonucleic Acid
dNTP: deoxynucleotide triphosphate
dRP: deoxyribose phosphate
DSB: Double Strand Break
dsDNA: Double Stranded DNA
E3330/APX3330: [(2E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl) methylene]-undecanoic acid, APE1 redox inhibitor
EcoRI: Restriction endonuclease that cleaves double stranded DNA at the sequence GTTAAC

ECL: Enhanced Chemiluminescence
ERCC1-XPF: Excision repair cross complementation group 1- Xeroderma Pigmentosum F
ETO: Etoposide (Topoisomerase II inhibitor)
Exo1: Exonuclease 1
FAM/5'-FAM: Fluorescein amidites, 6-carboxyfluorescein
FBS: Fetal Bovine Serum
FEN1: Flap Endonuclease 1 F
FITC: Fluorescein Isothiocyanate
FOLFIRINOX: A regimen (a combination of fluorouracil, leucovorin, irinotecan, and oxaliplatin)
GEM: Gemcitabine
H_2O_2: Hydrogen Peroxide
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR: Homologous Recombination
HRP: Horse Radish Peroxidase
HSS: High Speed Supernatant
Hus1: HUS1 checkpoint clamp component
IACUC: Institutional Animal Care and Use Committee
ICL: Interstrand Cross Link
IC50: The half maximal inhibitory concentration
IDCL: interdomain connector loop
IKK-α, IKK-β: IkappaB kinase complex (IKK) subunits
IPTG: Isopropyl β-D-1-thiogalactopyranoside kDa: kilo Dalton
KU55933: 2-(4-Morpholinyl)-6-(1-thianthrenyl)-4H-Pyran-4-one, 2-(Morpholin-4-yl)-6-(thianthrene-1-yl)-4H-pyran-4-one; ATM Inhibitor
LIG3: DNA Ligase 3
LSS: Low Speed Supernatant
MCM: Mini Chromosome Maintenance
MG-132: $C_{26}H_{41}N_3O_5$, Proteasome & Calpain inhibitor
MYH9: Myosin Heavy-Chain 9
MM: multiple myeloma
MMEJ: Microhomology-Mediated End Joining
MMR: Mismatch Repair
Mre11: MRE11 homolog, double strand break repair nuclease
MRN complex: Protein complex consisting of Mre11, Rad50 and Nbs1
MSN: Mesoporous silica nanoparticles
MTT: Thiazolyl blue tetrazolium bromide
Myc: Myc Proto-Oncogene Protein
NaF: Sodium Fluoride
Nbs1: Nijmegen breakage syndrome 1 (nibrin)
Nt.BstNB1: Restriction enzyme that catalyzes a single strand nick 3' 4 bases beyond its GAGTC recognition sequence
NU7441: 8-dibenzothiophen-4-yl-2-morpholin-4-ylchromen-4-one, DNA-PK inhibitor
OS: Oxidative Stress
p53: Tumor protein p53
p23: Prostaglandin E synthase 3 (PTGES3) enzyme

PARP: Poly (ADP-ribose) polymerase
PARP1: Poly (ADP-ribose) polymerase 1
PARP2: Poly (ADP-ribose) Polymerase 2
pBR322: A plasmid, one of the first widely used E. coli cloning vectors.
PBS: Phosphate Buffer Saline
PCNA: Proliferating Cell Nuclear Antigen
PCR: Polymerase Chain Reaction
PEO1: human ovarian cancer cell line
pGEX-4T1: Protein production plasmid with Ampicillin resistance
PIP: PCNA interacting peptide PNK: Polynucleotide kinase
PNKP: Polynucleotide Kinase 3'-Phosphatase
PNPK- Polynucleotide Kinase Phosphatase
pCDNA3: Mammalian expression vector with CMV promoter; amp resistance (neomycin for mammalian selection); restriction enzyme cloning
pS: Mutant pUC19 that has one Nt.BstNB1 recognition site
PVDF: Polyvinylidene Difluoride
Rad1: RAD1 checkpoint DNA exonuclease
Rad17:RAD17 checkpoint clamp loader component
Rad50: RAD50 double strand break repair protein
Rad9: RAD9 checkpoint clamp component
RECQ1: RecQ family member of DNA helicases
REV1: REV1 DNA directed polymerase
RNA: Ribonucleic Acid
rNMP: Ribonucleoside monophosphates
ROS: Reactive Oxygen Species
RPA: Replication Protein A
RS: Replication Stress
SCGE: single cell gel electrophoresis assay, COMET assay
S-CDK: S-phase cyclin-dependent kinase
SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SHM: Somatic hypermutation
SSB: Single Strand Break
SSBR: Single Strand Break Repair
siRNA: Small interfering ribonucleic acid
ssDNA: Single Stranded DNA
SUMO: Small Ubiquitin-like Modifier
SW620: p53 mutant
SYBR Gold: Nucleic Acid fluorescent stain
TAB004: A monoclonal antibody targeting tMUC1
TBE: Tris/Borate/EDTA
TBST: Tris Buffer Saline with Tween 20
TDP2: Tyrosyl-DNA phosphodiesterase 2
TE: Tris EDTA buffer
tMUC1: Tumor-associated form of MUC1
Top1: Topoisomerase 1
TopBP1: DNA topoisomerase 2-binding protein 1

VE822: Berzosertib; ATR inhibitor
Xho1: Restriction endonuclease that cleaves double stranded DNA at the sequence CTCGAG
XRCC1: X-Ray Cross Complimenting Protein 1
YFP: Yellow Fluorescent protein
Zf-GRF: Zinc finger (Glycine, Arginine, Phenylalanine)
γ-H2AX: Phosphorylated H2A Histone Family Member

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INTRODUCTION

DNA single-strand breaks (SSBs) occur more than 10,000 times per mammalian cell each day, representing the most common type of DNA damage. Unrepaired SSBs compromise DNA replication and transcription programs, leading to genome instability. Unrepaired SSBs are associated with diseases such as cancer and neurodegenerative disorders. Although canonical SSB repair pathway is activated to repair most SSBs, it remains unclear how unrepaired SSBs are sensed and signaled. In this review study, we propose a new concept of 4 step mechanism of SSB end resection for genome integrity that include SSB end sensing and processing as well as initiation, continuation, and termination of SSB end resection. We also compare SSB end resection with the well-known DSB end resection in DNA repair and DNA damage response (DDR) pathways. Overall, this study provides the first comprehensive perspective on SSB end resection in genome integrity.

APE2 (Apurinic/apyrimidinic endonuclease-2, also known as APEX2 or APN2) is an evolutionarily conserved protein with strong 3′-phosphodiesterase and 3′-5′ exonuclease activities but weak AP endonuclease activity and has been implicated in genome and epigenome integrity maintenance. Prior studies using different model systems have shown that APE2 plays crucial roles in DNA repair pathways including the base excision repair (BER), SSB repair, DSB repair & DDR pathways including the ATR-Chk1 DDR pathway and p53-dependent DDR pathway. Furthermore, APE2 has been implicated in development and growth as well as cancer etiology. A prior study has shown that APE2-knock out (KO) mice are viable but display growth retardation. This study provides evidence using cancer cell lines that activation of the ATR-Chk1 DDR pathway induced by hydrogen peroxide (H_2O_2), GEM, CPT, and ETO is compromised when APE2 is down-

regulated via siRNA. Furthermore, siRNA-mediated APE2-knockdown (KD) leads to a higher percentage of γH2AX-positive cells and micronuclei-positive cells. These results suggest that APE2 is a critical regulator of ATR-Chk1 DDR pathway to maintain genome integrity.

In this study, we found Celastrol, a natural compound derived from thunder god vine *Tripterygium wilfordii*, impaired APE2 interaction with ssDNA and APE2 3′-5′ exonuclease activity *in vitro* and also compromised the defined SSB-induced ATR-Chk1 DDR pathway in *Xenopus* egg extracts. But it remains elusive if the inhibitory function of Celastrol against APE2 is conserved in mammalian cells or not. Several library screens have identified NU7441 as a potential inhibitor of DNA-dependent protein kinase (DNA-PK) & preclinical studies showed that NU7441 significantly enhanced the sensitivity of DNA-PK-proficient V3-YAC cells to etoposide and promoted these cells to death. However, there is no report on the potential regulation of APE2 by NU7441 in its function in DDR pathway in a cellular context. The overall study is to elucidate the distinct functions and regulatory mechanisms of APE2 in DDR pathway in mammalian cells. In summary, I have demonstrated different approaches to target APE2 function in DDR pathway including but not limited to **a)** siRNA-mediated APE2 knockdown, **b)** Celastrol-mediated inhibition of APE2 catalytic functions, and **c)** NU7441-induced APE2 down-regulation. These findings will lay a solid foundation for future preclinical and clinical cancer treatment studies by targeting APE2 function and mechanism in DDR pathway. Most importantly, results and findings from this thesis project suggests that APE2 regulates the ATR DDR pathway in cancer cells and that targeting the novel function of APE2 in ATR DDR may open a new avenue for future therapeutics in cancers.

CHAPTER 1: SINGLE-STRAND BREAK END RESECTION IN GENOME INTEGRITY: FROM CONCEPT TO MECHANISM

ABSTRACT

DNA single-strand breaks (SSBs) occur more than 10,000 times per mammalian cell each day, representing the most common type of DNA damage. Unrepaired SSBs compromise DNA replication and transcription programs, leading to genome instability. Unrepaired SSBs are associated with diseases such as cancer and neurodegenerative disorders. Although canonical SSB repair pathway is activated to repair most SSBs, it remains unclear whether and how unrepaired SSBs are sensed and signaled. In this review, we propose a new concept of SSB end resection for genome integrity. We summarize the current understanding on molecular mechanism of SSB end resection that include SSB end sensing and processing as well as initiation, continuation, and termination of SSB end resection. We also compare SSB end resection with the well-known DSB end resection in DNA repair and DNA damage response (DDR) pathways. We further discuss how SSB end resection contributes to SSB signaling and repair. Finally, we identify areas of future study that may help us gain further mechanistic insight into the process of SSB end resection. Overall, this review provides the first comprehensive perspective on SSB end resection in genome integrity.

1.1. Introduction

DNA single-strand breaks (SSBs) are discontinuities in one strand of the DNA double helix, and are often associated with damaged or mismatched 5′- and/or 3′-termini at the sites of SSBs (1). SSBs can arise from oxidized nucleotides/bases during oxidative stress, intermediate products of DNA repair pathways (e.g., base excision repair (BER)), and aborted activity of cellular enzymes (e.g., DNA topoisomerase 1) (Fig. 1) (1, 2). Oxidative stress is an imbalance of the generation of reactive oxygen species (ROS) and anti-oxidant agents (2). It has been estimated that more than 10,000 SSBs are generated per mammalian cell each day, representing the most common type of DNA lesions (3, 4). Unrepaired SSBs are localized primarily in nucleus and mitochondria and may result in DNA replication stress, transcriptional stalling, and excessive PARP activation, leading to genome instability (Fig. 1) (1). Accumulating evidence suggests that SSBs are implicated in the pathologies of cancer, neurodegenerative diseases, and heart failure (Fig. 1) (1, 2, 5-7).

It is generally accepted that SSBs are repaired by various DNA repair mechanisms. Rapid global SSB repair mechanism includes SSB detection, DNA end processing, DNA gap filling, and DNA ligation, which is canonical SSB repair pathway (1). The SSB repair pathway is sometimes considered as a specialized sub-pathway of BER (8). Notably, PARP1 (Poly ADP ribose polymerase 1) and XRCC1 (X-ray repair cross-complementing protein 1) play essential roles in this canonical SSB repair pathway (9-11). Alternatively, recent evidence shows that SSBs can also be resolved by either homologous recombination (HR) or alternative homologue-mediated SSB repair pathway (11, 12). Unrepaired SSBs during DNA replication can be converted to more deleterious DNA double-strand breaks (DSBs) (13). The DNA replication-derived DSBs from SSBs result in chromosome

breakages and translocations, leading to severe genome instability (14), although cohesion-dependent sister-chromatid exchange is available for repairing SSB-derived DSBs (15). More details of various SSB repair pathways can be found from several recent reviews on the topic (8, 11, 16). However, understanding how SSBs are generated, sensed, repaired, and signaled remains incomplete, largely because of the lack of efficient in vivo or in vitro experimental systems.

In this review, we will introduce a new concept "SSB end resection" in the field of genome integrity, and summarize the current molecular understanding of SSB end resection. We compare the major features of SSB end resection with DSB end resection. We then focus on the critical roles of SSB end resection in SSB signaling and repair. Finally, we identify several outstanding questions for future studies of SSB end resection. This perspective serves the first comprehensive review of SSB end resection for mechanistic studies on this topic in the field of genome integrity.

1.2. Concept of SSB End Resection

In general, SSB end resection is defined as the enzymatic end processing at SSB sites. The directionalities of SSB end resection include 3′ to 5′ direction and 5′ to 3′ direction, which are designated as 3′–5′ SSB end resection and 5′–3′ SSB end resection, respectively. After SSB end resection, an ssDNA (single-stranded DNA) gap of context-specific length is generated. Due to the technical difficulty in determining whether the resection of the two SSB ends are dependent on or mutually exclusive to each other, we cannot exclude the possibility of bidirectional SSB end resection; for simplicity, we propose the two possible SSB end resection with different directionality (i.e., 3′–5′ SSB end resection and 5′–3′ SSB end resection).

Recent studies are in support of the concept of 3′–5′ SSB end resection. It has been demonstrated that oxidative DNA damage-derived indirect SSBs are processed by APE2 (AP endonuclease 2, also known as APEX2 or APN2) in the 3′ to 5′ direction to promote ATR-Chk1 DNA damage response (DDR) pathway in *Xenopus* cell-free egg extract system (17, 18). Interestingly, it has also been shown that a defined site-specific SSB structure can be resected in the 3′ to 5′ direction by APE2 in *Xenopus* system and reconstitution experimental system (19). Importantly, it was recently demonstrated that a 9nt-gap is formed in the 5′ side of a defined SSB structure for subsequent DNA repair in living cells (20). These findings are consistent with the critical roles of 3′–5′ SSB end resection in genome integrity. Some DNA metabolism enzymes, such as TDP2 (Tyrosyl-DNA phosphodiesterase 2) and APTX (Aprataxin), may digest SSB end in the 5′ to 3′ direction, suggesting a possible mechanism of 5′–3′ SSB end resection (8, 21).

However, there are almost no in-depth studies showing whether and how the 5′–3′ SSB end resection happens. Thus, the potential biological or physiological relevance of 5′–3′ SSB end resection remains unclear. The long-patch BER pathway involves PCNA (Proliferating cellular nuclear antigen)-mediated DNA repair synthesis and FEN1 (Flap structure-specific endonuclease 1)-mediated degradation of a DNA strand (22), which is excluded from our defined 5′–3′ SSB end resection. Future investigations are still needed to test whether SSB end can be resected in the 5′ to 3′ direction in various different model systems. Thus, we focus on the 3’–5’ SSB end resection processes in this review. Here, we propose a four-step molecular mechanism involved in the processes of 3′–5′ SSB end resection (Fig. 2): (I) Step 1 is SSB end sensing and processing, (II) Step 2 is the initiation phase of SSB end resection, (III) Step 3 is the continuation phase of SSB end resection and

(IV) Step 4 is the termination of SSB end resection. In the next section, we delineate the details of these four steps for SSB end resection.

1.3. Molecular Mechanism of SSB End Resection

1.3.1. SSB end sensing and processing

During the canonical SSB repair, SSB end sensing by sensor protein such as PARP1 is critical for the subsequent DNA repair process (1, 23). SSBs with the "-OH" groups at both ends are designated as SSBs with simple ends. On the other hand, SSBs with chemically heterogeneous structures, such as 3′-Top1 adduct, 3′-phosphate, 3′-phosphoglycolate, 5′-Top2 adduct, 5′-aldehyde, 5′-deoxyribose phosphate, or 5'-adenylate (AMP), are designated as SSBs with complex ends (8, 21). These complex ends of SSBs are recognized and processed or removed by various DNA metabolism enzymes such as TDP1 (Tyrosyl-DNA phosphodiesterase 1), APE1 (AP endonuclease 1), Polymerase beta, FEN1, and APTX, among others (8, 21). Such SSB end processing is important for canonical SSB repair pathway. It remains unclear how cells decide to proceed with the canonical SSB repair pathway, or alternatively, the SSB end resection-mediated non-canonical SSB repair pathway. Mechanistic studies are needed to find out whether the SSB end processing is critical for making decisions on choice of various SSB repair pathways.

1.3.2. Initiation of SSB end resection

It is critical for cells to resect SSBs in the 3′–5′ direction only when necessary, leading to a ssDNA gap. However, such a ssDNA gap is more deleterious than just a nick or 1-nt gap in genome. Thus, this initiation phase of SSB end resection must be highly regulated

via essential regulatory mechanisms. It has been demonstrated that several DNA metabolism enzymes may resect SSBs to initiate the SSB end resection process *in vitro*.

DNA exonucleases such as APE2, APE1, and Mre11 may be involved in SSB end resection initiation. APE2 has strong 3′–5′ exonuclease activity but weak AP endonuclease activity (24, 25). It has been shown that APE2 resects ~3nt on a defined SSB structure in the 3′–5′ direction even in the absence of PCNA in vitro (24). Notably, ~1–4 nt ssDNA gap structure will significantly enhance APE2's 3′–5′ exonuclease activity in vitro (24). However, a defined SSB structure is still resected into ~1–3 nt ssDNA gap in the 3'–5' direction when APE2 is absent in *Xenopus* cell-free system (19). These observations suggest that APE2 may contribute to the initiation of SSB end resection in in vitro assays, or alternatively, that other exonuclease resects SSB in the absence of APE2 using cell-free egg extracts. Considering the requirement of ssDNA for APE2's PCNA-mediated 3′–5′ exonuclease activity, APE2 may not be the exonuclease to initiate the SSB end resection process in vivo. Therefore, it remains unclear whether APE2 initiates SSB end resection in in vivo systems. APE1 (AP endonuclease 1, also known as Ref-1 or APN1) has weak 3′–5′ exonuclease activity but strong AP endonuclease activity (26). It has been demonstrated that APE1 can resect a SSB structure into ~1–3nt ssDNA gap structure in the 3′ to 5′ direction in vitro (19, 27). Of note, APE1 can also resect 1-nt gap or 2-nt gap structures in vitro. APE1's 3′–5′ exonuclease activity was shown to prevent trinucleotide repeat expansions (28). APE1 is also shown to remove mismatches at the 3′-end of SSB site (29). Interestingly, APE1 mutants at the F266 and W280 residues significantly enhance its 3′–5′ exonuclease activity (30). Structure determinant of such APE1's 3′–5′ exonuclease activity from a SSB structure has been recently elucidated in more details (31). However, it remains

elusive whether APE1 resects SSB in the 3′ to 5′ direction in vivo. Furthermore, Mre11's exonuclease from the Mre11-Rad50-Nbs1 (MRN) complex can resect SSB with simple end in the 3′–5′ direction in reconstitution system with purified proteins (32); however, the potential role of Mre11 in SSB end resection initiation requires a nearby DSB end (33, 34). Future studies are needed to determine whether SSB structure without a nearby DSB can be resected by Mre11's 3′–5′ exonuclease activity.

Furthermore, other type of DNA metabolism enzymes such as helicase and endonuclease may also be involved in the SSB end resection initiation. It has been reported that a 9-nt ssDNA gap is formed in the 3′–5′ direction of an oxidative or alkylation lesion in living cells (20). Mechanistic studies have revealed that the ssDNA gap formation is mediated by DNA helicase RECQ1 and endonuclease ERCC1-XPF in cooperation of PARP1 and RPA, and that the ssDNA gap formation in the 5′ side of DNA lesion promotes subsequent DNA repair (20). Consistent with this observation, Rad1-Rad10 nuclease in budding yeast (counterpart of human ERCC1-XPF) can remove 3' complex end of SSB and further resect SSB several nt in the 3′–5′ direction to promote the repair of hydrogen peroxide-induced SSBs (35). In addition, it is also possible that some previously unidentified DNA exonucleases and helicases/endonucleases can initiate the SSB end resection. Unbiased *de novo* identification and functional characterization of these DNA metabolism enzymes are needed to reveal more molecular details in the initiation phase of SSB end resection.

1.3.3. Continuation of SSB end resection

After the initiation phase, SSB end resection processing is continued by DNA metabolism enzymes with higher processivity of 3′–5′ exonuclease activities. The first

important player in continuation of SSB end resection is APE2, which has strong PCNA-mediated 3′–5′ exonuclease activity. Since the apparent outcome of SSB end resection is to generate a longer stretch of ssDNA gap, the APE2-mediated SSB end resection continuation must be under tight regulations, and such 3′–5′ SSB end resection only happens when it is necessary. At least three different types of regulatory mechanisms have been suggested to determine how APE2 contributes to SSB end resection continuation:

The first regulatory mechanism is to regulate how APE2 is recruited to SSB sites. APE2 is localized in nucleus and mitochondria (36), although there is no report on underlying mechanism of how exactly APE2 is imported into these organelles, respectively. APE2 interacts with PCNA via APE2's PIP (PCNA-interacting protein) box and PCNA's IDCL (interdomain connector loop) motif, which is designed as the first mode of APE2-PCNA interaction and is critical for the recruitment of APE2 to oxidative stress-damaged chromatin DNA (17, 24, 25, 37, 38). Therefore, PCNA may play an important role in the recruitment of APE2 to SSB sites on damaged chromatin.

The second regulatory mechanism is to enhance APE2's 3′–5′ exonuclease activity via APE2 interaction with ssDNA. APE2 interaction with PCNA is not sufficient for promoting its 3′–5′ exonuclease activity. Recent studies have demonstrated that a unique Zf-GRF motif within APE2 C-terminus plays an essential role for its recognition and binding to ssDNA region and associated 3′–5′ exonuclease activity (18). Once APE2 Zf-GRF interacts with ssDNA, the conformation of the catalytic domain within APE2 N-terminus may be changed for maximum 3′–5′ exonuclease activity. More structure/function analysis is needed to clarify how such ssDNA interaction within APE2 Zf-GRF promotes its 3′–5′ exonuclease activity.

The third regulatory mechanism is to promote APE2's 3′–5′ exonuclease activity via two distinct modes of the APE2-PCNA interaction. In addition to the first mode of APE2-PCNA interaction, APE2 Zf-GRF motif interacts with PCNA's C-terminus, which is designated as the second mode of APE2-PCNA interaction (19). Several separation-of-function mutants within APE2 have been characterized in *Xenopus* APE2 to distinguish the two modes of APE2-PCNA interaction (19). Notably, the two modes of APE2-PCNA interaction are neither dependent on nor exclusive to each other. Both modes of APE2-PCNA interaction are critical to promote APE2's 3′–5′ exonuclease activity in *Xenopus* (19). Similarly, yeast APE2 binds to the PCNA IDCL motif and C-terminus to enhance its 3′–5′ exonuclease activity (25). Based on the high similarity within APE2 Zf-GRF region among different species, the critical role of the second mode of APE2-PCNA interaction for APE2's exonuclease activity is likely conserved in mammalian cells.

Notably, APE2's 3′–5′ exonuclease activity has been demonstrated and characterized in vitro from experimental model organisms including *Arabidopsis thaliana*, *Trypanosoma cruzi*, *Ciona intestinalis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Xenopus laevis*, and *Homo sapiens (19, 36, 39-43)*, suggesting that the role of APE2's 3′–5′ exonuclease activity in SSB end resection is highly conserved during evolution. APE2 3'–5' exonuclease activity is important for the removal of 3'-blocked termini to repair DNA lesions from hydrogen peroxide treatment in *Saccharomyces cerevisiae* (38). In addition, *Ciona intestinalis* APE2 has 3′–5′ exonuclease activity and contributes to protection and survival from oxidative stress (43). Human APE2 is mostly localized in the nuclei and to some extent in the mitochondria (36). Furthermore, PCNA interacts with human APE2 to stimulate APE2's 3′–5′ exonuclease activity, which is important for

removing 3′-end adenine opposite from 8-oxoG (7,8-dihydro-8-oxoguanine) and subsequent 3′–5′ end resection (37). Notably, oxidative stress also promotes the colocalization of APE2 with PCNA in living cells (37).

1.3.4. Termination of SSB end resection

The apparent outcome of the 3′–5′ SSB end resection is DNA strand degradation, making it deleterious for genome stability if the 3′–5′ SSB end resection is not terminated when necessary. In vitro data have shown that a 3′ recessed ssDNA/dsDNA structure can be resected almost completely by PCNA-mediated APE2's 3′–5′ exonuclease activity (18). It is reasoned that some regulatory mechanisms are necessary to negatively regulate APE2's 3′–5′ exonuclease activity in vivo once sufficient ssDNA gap is generated. More studies are needed to dissect the molecular details of how SSB end resection is terminated.

1.4. SSB End Resection and DSB End Resection

It is well documented that DSB end resection in the 5′–3′ direction is critical for DSB repair and DSB signaling (44-46). Notably, Mre11 contributes to DNA repair of DSB ends or protein-DNA crosslinks via its 3′–5′ exonuclease activity (32, 47). Accumulating evidence suggests that Mre11's endonuclease activity is required for generating SSBs at DSB ends, which are further resected by 3′–5′ exonuclease activity of the MRN complex and 5′–3′ exonuclease activity of EXO1 (Exonuclease 1) (Fig. 3) (33, 34, 48). This mechanism is designated as bidirectional DSB end resection (49). After EXO1's initial end resection, the 5′–3′ DSB end resection is further continued by other DNA metabolism enzymes such as DNA2, which is known as the two-step mechanism for 5′–3′ DSB end resection (Fig. 3) (50).

In contrast to DSB end resection, SSB end resection has several distinctive features. First, the directionality of SSB end resection is in the 3′ to 5′ direction, whereas overall DSB end resection is in the 5′ to 3′ direction. It remains unclear whether a SSB can be resected in the 5′ to 3′ direction. The functionalities of DNA end resection are likely through different DNA metabolism enzymes involved in the two different DNA end resection (i.e., DSB and SSB end resection) pathways. Second, the ssDNA gap generated after 3′–5′ SSB end resection is relatively short (~18–26 nt), whereas the ssDNA after DSB end resection is large (~800 nt) (Fig. 3) (19, 48). Reconstitution evidence suggests that CtIP/Sae2 promotes endonuclease activity within Mre11 to make SSB from a nearby protein-occluded DSB end (51, 52). Although the size of ssDNA region generated from DSB end resection or SSB end resection is different, the RPA-coated ssDNA serves the platform for assembly of the DDR protein complex to trigger ATR-Chk1 DDR pathway activation (Fig. 4). Third, the 3′–5′ SSB end resection near a DSB end requires Mre11 exonuclease activity, whereas 3′–5′ SSB end resection without a nearby DSB end requires APE2 exonuclease activity (Fig. 3) (19, 53). Interestingly, it appears that while APE2 and Mre11 have related functions, they cannot compensate for the absence of each other in regards to 3′–5′ SSB end resection with or without DSB end, respectively.

1.5. Roles of SSB End Resection in SSB Signaling, SSB Repair, and Beyond

The 3′–5′ SSB end resection mediated by APE2 is essential for the ATR-Chk1 DDR pathway following oxidative stress in *Xenopus* egg extracts (17, 18, 54-57). Especially, APE2's 3′–5′ exonuclease activity is particularly critical for oxidative stress-induced DDR pathway activation (17). Furthermore, a defined site-specific SSB structure triggers ATR-Chk1 DDR pathway in a DNA replication-independent fashion in *Xenopus* egg extracts

(19). Notably, APE2's 3′–5′ exonuclease activity is essential for the defined SSB-induced ATR-Chk1 DDR pathway, whereas CDK (Cyclin-dependent kinase) kinase activity is dispensable for the SSB-induced DDR pathway (19). Furthermore, the APE2-mediated 3′–5′ SSB end resection is required for ssDNA generation and assembly of ATR, ATRIP, TopBP1, and the 9-1-1 (Rad9-Rad1-Hus1) complex onto SSB sites to trigger the ATR-Chk1 DDR pathway (Fig. 4) (19). Therefore, the APE2-mediated 3′–5′ SSB end resection is essential for SSB signaling.

Oxidative DNA damage-derived SSBs can trigger ATM-Chk2 DDR pathway activation in mammalian cells (58). Although unrepaired SSBs in XRCC1-deficient cells trigger ATM activation to prevent the generation of DSBs, the underlying mechanism of SSB-induced ATM DDR pathway activation remains unclear. It has been shown that one-end DSB is generated when DNA replication fork meets with SSB (Fig. 4) (13). Again, replication-derived BER-processed SSBs from methylation damage can trigger checkpoint signaling such as γ-H2AX, leading to chromatid breaks and chromosome translocations (14). It is conceivable that replication-derived DSBs from SSBs can activate the ATM-Chk2 DDR pathway (Fig. 4). In addition, such DSBs from SSBs during replication can be repaired by cohesion-dependent sister-chromatid exchange (15).

What is the role of 3′–5′ SSB end resection for SSB repair? XRCC1-mediated canonical SSB repair have been revealed in reconstitution system with recombinant human proteins or cultured mammalian cells (59, 60). XRCC1 promotes PNKP (Polynucleotide kinase phosphatase)-mediated SSB end termini processing, followed by gap filling by DNA polymerase beta and sealing by DNA ligase 3. More details of the canonical SSB repair can be found from recent comprehensive reviews (1, 11). It has been demonstrated

that defined plasmid-based SSB structure is repaired in about 30 min in the *Xenopus system* (19). Because of the involvement of 3′–5′ SSB end resection, this distinct repair pathway is designated as non-canonical SSB repair. The capacity of SSB repair in *Xenopus* system is comparable to that characterized in human SSB repair systems (59). However, it remains unclear how the XRCC1-mediated canonical SSB repair pathway and the APE2-mediated non-canonical SSB repair pathway contribute to the overall SSB repair. Do they work coordinately or independently? Notably, one distinct feature of the non-canonical SSB repair pathway is dependence on the ATR-Chk1 DDR pathway activation in *Xenopus* egg extracts (19).

Although the precise mechanism underlying the non-canonical SSB repair remains to be elucidated, we speculate two possible mechanisms: one or more SSB repair regulators are phosphorylated by ATR or Chk1 kinases, which are required for promoting SSB repair; alternatively, efficient SSB repair is suppressed by an inhibitory factor that can be phosphorylated by ATR or Chk1 to relieve the suppression. Although it is currently unknown whether the role of APE2 in SSB end resection-mediated non-canonical SSB repair is conserved in mammalians, previous studies have shown that APE2 is important for overall SSB repair following oxidative stress in mammalian cells (36, 37). The APE2-mediated non-canonical SSB repair in *Xenopus* has important implications in mammalian cells, especially in terminally differentiated cells such as neuron cells, most of which remain in the G0 or G1 phase of the cell cycle.

Unrepaired SSBs are implicated in human diseases such as neurodegenerative disorders, cancer, and heart failure (1, 5-7). SSB repair has been associated with hereditary genetic diseases including Ataxia-oculomotor apraxia 1 (AOA1) and spinocerebellar ataxia

with axonal neuropathy 1 (SCAN1)(60). Both germline and tumor-associated variants of genes encoding SSB repair proteins (e.g., XRCC1, APE1, and Polymerase beta) have been identified in humans, suggesting SSB repair as a tumor suppressor mechanism (61). However, it remains unclear whether the SSB-induced SSB signaling or the associated SSB repair pathways play direct or indirect roles in tumorigenesis. It has been shown recently in senescent epithelial cells that ROS induces more SSBs and downregulates PARP1 expression, leading to defective SSB repair and emergence of post-senescent transformed and mutated precancerous cells (5). Thus, the mutagenicity of accumulated unrepaired SSBs in epithelial cells is proposed as the driver of cancer development (62). APE2 mutants have been found in several cancer patients, suggesting that defective SSB end resection is implicated in cancer development (19).

Future mechanistic studies using mammalian cell lines and genetically engineered mouse models will allow us to better understand how APE2-medaited SSB end resection is involved in cancer development. Interestingly, accumulation of SSBs was found in cardiomyocytes of the failing heart and unrepaired SSB triggers DDR pathway, and increases inflammatory response through NF-κB signaling (6). In addition, newly defined distinct homology-dependent SSB repair pathways are proposed to support gene correction or editing using ssDNA donors at sites of SSBs (12, 63). The initiation of HR at SSBs is distinct from HR from DSB sites, and a current understanding of SSB-induced HR is summarized in a recent review (16). Together, findings from SSB end resection studies will contribute to the field of genome integrity.

1.6. Concluding Remarks and Perspectives for Future Studies

Although we have just begun to understand role and mechanism of SSB end resection in genome integrity, many significant questions in studies of SSB end resection remain unanswered. What is the molecular mechanism underlying the initiation phase of SSB end resection in vivo? Although a few DNA nucleases demonstrate 3′–5′ exonuclease activity in vitro, it is vital to determine how SSB end resection is initiated exactly. We speculate that type and complexity of SSB ends (e.g., simple ends or complex ends) may be important for the initiation of SSB end resection. How is SSB end resection terminated or negatively regulated? Whereas SSB end resection initiates and continues in the 3′–5′ direction, leading to ssDNA generation, some regulatory mechanisms should be in place to terminate SSB end resection when necessary. Otherwise, a longer stretch of ssDNA will be generated, leading to more severe genome instability such as DSBs and chromosome translocations.

How does cell make decisions to repair SSBs via canonical or non-canonical SSB repair pathway? We speculate that most SSBs are repaired via XRCC1-mediated canonical SSB repair pathway, and that APE2-mediate non-canonical SSB repair may take place only when the amount of SSBs is more than a repair threshold. Although non-canonical SSB repair requires ATR DDR pathway (19), future work is still needed to reveal molecular details of this non-canonical SSB repair. Will nucleosome and chromatin remodeling complex at or near SSB sites regulate SSB end resection? A recent report has demonstrated that PARP3 recognizes site-specific SSB in nucleosome and monoribosylates Histone 2B in DT40 cells (64). It is also shown that SNF2 chromatin remodeling protein ALC1 is important for chromatin relaxation and SSB repair (65). Thus, it is important to determine

whether SSB end resection is regulated by the context in chromatin including nucleosome and chromatin remodeling complex.

Various experimental systems including the *Xenopus* egg extract system and mammalian cells in culture have been developed to study SSB repair and signaling. The *Xenopus* egg extracts system has been utilized and optimized to dissect different aspects of SSB end resection directly: hydrogen peroxide-induced indirect SSBs on chromatin DNA in *Xenopus* low speed supernatant and defined site-specific plasmid-based SSBs in *Xenopus* high-speed supernatant (17-19). Thus, *Xenopus* egg extracts system provides an excellent experimental system to reveal the molecular details of replication-dependent and -independent SSB end resection in SSB repair and signaling.

In addition, SSBs are accumulated after treatment of DNA damaging agents (e.g., methyl methane-sulfonate and hydrogen peroxide) in mammalian cells such as terminally differentiated muscle cells and cardiomyocyte (6, 66). SSBs are also generated when BER proteins such as XRCC1 is knocked down or deficient in mammalian cells (6, 58). SSBs, but not DSBs, can be induced after local UVC irradiation in XPA-UVDE cells which express UV damage endonuclease (UVDE), but which are deficient in nucleotide excision repair protein XPA (67, 68). Site-specific SSB can be introduced by transient transfection with Cas9 and gRNA expression vectors in human osteosarcoma cells (U2OS-DR-GFP) and mouse embryonic stem cells (ES-DR-GFP) harboring a single genetically integrated copy of the DR-GFP reporter (16, 69). A recent study has demonstrated that a small ssDNA gap is generated in the 5' side of SSB in plasmid-transfected cells, suggesting that the 3′–5′ SSB end resection is conserved in mammalian cells (20). Future studies of the

outstanding questions using these various experimental systems will provide a better understanding of all aspects of SSB end resection in genome integrity.

Taking together, we introduce the concept and mechanism of SSB end resection and summarize the current understanding on the biological significance of SSB end resection in genome integrity.

CHAPTER 2: APE2 IS A CRITICAL REGULATOR OF THE DNA DAMAGE RESPONSE TO MAINTAIN GENOME INTEGRITY IN PANCREATIC CANCER CELLS

ABSTRACT

The maintenance of genome integrity and fidelity is vital for the proper function and survival of all organisms. Recent studies have revealed that APE2 is required to activate an ATR-Chk1 DNA damage response (DDR) pathway in response to oxidative stress and a defined DNA single-strand break (SSB) in *Xenopus laevis* egg extracts. However, it remains unclear whether APE2 is a general regulator of the DDR pathway in mammalian cells. Here, we provide evidence using human pancreatic cancer cells that APE2 is essential for ATR DDR pathway activation in response to different stressful conditions including oxidative stress, DNA replication stress, and DNA double-strand breaks. Fluorescence microscopy analysis shows that APE2-knockdown (KD) leads to enhanced γH2AX foci and increased micronuclei formation. In addition, we identified a small molecule compound Celastrol as an APE2 inhibitor that specifically compromises the binding of APE2 to ssDNA, its 3′-5′ exonuclease activity, and the defined SSB-induced ATR Chk1 DDR pathway in *Xenopus* egg extracts. Notably, cell viability assays demonstrate that APE2-KD or Celastrol sensitizes pancreatic cancer cells to chemotherapy drugs. Overall, we propose APE2 as a general regulator for the DDR pathway in genome integrity maintenance.

2.1. Introduction

Cells undergo continuous bombardments of exogenous and endogenous factors that can lead to genomic instability. It is critical for a cell to maintain genome integrity and fidelity for proper cellular function and survival in stress conditions. This task is daunting due to constant insults on the DNA by genotoxic agents, nucleotide mis-incorporation or deprivation during DNA replication, and the intrinsic biochemical instability of the DNA itself (70). Both exogenous and endogenous sources can result in DNA replication stress and/or DNA lesions that include DNA double-strand breaks (DSB), DNA single-strand breaks (SSBs), and oxidative DNA damage (2-4). Although cells have evolved several different DNA repair pathways to resolve DNA lesions, deficiency in DNA repair pathways or failure to resolve replication stress may result in blockage or collapse of replication and transcription machinery, leading to cellular cytotoxicity, mutagenesis, and/or cell death (2, 71). In humans, DNA lesions are involved in numerous genetically inherited disorders, aging and carcinogenesis (4, 71). In response to DNA damage, cells have also evolved the DNA damage response (DDR) pathways to coordinate DNA repair, transcription activation, cell cycle progression, and cell death (3, 72). ATM (Ataxia telangiectasia mutated) and ATR (ATM & Rad3-related) kinases are the key regulators in DDR pathways. Whereas ATM-mediated DDR pathway is primarily activated in response to DSBs, ATR-mediated DDR pathway is triggered by several types of stressful conditions, including DNA replication stress, oxidative stress, SSBs, and DSBs (73-75). The ATR DDR pathway is critical for duplicating DNA under stressful conditions (76), and ATR inhibitors as either monotherapy or combination therapy have been in different phases of clinical trials of cancer patients (77, 78).

Depending on the nature and context of DNA damage or replication stress, the ATR DDR pathway is activated by different regulatory mechanisms. It has been proposed that single-strand DNA (ssDNA) coated with RPA (i.e., RPA-ssDNA) together with a 5′-ssDNA/dsDNA junction may serve as a platform to recruit ATR DDR complexes including ATR, ATRIP, TopBP1, and the Rad9-Rad1-Hus1 (9-1-1) complex for ATR activation (75, 79). In DNA replication stress, stalled DNA replication forks induced by aphidicolin or gemcitabine (GEM) uncouple helicase and DNA polymerases, generating RPA-ssDNA for ATR activation (80-82). In response to DSBs induced by γ-radiation, Topoisomerase I inhibitor Camptothecin (CPT), and Topoisomerase II inhibitor Etoposide (ETO), ATR can also be activated by ssDNA derived from bidirectional DSB end resection by different endonucleases and exonucleases such as Mre11 and Exo1 (48, 83, 84). Oxidative DNA damage induced by hydrogen peroxide (H_2O_2) also activates ATR DDR pathway by generating ssDNA at oxidative damage sites (17, 18). Recent studies have demonstrated that defined SSB structures can activate the ATR DDR pathway via a distinct 3′-5′ end resection mechanism that generates necessary short ssDNA (19, 85).

APE2 (Apurinic/apyrimidinic endonuclease-2, also known as APEX2 or APN2) is an evolutionarily conserved protein with strong 3′-phosphodiesterase and 3′-5′ exonuclease activities but weak AP endonuclease activity and has been implicated in genome and epigenome integrity maintenance (37, 86). Prior studies using different model systems have shown that APE2 plays crucial roles in DNA repair pathways including the base excision repair (BER) pathway, SSB repair pathway, DSB generation and DSB repair pathway, DDR pathways including the ATR-Chk1 DDR pathway and p53-dependent DDR pathway, immune responses including immunoglobulin somatic hypermutation (SHM) and class

switch recombination (CSR), and active DNA demethylation (17, 19, 24, 37, 38, 87-94). Furthermore, APE2 has been implicated in development and growth as well as cancer etiology. A prior study has shown that APE2-knock out (KO) mice are viable but display growth retardation (95). Accumulating evidence has shown genomic alterations and abnormal expression of APE2 expression in multiple cancer tissues, including pancreatic cancer and multiple myeloma (MM), and APE2 is proposed to function as an oncogene in liver cancer (96-98).

Although the underlying molecular mechanism remains to be determined, recent genetic screens identified APE2 as a synthetic lethal target in BRCA1- or BRCA2-deficient human colon cancer cell line DLD-1, human ovarian cancer cell line PEO1, or engineered human epithelial cell line RPE1-hTERT under unperturbed conditions (90, 99). It has been demonstrated in recent series of studies using *Xenopus* egg extracts that APE2 is critical for the ATR-Chk1 DDR pathway in response to oxidative DNA damage and defined SSB structures (17-19). Mechanistically, APE2 is recruited to oxidative stress-derived SSB sites or defined SSB structures for a distinct 3′-5′ SSB end resection via its 3′-5′ exonuclease activity, leading to RPA-ssDNA, assembly of the ATR DDR complex including ATR, ATRIP, TopBP1, and the 9-1-1 complex, and activation of the ATR DDR pathway (17-19, 85). Moreover, APE2 recruitment and activation require its interaction with ssDNA via its C-terminal Zf-GRF motif and two modes of association with PCNA via its Zf-GRF motif and PCNA-Interacting Protein box (PIP) (18, 19). APE2 directly associates with and brings Chk1 to the activated ATR for phosphorylation (17). However, it remains largely unknown whether and how APE2 regulates the ATR DDR pathway in response to different stressful conditions in mammalian cells.

With a ~9% five-year survival rate for all stages combined, pancreatic cancer ranks the fourth most common form of cancer-related deaths in the US, nearly 57,600 estimated new cases and over 55,000 estimated deaths in 2020 (100). Although GEM has been the standard treatment of pancreatic cancer, the clinical effect of GEM monotherapy remains limited due to low overall survival months and efficacy (101). In contrast, new therapy regimen such as a modified FOLFIRINOX regimen (a combination of fluorouracil, leucovorin, irinotecan, and oxaliplatin) as an adjuvant therapy after surgical resection of pancreatic cancer is still developing (102). A combination of GEM with radiotherapy or other chemotherapy drugs such as ATR inhibitor AZD6738 shows great premise in pancreatic cancer regression (103). Targeting ATR has emerged as a new area of research for cancer treatment (77, 78, 104), it is reasonable to investigate and explore innovative therapy via targeting the ATR-Chk1 DDR pathway's regulatory mechanisms to increase efficacy and/or reduce the toxicity of chemotherapy drugs in pancreatic cancer treatment.

This study provides evidence using pancreatic cancer cell lines that activation of the ATR-Chk1 DDR pathway induced by hydrogen peroxide (H_2O_2), GEM, CPT, and ETO is compromised when APE2 is down-regulated via siRNA. Furthermore, siRNA-mediated APE2-knockdown (KD) leads to a higher percentage of γH2AX-positive cells and micronuclei-positive cells. These results suggest that APE2 is a general regulator of the ATR-Chk1 DDR pathway to maintain genome integrity. In addition, we found that Celastrol, a natural compound derived from thunder god vine *Tripterygium wilfordii* (105), impaired APE2 interaction with ssDNA and APE2 3′-5′ exonuclease activity *in vitro* and also compromised the defined SSB-induced ATR-Chk1 DDR pathway in *Xenopus* egg extracts. Notably, the ATR-Chk1 DDR pathway activation induced by H_2O_2, GEM, CPT,

and ETO in pancreatic cancer cells was compromised by the addition of Celastrol. Cell viability assays demonstrated that APE2 suppression via siRNA-mediated KD or the addition of Celastrol sensitized pancreatic cancers to chemotherapy drugs. Our evidence suggests that APE2 regulates the ATR DDR pathway in pancreatic cancer cells and that targeting the novel function of APE2 in ATR DDR may open a new avenue for future therapeutics in pancreatic cancers.

2.2. Materials and methods

Cell culture, treatments and cell lysate preparation

PANC1 and MiaPaCa2 cells were purchased from ATCC (Cat#CRL-1469 and CRL-1420) and cultured in complete media (DMEM (Corning) supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin (Gibco)) for PANC1 or completed media with 2.5% Horse Serum (Sigma) for MiaPaCa2, respectively. To treat cells, H_2O_2 (Sigma Cat#HX0635, 100-1250μM), Gemcitabine (GEM, Sigma Cat#G6423, 1-500μM) Camptothecin (CPT, Calbiochem Cat#208925, 1-5μM), Etoposide (ETO, Calbiochem Cat#341205, 1.25-50μM), VE-822 (Selleckcham Cat#S7102), KU55933 (EMD Millipore Cat#118500), or Celastrol (Sigma Cat#219465, 0.5μM-1mM) was dissolved in water or DMSO depending on the solubility of the small molecule or reagent, and added to complete media to the final concentrations as indicated in the individual experiments.

Briefly, cells were washed with phosphate-buffered saline, PBS (Gibco Cat#10010023) and trypsinized (Corning Cat#25-053-CI). The cells were collected by centrifugation and resuspended in ice-cold PBS followed by centrifugation. Cultured cells were lysed with in lysis buffer (20 mM Tris-HCL pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40,

0.5 mM Na_3V0_4, 5 mM NaF, 5 μg/mL of Aprotinin and 10 μg/mL of Leupeptin). Lysates were centrifugated at 13,000 rpm for 30 minutes at 4°C. The supernatants were transferred into fresh tubes for measuring protein concentrations via Bradford assays (BIO-RAD Cat#5000205) and subsequent immunoblotting analysis.

Recombinant DNA, plasmid DNA, and FAM-labeled DNA structures, and recombinant proteins

pcDNA3-YFP was a gift from Doug Golenbock (Addgene plasmid Cat#13033; http://n2t.net/addgene:13033; RRID: Addgene_13033). Recombinant pcDNA3-YFP-xAPE2 was prepared by subcloning the full-length of xAPE2 into pcDNA3-YFP at EcoR1 and XhoI sites. Briefly, the coding region of xAPE2 was amplified by PCR with a forward oligo (5'-GGGGGGAATTCATGAAGATTGTGAGCTGGAACATCAATG-3') and a reverse oligo (5'-GGGGGCTCGAGGTCCTCACATCCAGCTTTTTTGGTGAG-3'). Purified PCR product and pCDNA3-YFP were catalyzed by EcoRI (New England Biolabs Cat#R3101) and XhoI (New England Biolabs Cat#R0146) and ligated together by T4 DNA ligase (New England Biolabs Cat#m0202). After transformation into DH5alpha *E. coli*, plasmids were prepared via QIAprep Spin Miniprep Kit (QIAGEN Cat#27106) following vendor's protocol. In addition, the control (CTL) plasmid, SSB plasmid, FAM-labeled 70-nt ssDNA, and FAM-labeled 70-bp dsDNA with a gap structure, were described previously (19, 87, 106). The pET32a-hAPE2 was described previously (36). The expression and purification of recombinant protein GST-Zf-GRF, GST-xAPE2, and His-tagged xPCNA has been described recently (17, 19, 87). The His-tagged human APE2 recombinant protein was expressed and purified as described previously (36).

Protein-DNA interaction assays and in vitro exonuclease assay

The method for the ssDNA-bead binding assays in Fig. 6D & 9C was described recently (19). The Input and Bead-bound fractions were analyzed via immunoblotting analysis. For the Electrophoretic mobility shift assays (EMSA) assays in Fig. 9D and 9E, similar method has been described previously (19, 87). The method for the *in vitro* exonuclease assay of APE2 in Fig. 9F was described previously (19).

Experimental procedures for Xenopus egg extracts & SSB-induced DDR pathway assays

The Institutional Animal Care and Use Committee (IACUC) at University of North Carolina at Charlotte approved the care and use of *Xenopus laevis*. The preparation of *Xenopus* HSS and the setup of SSB-induced DDR pathway assays were described previously (19, 54, 55, 57, 106, 107).

Immunoblotting analysis and antibodies

Immunoblotting analysis of cell lysates or *Xenopus* egg extracts was carried out similarly as we described previously (17-19, 87). Primary antibodies against Chk1 (Santa Cruz Biotechnology Cat#sc-8408), Chk1 phosphorylation Ser345 (Cell Signaling Technology Cat#133D3), RPA32 (ThermoFisher Scientific Cat#MA1-26418), RPA32 phosphorylation Ser33 (Bethyl Laboratories Cat#A300-246A), p53 phosphorylation Ser15 (Cell Signaling Technology Cat#9284S), and Tubulin (Santa Cruz Biotechnology Cat#sc-8035) were purchased from various vendors. Anti-human APE2 antibodies were prepared as described previously (36).

Transfection and siRNA-mediated APE2-KD assays

For siRNA experiments, APEX2 siRNA (Dharmacon-HorizonDiscovery ON-TARGETplus Human APE2 siRNA Cat#L-013730-01-0005) or control siRNA (Dharmacon-HorizonDiscovery ON-TARGETplus non-targeting siRNA Cat#D-001810-01-05) was mixed with Lipofectamine^{R} RNAiMAX (ThermoFisher Scientific Cat#13778100) in Opti-MEM I Reduced Serum Medium (Gibco Cat#31985070) and incubated for 3-5 days according to the manufacture's protocol. The target sequences of the Dharmacon APE2 siRNA include 5'-GAGCCAUGUGAUGCGUA-3', 5'-CAACAAUCAAACCCGGGUA-3', 5'-GGACGAGCUGGAUGCGGAU-3', & 5'-GAGAAGGAGUUACGGACCU-3', whereas the non-targeting siRNA sequence is 5'-UGGUUUACAUGUCGACUAA-3'. For the rescue experiments in Fig. 1A and S1B, after siRNA-mediated APE2-KD, transfecting control plasmid pcDNA3-YFP (Addgene Cat#13033) or pcDNA3-YFP-xAPE2 with Lipofectamine 2000 (ThermoFisher Scientific Cat#116680019) in Opti-MEM I Reduced Serum Medium. After different treatment and incubation, cells were imaged via fluorescence microscopy to ensure YFP or YFP-xAPE2 was expressed in cells.

FACS analysis

Samples were prepared following standard cell cycle analysis by DAPI staining. Briefly, isolated resuspended in PBS, and fixed by cold 70% ethanol overnight at 4°C. After centrifugation at 3,000 rpm for 5 minutes at 4°C, the fixed cells were resuspended in DAPI staining buffer (50µg/mL of DAPI and 20 µg/mL of RNase A in 1x PBS) for 30 minutes at room temperature and analyzed via FACS analysis (FORTESSA FLOW-

CYTOMETER).

Immunofluorescence analysis

Cells were fixed in 3% formaldehyde solution for 15 min at room temperature and permeabilized with 2% Triton-X 100. Cells were then incubated with antibodies again γH2AX (EMD Millipore Cat#05-636-AF488, anti-phospho Histone H2AX Ser139-Alexa Fluor 488 conjugate) or APE2 (GeneTex Cat#GTX80642) overnight at 4°C. For APE2 experiment, goat anti-rabbit IgG H&L-conjugated with Alexa Fluor 594 (Abcam Cat#ab150080) was probed as the secondary antibodies. Then cells were mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen Cat#36941) before immunofluorescence imaging by confocal laser scanning microscope (Olympus Fluoview FV1000) or upright fluorescence microscope (Leica DM6 B) analyses.

Cell viability assay

Cell viability assay was carried out to assess percentage of viable cells via CellTiter-GLO 2.0 assays (in experiments in Fig. 11B, 11E, and 12G) or MTT (Thiazolyl blue tetrazolium bromide) assays (other cell viability assay experiments). We performed both of the techniques and got similar kind of results by analyzing the raw data of absorbance values (in MTT) or luminescence values (in CellTiter-GLO 2.0) using Microsoft Excel Spreadsheet. Pancreatic cells were seeded at 3,000 cells/well in transparent 96-well plats for MTT assays or Opaque 96-well plates for CellTitreGLO-2.0 assays. 20 µl of MTT reagent (Acros Organics Cat#158992500) was added per well and incubated at 37°C for 3.5 hours, whereas 100 µl of CellTiter-GLO 2.0 reagent (Promega) was incubated at each well at room temperature for 10 minutes. For MTT assays, cell medium was removed and

150 µl of MTT solvent (VWR Chemicals, Isopropyl ethanol and 37M Hydrochloric acid) was added to each well for a 10-minute incubation with rocking and a subsequent 5-minute incubation without rocking. MTT (absorbance, abs) and CellTiter-GLO 2.0 (luminescence, lum) values were determined by SpectraMAX iD5 Multiplate Reader (Thermoscientific). The MTT/CellTiter-GLO 2.0 values were calculated based on Percentage (%) = [100 × (sample abs/lum)/ (control abs/ lum)]. MTT/CellTiter-GLO 2.0 assay analyses using Microsoft Excel were performed in triplicates (n=3). Data are presented as mean ± SD for the error bars and normalized with no treatment group.

DNA-Bead binding assays

ss80nt DNA-bead binding assays showed in Fig 3E, 20 µl of beads (QIAGEN, Ni-NTA Agarose beads, 25ml, Cat#018244) are washed in 1ml of B&W buffer (3 times), then concentrated on magnet stand and then resuspended in 40 µl 2X B&W buffer to start for the bead processing part of this assay. 3µl of B-80 (ssDNA 80nt) and 37 µl of H_2O was added for pulldown at room temperature in the rotating stand for 15 mins, then washed 3 times in 1 ml B&W buffer and concentrated in Magnet stand and lastly resuspended with 100 µl Buffer A plus. During that time, samples were prepared parallelly such as 1µl of sample protein (purified human APE2, stock 5µg/µl) is added into 49 µl of H_2O to make a 50-fold dilution to get the 200ng/µl final conc. of human APE2 protein. From 50 µl mixed sample, 5 µl was taken for the inputs where treatments were given according to the experimental design and left over 45 µl of mixed sample was added to the 100 µl processed beads in buffer A plus solution. After incubation at 4^{o}C for 30 min, aliquots of sample and bead mixture were collected as Input, and the beads were washed with Buffer A for three times. The Input and Bead-bound fractions were analyzed via immunoblotting analysis.

Statistical analyses

GraphPad PRISM 8 statistical analysis software was used to perform statistical analysis of γH2AX/micronuclei-positive cells in Fig. 7C, 7D, 7F, 8C, 8D, and 8H. Data were presented as mean ± SD from three experiments. A paired two-sided t-test was conducted to determine significance of difference. $P<0.05$ is considered significant and $P<0.01$ is considered highly significant. MTT/CellTiter-GLO 2.0 assay (Cell Survival assays) analyses using Microsoft Excel were performed in triplicates (n=3). Data are presented as mean ± SD for the error bars and normalized with no treatment group.

2.3. Results

APE2 is important for the ATR-Chk1 DDR pathway in different stressful conditions in pancreatic cancer cells

Our series of studies using *Xenopus* egg extracts have demonstrated that APE2 is important for the ATR DDR pathway in oxidative stress (17, 18). To determine the role of APE2 in the ATR DDR pathway in pancreatic cancer cells, we first established that H_2O_2 triggered Chk1 and RPA32 phosphorylation in pancreatic cancer PANC1 cells and that ATR-specific inhibitor VE-822 prevented H_2O_2-induced Chk1 and RPA32 phosphorylation (Fig. 6A). Notably, the H_2O_2-induced Chk1 phosphorylation and RPA32 phosphorylation were compromised in APE2-KD cells (Lane 2 vs. Lane 4, Fig. 5A). To validate the phenotype of oxidative stress-induced ATR DDR pathway is due to APE2 reduction, we performed complementation assays by transfecting recombinant plasmid of full-length *Xenopus* APE2 tagged with YFP (YFP-xAPE2), that cannot be targeted for protein reduction by APE2-siRNA, or control plasmid of YFP in APE2-KD PANC1 cells

(Lane 5-8, Fig. 5A). Using this siRNA-resistant YFP-xAPE2 approach, we showed that YFP-xAPE2 but not YFP rescued the H_2O_2-induced Chk1 and RPA32 phosphorylation in APE2-KD PANC1 cells (Lane 4, 6, and 8, Fig. 5A). Our control experiment showed that the expression of YFP-xAPE2 and YFP was similar in APE2-KD PANC1 cells regardless of H_2O_2 treatment (Fig. 5B). These observations suggest that APE2 is critical for the ATR-Chk1 DDR pathway in oxidative stress in PANC1 cells. To exclude the possible cell-specific role of APE2 in the ATR DDR pathway, we performed similar experiments in MiaPaCa2 cells and found that APE2 is also essential for the H_2O_2-induced ATR-Chk1 DDR pathway in MiaPaCa2 cells (Fig. 6B-C). Thus, the above findings demonstrate the critical role of APE2 in the ATR-Chk1 DDR pathway following oxidative stress in pancreatic cancer cells.

To test whether APE2 is a general regulator in the activation of the ATR-Chk1 DDR pathway, we investigated other stressful conditions such as GEM-induced stalled DNA replication forks and CPT/ETO-induced DSBs. Consistent with the ATR DDR pathway by H_2O_2-induced oxidative stress, Chk1 and RPA32 phosphorylation was triggered by GEM, CPT, or ETO in PANC1 cells with the treatment of control (CTL) siRNA but not APE2-siRNA, suggesting that APE2 has a role in ATR DDR under various stressful conditions in PANC1 cells (Fig 5C). We also noted similar findings of APE2 in the regulation of the ATR-Chk1 DDR pathway under these different stressful conditions in MiaPaCa2 cells (Fig. 5D). Overall, these observations suggest that APE2 regulates the ATR DDR pathway in response to different stressful conditions in human pancreatic cancer cells.

APE2-KD by siRNA leads to severe DNA damage and more micronuclei in pancreatic cancer cells

To determine the role of APE2 in protecting cells from various stressful conditions, we chose to measure γH2AX status in pancreatic cancer cells under normal or damaging environments (e.g., treatment of H_2O_2, GEM, CPT, or ETO). Our fluorescence microscopy analysis shows that the percentage of γH2AX-positive cells in APE2-KD PANC1 cells was higher than that in control siRNA PANC1 cells regardless of the treatment of H_2O_2 or GEM (Fig. 7A-C). We also noted similar observations from the treatment of CPT or ETO (Fig. 7D). Furthermore, we found that APE2-KD by siRNA led to severe γH2AX in MiaPaCa2 cells under normal conditions or after treatment of H_2O_2, CPT, or ETO (Fig. 8A-F). These observations suggest that APE2 may protect pancreatic cancer cells from DNA damage such as SSBs and DSBs from both endogenous and exogenous sources.

A recent study has shown the critical function of APE2 in the regulation of homologous recombination-mediated DSB repair in MM (96). Micronuclei, a common feature of chromosome instability, are formed due to mitotic errors that mis-segregate intact chromosomes, errors in DNA replication, or repair defects that generate acentric chromosome fragments (108, 109). To further validate the critical role of APE2 in DSB repair, we examined the micronuclei formation in pancreatic cancer cells under normal or DSB-generating conditions. Our microscopy analysis demonstrates that more percentage of micronuclei-positive cells were observed in APE2-KD PANC1 cells regardless of the treatment of CPT or ETO (Fig. 7E, 7F). We also observe similar results on the role of APE2 in micronuclei formation in MiaPaCa2 cells (Fig. 8G-H). These observations of severe γH2AX and micronuclei formation in APE2-KD cells suggest APE2's function in resolving the stressful environments, consistent with APE2's function in the DDR pathway.

Function of APE2 in the SSB-induced ATR DDR pathway is compromised by a distinct APE2 inhibitor Celastrol in Xenopus egg extracts

To translate the basic mechanisms of APE2 function in the DDR pathway into future cancer therapy, we sought to identify small molecule inhibitors of APE2 functions. From an unbiased screen of 9,195 compounds, four small-molecule compounds (Dihydrocelastryl, Anthothecol, Erysolin, and MARPIN) were identified to selectively inhibit Chk1 phosphorylation induced by stalled DNA replication forks in p53-deficient cells (110). Although the underlying mechanism remains unclear, Dihydrocelastryl is structurally similar to Celastrol, a natural compound derived from thunder god vine and has been implicated in pancreatic and prostate cancer chemotherapies as a HSP90 modulator and/or proteosome inhibitor (Fig. 9A) (111, 112). Recently, we characterized the requirement of APE2 in the ATR-Chk1 DDR pathway activation in response to defined SSB structures in the *Xenopus* high-speed supernatant (HSS) system (19, 55, 106, 107). The Celastrol treatment compromised Chk1 phosphorylation induced by defined SSB plasmid but not in control (CTL) plasmid in the *Xenopus* HSS system (Fig. 9B).

Next, we sought to elucidate how Celastrol regulates the ATR-Chk1 DDR pathway. Due to the significance of the C-terminal Zf-GRF motif of APE2 in the SSB-induced ATR DDR pathway, we first tested whether Celastrol affects the binding of APE2 Zf-GRF motif to ssDNA. Our GST-pulldown experiments show that GST-Zf-GRF but not GST binds to bead coupled with ssDNA, consistent with the finding of Zf-GRF-ssDNA interaction from previous studies (18, 19). Notably, the binding of Zf-GRF to ssDNA was compromised by Celastrol (Fig. 9C). Furthermore, EMGS assays demonstrated that GST-Zf-GRF but not GST can associate with 70-nt ssDNA in vitro (Fig. 9D), and that such Zf-GRF-ssDNA

association was compromised by Celastrol (Fig. 9E). Notably, Celastrol impaired the 3′-5′ SSB end resection of dsDNA with a gapped structure by recombinant APE2 and PCNA in *in vitro* exonuclease assays, suggesting that APE2 3′-5′ exonuclease activity is inhibited by Celastrol (Fig. 9F). Thus, we identified Celastrol as an uncharacterized small molecule inhibitor of APE2 for its function in SSB end resection and SSB signaling function.

Celastrol impairs the ATR-Chk1 DDR pathway in pancreatic cancer cells

As the binding of recombinant human APE2 protein to ssDNA was compromised by Celastrol (Fig. 6D), we tested whether the inhibitory function of Celastrol in the ATR-Chk1 DDR pathway is conserved in pancreatic cancer cells. Particularly, Chk1 phosphorylation and RPA32 phosphorylation induced by H_2O_2, GEM, CPT, or ETO were impaired by Celastrol in PANC1 cells and MiaPaCa2 cells (Fig. 10A-B). Furthermore, FACS analysis shows that H_2O_2-induced oxidative stress may lead to S phase arrest. Consistent with the inhibitory effect of Celastrol in the checkpoint function of the ATR DDR pathway, the treatment of Celastrol in PANC1 cells impaired the S phase arrest by oxidative stress (Fig. 10C-10D).

APE2-KD by siRNA or APE2 inhibition by Celastrol sensitizes pancreatic cancer cells to chemotherapy drugs

Previous studies show that ATR inhibitor VE-822 sensitizes cancer cells to radiation or chemotherapy drugs such as CPT (104, 113). Chk1-KD by siRNA or Chk1 inhibition by small molecule inhibitor AZD7762 has been shown to function in a synthetically lethal manner with GEM in pancreatic cancers (114). Our findings on APE2 in the ATR-Chk1 DDR pathway in both the *Xenopus* system and pancreatic cancer cells prompt us to target

the function and regulatory mechanism of APE2 in the ATR DDR pathway that may sensitize cancer cells to chemotherapy drugs. To test this directly, we took two strategies: APE2 suppression by siRNA-mediated knockdown and APE2 inhibitor Celastrol. Notably, cell viability assays show that APE2-KD PANC1 cells are more sensitive to H_2O_2, GEM, CPT, or ETO than CTL-KD PANC1 cells that APE2 suppression sensitized PANC1 cells to DNA damaging conditions (Fig. 11A-11D). Similarly, APE2 inhibition by Celastrol also sensitized PANC1 cells to H_2O_2-induced oxidative stress and chemotherpay drugs GEM, CPT, and ET in a dose-dependent manner (0.5 μM, 0.75 μM, and 1 μM) (Fig. 11E-11H). Furthermore, APE2 suppression by siRNA-mediated KD or Celastrol-mediated inhibition also sensitized MiaPaCa2 cells to oxidative stress or chemotherapy drugs (Fig. 12A-12H). These observations suggest that pancreatic cancer cells may rely to APE2-mediated ATR DDR pathway and DNA repair mechanisms to protect from various different stressful conditions, including chemotherapy drugs, replication stress or oxidative stress.

2.4. Discussion

Accumulating evidence suggests that APE2 plays various critical roles in maintaining genome and epigenome integrity (86). However, it remains unclear whether APE2 is required for the ATR DDR pathway in mammalian cells. This study demonstrated that APE2 is vital for the ATR-Chk1 DDR pathway in response to different stress conditions including oxidative stress, DNA replication stress, and DSBs in pancreatic cancer cells (Fig. 5 and 6). The reasoning behind the usage of 4 different types of DNA damaging agents such as Replication Stress inducers (GEM, 3-AP); Oxidative stress inducer (H_2O_2); PARP1 inhibitor (INI) & DSB inducers (CPT & ETO) for this study is to induce intermediary DNA SSBs in cells in shorter incubation periods of treatment to exclude the

possibility of SSBs converting to DSBs which is the actual case if we treat the cells with DNA damaging agents for longer incubation periods such as more than 6 hours. For our study, those SSBs would induce ATR-Chk1 DDR pathway activation by phosphorylating Chk1 & RPA proteins. Since we treated the cells with different DNA damaging agents with their optimized working concentrations in the same experiment to get a full picture of ATR-Chk1 DDR pathway activation, we saw few discrepancies in the Chk1 & RPA2 phosphorylation signal bands from western blot analyses quantitively. Luckily, during quantification we did consider normalizing the signals with loading control Tubulin and the total protein Chk1 & RPA controls to exclude the errors in visibly discrepant Chk1 & RPA phosphorylation bands quantitively. Most importantly,

Fluorescence microscopy analysis shows that APE2-KD by siRNA leads to a higher percentage of γH2AX and more micronuclei under normal or stress conditions in pancreatic cancer cells (Fig. 7 and 8). Furthermore, we identified a small molecule Celastrol as the first APE2 inhibitor that prevents the binding of APE2 Zf-GRF to ssDNA, APE2's 3′-5′ exonuclease activity, and the SSB-induced ATR-Chk1 DDR pathway in the *Xenopus* HSS system (Fig. 9). Notably, Celastrol treatment impairs the ATR-Chk1 DDR pathway in pancreatic cancer cells (Fig. 10). Finally, APE2 suppression by siRNA-mediated knockdown or APE2 inhibition by small molecule inhibitor Celastrol can sensitize pancreatic cancer cells to chemotherapy drugs including GEM, CPT, and ETO (Fig. 11 and 12). These observations from this study indicate the critical role of APE2 in the DNA damage response to maintain genome integrity in mammalian cells and a schematic model role of APE2 can be illustrated in a cellular context (Fig. 13). Previous studies have demonstrated that APE2 is required for the ATR DDR pathway in response

to oxidative stress and defined SSB structures in *Xenopus* egg extracts (17-19). Our observations in this study support the critical role of APE2 in the ATR-Chk1 DDR pathway in pancreatic cancer cells (Fig. 5 and 6). Furthermore, the role of APE2 in ATR DDR is also applied to GEM-induced DNA replication stress and CPT/ETO-induced DSBs, suggesting that APE2 is a more general regulator of the ATR DDR in various stress conditions in mammalian cells. Future studies are needed to test whether the role of APE2 in the ATR DDR pathway is conserved in other cancer-type cells or under other DNA damaging conditions.

Our data demonstrate the critical function of APE2 protecting pancreatic cancer cells from DNA damaging conditions (Fig. 7). Previous studies have shown that APE2 is critical for the SSB repair pathway in *Xenopus* egg extracts, and homologous recombination-mediated DSB repair pathway in MM cells (88, 96). A recent CRISPR/Cas9-mediated genetic screen identified APE2 as a synthetic lethal target of BRCA2 in human colon epithelial cell line DLD-1 cells and human ovarian cancer cells PEO1 cells (99). Although the underlying mechanism remain unknown, more γH2AX under normal condition in APE2-knockout (KO) PEO1 cells (99) is consistent with our observation of more γH2AX and micronuclei in APE2-KD PANC1 and MiaPaCa2 cells (Fig. 7 and 8). Furthermore, the function of APE2 in protecting cells from DNA damage and micronuclei under different stress conditions (Fig. 7) suggests that APE2 may facilitate several different DNA repair pathways including SSB repair and DSB repair mechanisms to promote survival in cancer cells. Alternatively, the protection of cancer cells from DNA damaging conditions by APE2 may be mediated from APE2's critical function in the ATR-Chk1 DDR pathway indirectly due to the critical function of the ATR DDR pathway in genome integrity.

Small molecule inhibitors targeting multi-function protein APE1 in DNA repair and redox signaling (e.g., Methoxyamine, AR03, APE1 inhibitor III, and E3330/APX3330) have been identified and characterized, and E3330/APX3330 as APE1 redox inhibitor has entered and completed Phase I clinical trials in patients with advanced solid tumors (NCT03375086) (115, 116). However, there is no specific and/or non-specific small molecule inhibitor targeting APE2 functions from the literature. To the best of our knowledge, Celastrol is the first characterized APE2 inhibitor that compromises the binding to DNA and catalytic function of APE2, and APE2's function in the ATR DDR pathway both in the *Xenopus* system and pancreatic cancer cells. More importantly, Celastrol treatment can sensitize pancreatic cancer cells to chemotherapy drugs including GEM, CPT, and ETO (Fig. 11, 12), which is similar to the phenotype of APE2-KD cells as expected. Future translational studies targeting APE2 function in ATR DDR pathway by Celastrol using mouse models will provide new insight into how function and mechanism of APE2 can be targeted for cancer therapy. Although Celastrol exhibits anti-cancer and anti-inflammation activities in previous studies, the translational implication of Celastrol remains limited due to toxicity and narrow therapeutic window (117, 118). Therefore, we identified and characterized the first small molecule inhibitor of APE2 with a good potential of targeted cancer therapy in this study.

Recent accumulating evidence has suggested that, in addition to targeting ATR protein itself (78, 103, 113), regulators/modulators of the ATR-Chk1 DDR pathway have also been targets for cancer therapy. For example, a negative selection RNAi screen from over 10,000 genes in pancreatic cancer BxPC-3 cells identified Rad17, an important regulator of the ATR-Chk1 DDR pathway (75, 119), as the most significant synthetic lethal

target with GEM treatment, and validation experiments showed that Rad17-KD sensitizes pancreatic cancer cells including BxPC-3, MiaPaCa2, and JoPaca-1 to GEM (80). Whereas TopBP1 is a well-established regulator of the ATR-Chk1 DDR pathway (75, 81), recent studies have demonstrated that TopBP1 promotes prostate cancer progression and that down-regulation of TopBP1 significantly suppressed the proliferation of prostate cancer 22RV1 & LNCaP cells via an apoptosis-mediated mechanism (120). Rad9-KD via siRNA enhanced sensitivity of breast cancer cell MCF-7 and MDA-MB-231 to doxorubicin that induces DSBs (121). Downregulation of Rad17, Rad9, TopBP1, or Claspin can drastically sensitize human cervical cancer HeLa cells to hyperthermia (122). Consistently with the idea of suppressing ATR DDR to enhance chemotherapy drugs, our findings have demonstrated the enhanced sensitivity of pancreatic cancer cells to chemotherapy drugs by siRNA-mediated APE2-KD or Celastrol-mediated APE2 inhibition.

What are the roles of APE2 in cancer etiology and therapeutics? Recent studies have shown abnormal expression of APE2 in various cancer patients (96, 97), and APE2 has also been suggested as an oncogene in liver cancer (98). Particularly, APE2 was recently identified as a synthetic-lethality target in BRCA1/2-deficient cells from a couple of CRISPR-mediated genetic screens, although the exact underlying mechanism remains to be elucidated (90, 99). Our finding on the critical role of APE2 in the ATR DDR pathway in pancreatic cancer cells in this study provides vital knowledge for future translational studies targeting APE2 in various mice models with a different genetic background such as deficiency of BRCA1/2 or ATM. Overall, we have demonstrated the critical function and role of APE2 in the ATR-Chk1 DDR pathway in pancreatic cancer cells, which can be designed for future combination or synthetic lethality therapies for cancers.

CHAPTER 3: DISTINCT FUNCTION OF APE2 IN DNA DAMAGE RESPONSE IN MAMMALIAN CELLS

ABSTRACT

The maintenance of genome integrity and fidelity is vital for the proper function and survival of all organisms. Recent studies from Chapter#2 have revealed that APE2 is required to activate an ATR-Chk1 DNA damage response (DDR) pathway in response to different stressful conditions including oxidative stress, DNA replication stress, and DNA double-strand breaks in pancreatic cancer PANC1 & MiaPaCa2 cell models and a defined DNA single-strand break (SSB) in *Xenopus laevis* egg extracts. However, it remains unclear whether distinct function of APE2 as a critical regulator of the DDR pathway is conserved in different types/species of mammalian cells. Here, we provide evidence using few other human cancer and normal cells with different morphological characterization, genetic background & mutation status of key DDR genes that APE2 is essential for ATR DDR pathway activation in response to different stressful conditions including oxidative stress, DNA replication stress, and DNA double-strand breaks. Fluorescence microscopy analysis shows that APE2-knockdown (KD) leads to enhanced γH2AX foci and increased micronuclei formation. In addition to our previously found small molecule inhibitor Celastrol we identified another small molecule inhibitor NU7441 as a potential APE2 regulator that may lead to APE2 abundance/expression reduction. Notably, cell viability assays demonstrate that Celastrol or NU7441 sensitizes cancer cells to chemotherapy drugs. Whereas, APE2-KD also sensitizes cancer cells but not normal cells to chemotherapy drugs. Overall, we propose APE2 as a critical regulator for the DDR pathway in genome integrity maintenance in mammalian cells.

3.1. Introduction

A recent bioinformatic study has demonstrated that APE2 mRNA expression from tumor tissues is elevated in comparison to matched non-malignant tissues in 5 cancer types such as kidney, lung, breast, liver and uterus cancer (97). This pattern of APE2 overexpression is also similar to that of PCNA, BRCA2, and XRCC1. Furthermore, APE2 expression is positively correlated with the expression of PCNA, APE1, PARP1, XRCC1, Chk1, and Chk2 across six cancer types (97). These observations are consistent with the role of APE2 in BER and SSB repair pathways that include PCNA, APE1, XRCC1, and PARP1 (86). Furthermore, Kumar *et al*. recently reported that APE2 is overexpressed at the levels of mRNA and protein in cultured MM cell lines and 112 MM patients from two datasets (96). Interestingly, it is recently reported that cisplatin-treatment can increase APE2 expression in kidney cells in mice (123). Therefore, we sought to determine the abundance and expression of APE2 and other DNA repair and DDR proteins in various cancer & non-cancer cells. Furthermore, findings from our Chapter #2 on the role and mechanism of APE2 in pancreatic cancer PANC1 & MiaPaCa2 cells prompt us to further explore function of APE2 in mammalian cells in more general setting, such as other pancreatic cancer cells with different genetic & morphological background, or different mutation & transformation status, a normal pancreatic epithelial cell, as well as cancer cells from different cancer types.

To translate basic mechanisms of APE2 function in DDR pathway to cancer therapy, we sought to identify small molecule inhibitors of APE2 functions. Celastrol, a natural compound derived from thunder god vine and originally identified as HSP90 modulator, has been implicated in cancer therapies, such as pancreatic cancer and prostate cancer (104,

124). Although Celastrol exhibits anti-cancer and anti-inflammation activities, translational implication of Celastrol remains limited due to toxicity and narrow therapeutic window. Celastrol has been shown to exhibit anti-proliferative activity against a variety of tumor cells, including leukemia and prostate cancer (124). It also modulates the expression of pro-inflammatory cytokines (125), inducible nitric oxide synthase, adhesion molecules in endothelial cells (126), proteasome activity (124), topoisomerase II (127), and heat shock response (128). Numerous molecular targets of Celastrol have been identified, including IKK-α, IKK-β (129), cdc37 (130), p23 (131) , heat shock factor-1 (128), and proteasomes (124). In Chapter 2, I have shown that Celastrol affects ATR DDR activation in PANC1 & MiaPaCa2 pancreatic cancer cells. However, it remains unclear whether Celastrol plays a more general role in the regulation of ATR-Chk1 DDR pathway in mammalian cells. Thus, I have examined the role of Celastrol in the regulation of APE2 functions in other pancreatic cancer cells including Panc10.05 andCAPAN-1, normal human pancreatic duct epithelial (hPDE) cells and human bone osteosarcoma U2OS cells.

Several library screens have identified NU7441 as a potential inhibitor of DNA-dependent protein kinase (DNA-PK) with IC50 value of 13 nM (132, 133). NU7441 is a selective ATP-competitive inhibitor of DNA-PK as NU7441 shows no inhibition effect on the DNA-PK-related enzymes ATM and ATR at concentration of 100 μM. Further preclinical studies have demonstrated that NU7441 at a concentration of 100 nM significantly enhanced the sensitivity of cells to etoposide and promoted cells to death in DNA-PK-proficient V3-YAC cells but not in DNA-PK-deficient cell V3 cells (134). More mice cancer model studies also support the benefits of combining NU7441 with chemotherapy or radiotherapy for cancer treatment (134, 135). However, there is no report

on the potential regulation of APE2 by NU7441 in its function in DDR pathway in a cellular context. Notably, my study has shown that NU7441 may lead to APE2 expression and/or abundance reduction.

The overall study of Chapter 3 is to elucidate the distinct functions and regulatory mechanisms of APE2 in DDR pathway in mammalian cells including cancer and non-cancer cells. In summary, I have demonstrated several different approaches to target APE2 function in DDR pathway including but not limited to **a)** siRNA-mediated APE2 knockdown, **b)** Celastrol-mediated inhibition of APE2 catalytic functions, and **c)** NU7441-induced APE2 down-regulation. These findings will lay a solid foundation for future preclinical and clinical cancer treatment studies by targeting APE2 function and mechanism in DDR pathway.

3.2. Materials and methods

Cell culture, treatments and cell lysate preparation

PANC1 and MiaPaCa2 cells were purchased from ATCC (Cat#CRL-1469 and CRL-1420) and cultured in complete media, DMEM (Dulbecco's modified Eagle Medium, Corning) supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin (Gibco) for PANC1 & U2OS or DMEM complete media with 2.5% Horse Serum (Sigma) for MiaPaCa2, respectively. MEM (Minimum Essential Medium, Corning, Cat#10-009-CV), RPMI 1640 (Roswell Park Memorial Institute Medium, Gibco, Cat#11875-093), IMDM (Iscove's Modified Dulbecco's Media, Gibco, Cat#12440-053) supplemented with 10% FBS & 1% Pen-Strep solution were used to culture CAPAN-1, Panc10.05 & U87-MG, hHμ cells, respectively.

Cell lysate preparation, and cell treatments with H_2O_2, Gemcitabine (GEM), Camptothecin (CPT), Etoposide (ETO), KU55933, and VE-822 have been described in Chapter 2. Iniparib (INI, PARP inhibitor, Selleckchem Cat#S1087, 20μM-100μM), NU7441 (DNA-PK inhibitor, Selleckchem Cat#S2638, 50nM-100μM) or MG-132 (Proteasome inhibitor, EMD Millipore, Cat#474790, 100nM-1μM) was dissolved in DMSO, and added to complete media to the final concentrations as indicated in the individual experiments.

Immunoblotting analysis and antibodies

Immunoblotting analysis of cell lysates was carried out similarly as we described previously (17-19, 87). Primary antibodies against Chk1 (Santa Cruz Biotechnology Cat#sc-8408), Chk1 phosphorylation Ser345 (Cell Signaling Technology Cat#133D3), RPA32 (ThermoFisher Scientific Cat#MA1-26418), RPA32 phosphorylation Ser33 (Bethyl Laboratories Cat#A300-246A), p53 phosphorylation Ser15 (Cell Signaling Technology Cat#9284S), ATM (ATM 2C1, GENETEX, Cat# GTX 70103), ATM phosphorylation at Ser1981 (Rockland, Cat#200-301-500), ATR (Santa Cruz Biotechnology, Cat# sc-515173), PARP1 (Santa Cruz Biotechnology, Cat# sc-8007), γH2AX phosphorylation at Ser139 (Cell Signaling, Cat#25775), H2AX (Cell Signaling, H2AX-D17A3-XP, Cat#76315), UNC371 purified Xenopus APE2 (Yan Lab), Human APE2 for microscopic studies (GENETEX APEX2, Cat# GTX 80642) ; APEX2 polyclonal, (Abcam, Cat#135742), APEX2 polyclonal (BIOSS, Cat#bs-6587R), BRCA (EMD Millipore Anti-BRCA2 (Ab-1), Cat#OP95), PCNA (Santa Cruz Biotechnology, Cat# sc-56) and Tubulin (Santa Cruz Biotechnology Cat#sc-8035) were purchased from

various vendors. Anti-human APE2 antibodies were a gift from Drs. Daisuke Tsuchimoto and Yusaku Nakabeppu and also described previously (36).

After primary antibody incubation overnight, secondary antibodies (Cell Signaling, HRP-linked conjugate Anti-Mouse-IgG, Cat#7076S, Anti-rabbit-IgG, cat#7074S) were given to the membranes after washing them 3 times with TBST buffer, 10 mins each. Secondary antibody incubation period was 1 hr in room temperature rocker. Then they were washed again 3 times with TBST buffer, 10 mins each. Thus, we prepare the C reagents (ADVANSTA, Western Bright ECL, Western Blot Detection Kit, Cat#K-12045-D50) diluted them into DI H_2O and stain the membranes from 5-10 mins and then made them ready for imaging via BIORAD imager. Immunoblotting analysis & quantification was performed via BIORAD software.

Transfection and siRNA-mediated APE2-KD assays

For siRNA experiments, APEX2 siRNA or control siRNA was transfected into cells with Lipofectamine^R RNAiMAX in Opti-MEM I Reduced Serum Medium as described in Chapter 2. For the rescue experiments, after siRNA-mediated APE2-KD, transfecting control plasmid pcDNA3-YFP or pcDNA3-YFP-xAPE2 with Lipofectamine 2000 in Opti-MEM I Reduced Serum Medium. After different treatment and incubation, cells were imaged via fluorescence microscopy to ensure YFP or YFP-xAPE2 was expressed in cells.

Immunofluorescence analysis and cell viability assays

Cell sample preparation and immunofluorescence analysis as well as cell viability assays have been described in Chapter 2.

Statistical analyses

GraphPad PRISM 8 statistical analysis software was used to perform statistical analysis of γH2AX/micronuclei-positive cells. Data were presented as mean ± SD from three experiments. A paired two-sided t-test was conducted to determine significance of difference. $P<0.05$ is considered significant and $P<0.01$ is considered highly significant. MTT/CellTiter-GLO 2.0 assay (Cell Survival assays) analyses using Microsoft Excel were performed in triplicates (n=3). Data are presented as mean ± SD for the error bars and normalized with no treatment group.

3.3. Results

ATR inhibitor compromises H_2O_2-induced ATR-Chk1 DDR activation & sensitizes cancer cells to oxidative stress conditions

In Chapter 2, H_2O_2 was utilized as an oxidative stress inducing agent to trigger ATR-Chk1 DDR in pancreatic cancer cells. To determine the role of APE2 in ATR DDR pathway in U2OS cancer cells, we established that H_2O_2 triggered Chk1 and RPA32 phosphorylation in U2OS cells and that ATR specific inhibitor VE-822 compromised H_2O_2-induced Chk1 and RPA32 phosphorylation (Fig. 14A). Expectedly, ATM inhibitor KU55933 couldn't alone compromise ATR-Chk1 DDR activation in U2OS cells (Fig. 14B). Furthermore, ATR inhibitor VE-822 sensitizes U2OS cells to oxidative stress conditions in a dose-dependent manner (Fig. 14C). These observations suggest that targeting ATR but not ATM may provide better strategy to sensitize U2OS cells to oxidative stress. And the literatures also documenting an emerged new era of research to treat cancer and tumors by targeting ATR DDR pathway and their key DDR proteins.

APE2 protein abundance in a panel of pancreatic cancer cells

For this study, we have prepared cell lysates from a panel of different pancreatic cancer cell lines with different p53 mutation status and non-cancer pancreatic cells, and have quantified APE2 & other DDR proteins' abundance via Western blot analysis. For this analysis, equal total proteins of cell lysates were loaded and tubulin was used as loading control. APE2 protein level quantification was normalized against tubulin. My preliminary data show that APE2 is expressed in all pancreatic cancer cells tesed (i.e., Panc10.05, MiaPaCa2, PANC1, and CAPAN-1 cells) and that APE2 expression in pancreatic cancer cells may not be significantly up-regulated or down-regulated in these four pancreatic cancer cell lines in comparison to non-cancer hPDE cells (Fig. 15A-B). It seems that APE2 abundance in PANC1 and CAPAN-1 is sort of reduced but future studies are required to ensure the difference is statistically significant or not. As expected, BRCA2 was almost depleted in CAPAN-1 cells. Of note, expression of PARP1, ATM, and ATR is reduced in PANC1 and CAPAN-1 with comparison to hPDE cells (Fig. 15A-B).

APE2 is important for the ATR-Chk1 DDR pathway under different stress conditions in cancer cells with different genetic background but not in normal cells

In Chapter 2, I have demonstrated evidence that APE2 is vital for the ATR-Chk1 DDR pathway in PANC1 and MiaPaCa2 cells. To validate the critical function of APE2 in DDR pathway in other mammalian cells, we repeated the similar APE2-KD and rescue experiments in Panc10.05 cells and found that APE2 is also critical for the ATR-Chk1 DDR pathway in oxidative stress in Panc10.05 cells (Fig. 16A-16C). To determine the role of APE2 in ATR DDR pathway in different stress conditions, we performed similar experiments in Panc10.05, U2OS & CAPAN-1 & normal hPDE cells and found that APE2

is also important for the ATR-Chk1 DDR pathway caused by hydrogen peroxide-induced oxidative stress, GEM-induced DNA replication stress, and CTP/ETO-induced DSBs in Panc10.05, U2OS & CAPAN-1 (Fig. 17) but not in normal hPDE cells (Fig. 18). Thus, the above observations demonstrate the critical role of APE2 in ATR-Chk1 DDR pathway following stress conditions in cancer cells but not in non-cancerous cells.

APE2-KD by siRNA leads to severe DNA damage and more micronuclei in cancer cells irrespective of cancer type

For this study, we utilized both Confocal microscopy analysis & Leica-LASX microscopy analysis to visualize & quantify DNA DSBs marker γH2AX foci & our protein of interest, APE2 foci and also micronuclei formation due to chromosomal instability during cancer cell division cycles. To determine the role of APE2 in protecting cells from various stressful conditions, we chose to measure γH2AX status in pancreatic (PANC1 and Panc10.05) & osteosarcoma cancer (U2OS) cells under normal or damaging environments (e.g., treatment of H_2O_2, GEM, CPT, ETO & INI). Our fluorescence microscopy analysis shows that the percentage of γH2AX-positive cells in APE2-KD PANC1 cells was higher than that in control siRNA PANC1 cells regardless of treatment of H_2O_2, GEM, CPT, ETO or INI (Fig 19C, 20C-20D). We also noted similar observations in Panc10.05 cells from treatment of H_2O_2 and CPT (Fig. 20A-20B). Furthermore, we found that APE2-KD by siRNA led to severe γH2AX in U2OS cells under normal condition or after treatment of CPT, or ETO (Fig. 21A-21B). These observations suggest that APE2 may protect pancreatic cancer cells from DNA damage such as SSBs and DSBs from both endogenous and exogenous sources. To further validate the critical role of APE2 in DSB repair, we examined the micronuclei formation in PANC1 & U2OS cells under normal, replication

stress, PARP1 inhibitory or DSB-generating conditions. Our microscopy analysis demonstrates that more percentage of micronuclei-positive cells were observed in APE2-KD PANC1 cells regardless of the treatment of H_2O_2, GEM, CPT, ETO & INI (Fig. 19A-19B). We also observed similar results on the role of APE2 in micronuclei formation in U2OS cells (Fig. 21C-21D). These observations of severe γH2AX and micronuclei formation in APE2-KD cells suggest APE2's function in resolving the stressful environments, consistent with APE2's function in DDR pathway.

Celastrol impairs the ATR-Chk1 DDR pathway in different cancer and normal cells

From my findings in Chapter 2, we showed that 2.5μM Celastrol dose for 5hrs was enough to inhibit the DNA binding function of APE2 without being reduced in abundance rendering the inactivation of ATR-Chk1 DDR pathway. Parallelly, we did the same setup of experiments but with 1μM & 5μM dose of Celastrol for 5 hrs and unexpectedly observed that 1μM Celastrol concentration couldn't inactivate ATR-Chk1 DDR pathway (Fig. 22A). More importantly, we have noted that 5μM dose of Celastrol for 5 hrs was high enough to encourage APE2 abundance reduction via proteasomal degradation rendering severe inactivation of ATR-Chk1 DDR pathway as well as few other DDR protein degradation such as ATM, ATR, etc. (Fig. 22B). We also tested whether the inhibitory function of Celastrol in ATR-Chk1 DDR pathway is conserved in other cancer and normal cells. Notably, Chk1 phosphorylation and RPA32 phosphorylation induced by CPT or ETO were impaired by Celastrol in Panc10.05 cells (Fig. 22C) and Chk1 phosphorylation and RPA32 phosphorylation induced by H_2O_2, GEM, CPT, or ETO were impaired by Celastrol in CAPAN-1 (Fig. 22D) & normal hPDE (Fig. 22E) cells. We also noted similar findings in U2OS cells where Chk1 phosphorylation and RPA32 phosphorylation induced by H_2O_2 or

CPT & ETO were impaired by Celastrol (5μM) (Fig. 23A-B). Because Celastrol has been shown as Hsp90 inhibitor, I wanted to test whether Hsp90 inhibitor such as17-AAG (Tanespimycin) has a role in the ATR DDR pathway. Interestingly, the addition of 17-AAG impaired the Chk1 phosphorylation and RPA32 phosphorylation induced by hydrogen peroxide in U2OS cells (Fig. 23C). Unlike Celastrol, 17-AAG treatment resulted in the reduction of total Chk1 (Fig. 23C). Pretreatment of MG-132 reversed the 17-AAG-induced reduction of total Chk1, but not the impairment of Chk1 and RPA32 phosphorylation by 17-AAG (Fig. 23C).

APE2-KD by siRNA sensitizes cancer cells but not normal cells and APE2 inhibition by Celastrol sensitizes both cancer and normal cells to chemotherapy drugs

Our findings on APE2 in ATR-Chk1 DDR pathway in both *Xenopus* system and pancreatic cancer PANC1 & MiaPaCa2 cells (Fig. 9, 11, 12) prompt us to reason that targeting the function and regulatory mechanism of APE2 in ATR DDR pathway may sensitize cancer cells to chemotherapy drugs in other pancreatic cancer cells such as Panc10.05 & CAPAN-1 or other type of cancer cells such as human osteosarcoma U2OS, mouse astrocyte U87 glioblastoma cells, or in normal pancreatic cells hPDE. To test this directly, we took two strategies: APE2 suppression by siRNA-mediated knockdown and APE2 inhibitor Celastrol. Notably, cell viability assays show that APE2-KD Panc10.05 & APE2-KD CAPAN-1 cells are more sensitive to H_2O_2, GEM, CPT, or ETO compared with CTL-KD Pan10.05 (Fig. 24A-C) & CAPAN-1 cells (Fig. 25A-D), suggesting that APE2 suppression sensitize Panc10.05 & CAPAN-1 cells to DNA damaging conditions. Furthermore, we noted similar outcomes using U2OS cells & U87-MG cells that APE2 suppression sensitized U2OS (Fig. 27A-D) & U87-MG (Fig. 28A) cells to DNA damaging

conditions. Notably, siRNA-mediated APE2-KD had almost no noticable effect on hPDE cells sensitivity to H_2O_2, GEM, CPT, or ETO (Fig. 26A-D). All the above observations suggest that regulating APE2 abundance via siRNA can sensitize cancer cells but not normal cells to chemotherapy drugs.

Concordantly, APE2 inhibition by Celastrol sensitized Panc10.05 (Fig. 24D-F), CAPAN-1 (Fig. 25E-H), U2OS (Fig. 27E-H), U87-MG (Fig. 28B-C) and even normal hPDE (Fig 26E-H) cells to H_2O_2-induced oxidative stress and chemotherpay drugs GEM, CPT, and ET in a dose-dependent manner (Celastrol 0.5 μM or 0.75 μM or 1 μM). These observations suggest that Celastrol sensitized all cancer cells incluidng Pan10.05, CAPAN-1, U2OS, and U87-MG. My interpretation to the discrepancy of differential role of APE2-KD and APE2 inhibitor Celastrol in hPDE cells is that Celastrol may impair the activity of other proteins in addition to APE2, such as Hsp90, among others.

NU7441 compromises the ATR-Chk1 DDR pathway activation in mammalian cells by regulating APE2 protein abundance

The findings from the regualtion of APE2 in the ATR DDR pathway in cancer cell and non-cancer cells prompted us to adjust our exeprimental strategy to focus on more the regulatory mechanisms of how APE2 protein expression and/or stability is regulated. A recent study has demonstrated that DNA-PK is implicated in rRNA biosynthesis, and that DNA-PKcs inhibitor NU7441 leads to ~45% reduction of global protein production in mouse cells (136). Preliminary studies from our collaborator suggest that APE2 protein abundance may be regulated by NU7441 in mouse cells. To test whether NU7441 regulates APE2 protein abundance and functions in human cancer and normal cells, I first tested the cell viability of PANC1 cells after 3-day treatment of NU7441 and found that IC50 of

NU7441 is ~5 μM (Fig. 29A). Next, PANC1 cells were treated with NU7441 at the concentration of 10 μM for different days (from 1 d to 5 d), and immunoblotting analysis of total cell lysates show that APE2 abundance is gradually reduced starting at 2d timepoint (Fig. 29B). The abundance of tubulin was still stable within 3d-treatment. Thus, we designed our follow-up experiments within 3d treatment of NU7441.

Next, I sought to test whether NU7441 affects ATR-Chk1 DDR pathway in cancer and normal cells of different genetic background by regulating APE2 abundance. Notably, Chk1 phosphorylation and RPA32 phosphorylation induced by H_2O_2 or CPT were compromised to some extent by 3d treatment of NU7441 at the concentration of 10μM in PANC1 cells (Fig. 30A). This phenotype is likely due to the APE2 reduction induced by NU7441 (Fig. 30A). Furthermore, I tested whether acute treatment of NU7441 (i.e., 10μM for 4 hours) also resulted in APE2 abundance reduction, leading to defects of Chk1 phosphorylation and RPA32 phosphorylation induced by H_2O_2 or CPT (Fig. 30B). To test whether NU7441-induced APE2 protein reduction is due to proteosome-mediated degradation, I pre-treated PANC1 cells with MG-132, an inhibitor of proteosome, and found that MG-132 treatment can partially rescued APE2 abundance, and the defect of Chk1 phosphorylation and RPA32 phosphorylation induced by H_2O_2 or CPT in the presence of NU7441 (Fig. 31A). I found similar observations using MiaPaCa2 cells and hPDE cells (Fig. 31B-31C). Collectively, our observations suggest that NU7441 may lead to APE2 protein reduction likely through proteosome-mediated degradation.

NU7441 sensitizes cancer cells to chemotherapy drugs

NU7441 treatment (at the concentration of 5μM) sensitized PANC1 (Fig. 32A-C), MiaPaCa2 (Fig. 32D-F) & U2OS (Fig. 32G-I) cells to H_2O_2-induced oxidative stress and

chemotherpay drugs GEM and CPT. These observations suggest that cells may rely on APE2-mediated ATR DDR pathway to protect themselves from various different stress conditions. The regulation of APE2 by NU7441 may be one of several mechanisms contributing to the synegistic effect of NU7441 with chemotherapy drugs.

3.4. Discussion

In Chapter 2, a working model for the role of APE2 in the ATR-Chk1 DDR pathway in pancreatic cancer cells including PANC1 and MiaPaCa2 has been proposed (Fig. 13). In Chapter 3, I extended our understanding of APE2 functions in DDR pathway to other pancreatic cancer cells different genetic background such as Panc10.05 (p53-deficient) and CAPAN-1 (BRCA2-deficient) (Figs. 16-17). BRCA1 and BRCA2-deficient cells are exquisitely sensitive to treatment with inhibitors of poly(ADP-ribose) polymerase (PARP) (137, 138). Interestingly, ATR DDR pathway induced by PARP1 inhibitor Iniparib (INI) in CAPAN-1 cells was impaired in the abence of APE2 (Fig. 17D). These observations suggest that the critical role of APE2 for DDR in stress conditions is conserved in various cancer cells regardless of their genetic background and DDR gene mutation status.

I also examined the function of APE2 in other cancer types such as human bone osteosarcoma U2OS cells and found that APE2 has the similar function in DDR pathway (Figs. 14, 17, and 23). These observations indirectly suggest that APE2 promotes cell survial via ATR-Chk1 DDR pathway in different stress conditons in mammalian cancer cells. Future studies may also test whether APE2 plays an important role in other cancer types. Interestingly, APE2-KD had almost no effect on the ATR-Chk1 DDR pathway under stress conditions in hPDE cells (Fig. 18), and APE2 is dispensable for the sensitization of hPDE cells to chemotherpay drugs (Fig. 26A-D). I intepret this observation that normal

cells may surve these stress conditions via different signaling pathway other than APE2-mediated ATR DDR pathway as they have wild type of DNA repair and DDR pathways such as p53, BRCA1, BRCA2, and ATM etc. But adding to this, we have noted that these results from the experiments was conducted only in one type of normal cells which is hPDE, pancreatic ductal epithelial cells in this study. So we can't exclude the possibility that APE2-KD might have some effects on the ATR-DDR pathway in other types of tissue specific normal cells such as bone, kidney, breast or brain astrocyte normal cells which we can prioritize for future studies to investigate distict function of APE2 in mammalian normal cells. Most importantly, we can compare APE2 function in cancer vs normal cells.

Furthermore, I examined two potential small-molecule regulators for APE2 functions: Celastrol and NU7441. Combining findings from Chapter 2 and Chapter 3, it seems that Celastrol functions more like an inhibitor of APE2's catalytic function such as 3′-5 exonuclease activity more or less in a dose-dependent manner to negatively regulate APE2 function in the ATR DDR pathway. However, Celastrol may regulate APE2 abundance in both cancer & normal cells at certain higher doses, which may be due to its indirect role in regulating other target proteins such as Hsp90. On the other hand, NU7441 seems has two potential mechanisms in the regulation of APE2: its inhibition of global protein expression that include APE2, or proteosome-mediated APE2 degradation. These two scenarios are neither exclusive to each nor parallel to each other. Future studies are needed to determine the exact mechanism of the regulation of APE2 by NU7441. Nonetheless, synergistic effect of NU7441 with chemotherapy drugs in both cancer & normal cells could be partially attributed to its regulation of APE2 abundance and functions in ATR DDR pathway.

Two recent genetic screens have identified APE2 as a synthetic lethality target in BRCA2-deficient cells (90, 99). It was proposed that APE2 processes AP sites at replication forks, preventing blockage in fork progression and formation of DNA strand breaks, requiring BRCA2 activity for repair and restoration of DNA synthesis. In our study, we have provided support in this context that siRNA-mediated APE2-KD or APE2 inhibition by Celastrol impaired the ATR-Chk1 DDR pathway (Fig. 17D, 22D, 25A-H), suggesting the critical function of APE2 for BRCA2-deficient CAPAN-1 cells to survive endogenous or exogenous DNA damage.

Newly-formed micronuclei do not have marked levels of DNA damage in G1, but damaged micronuclei accumulate as cells progress into the S and G2 phases of the cell cycle (109, 139), suggesting a link between DNA damage and DNA replication. Hereby, we proposed a new feature of APE2 in mammalian cell system is that APE2 also protects cells in stress conditions (especially DSB damage) by preventing chromosomal damage during their mitotic division thus reducing the formation of Micronuclei. Based on our previously mentioned hypothetical model, we defined our experimental designs, after follow-thorough experiments we have concluded that targeting APE2's function (siRNA mediated knockdown of APE2) results in an increase of Micronuclei formation (Fig. 19A-B, 21C-D) in PANC1 & U2OS cells in DSB stress conditions (CPT or ETO) implying compromised ATR-Chk1 activation in cells siRNA with APE2 compared to control siRNA cells. Consistent with this, we can infer that APE2 can protect cells in stress conditions by preventing chromosomal mis-segregation and re-incorporation of micronuclei DNA into daughter nuclei, therefore, maintaining genome integrity.

CHAPTER 4: BROADER IMPLICATIONS OF ATR DDR PATHWAY AND DISCUSSION FOR FUTURE STUDIES

4.1. Overall Conclusions

Combining all our studies, we introduce the concept and mechanism of SSB end resection and summarize the current understanding on the biological significance of SSB end resection in genome integrity & we provide evidence using human cancer and normal cells with different morphological characterization, genetic background & mutation status of key DDR genes that APE2 is essential for ATR DDR pathway activation in response to different stressful conditions including oxidative stress, DNA replication stress, and DNA double-strand breaks. Fluorescence microscopy analysis shows that APE2-knockdown (KD) leads to enhanced γH2AX foci and increased micronuclei formation. Furthermore, we identified a small molecule compound Celastrol as an APE2 inhibitor that specifically compromises the binding of APE2 to ssDNA, its 3′-5′ exonuclease activity, and the defined SSB-induced ATR Chk1 DDR pathway in *Xenopus* egg extracts. In addition, we identified another small molecule inhibitor NU7441 as a potential APE2 regulator that may lead to APE2 abundance/expression reduction in mammalian cells. Notably, cell viability assays demonstrate that Celastrol or NU7441 sensitizes cancer cells to chemotherapy drugs. Whereas, APE2-KD also sensitizes cancer cells but not normal cells to chemotherapy drugs. Overall, we propose APE2 as a critical regulator for the DDR pathway in genome integrity maintenance in mammalian cells.

Although it has been well known that ATR DDR pathway regulates cell cycle progression, transcriptional activation, DNA damage repair, and apoptosis or senescence

(3, 75), hundreds and thousands of ATR substrates have been identified and characterized that are not only in these characterized pathways (140), suggesting that ATR DDR pathway may have broader implications. Furthermore, targeting ATR DDR pathway separately or in combination with chemotherapy and/or radiotherapy has increased attention for cancer treatment (77, 78, 141). In addition, nanoparticle-based drug delivery system has also been implicated in the targeted cancer therapeutics (142). Thus, it is significant to determine if targeting ATR DDR and/or nanotechnology-based targeted delivery system improve efficiency and efficacy of cancer therapy.

4.2. Role of ATR DDR in microparticles-based sepsis treatment

Microparticles are intact vesicles derived from the plasma membrane during cellular activation and apoptosis (143), and have both physiological and pathological functions in the endothelial and haemostatic responses to sepsis. The collaborative project with Dr. Clemens lab gave us the opportunity to work with human microvascular endothelial cell (HMEC) in a microparticles-based sepsis treatment approach and investigate the potential function of ATR DDR pathway in sepsis. To test this, we performed immunoblotting analyses to determine whether the ATR DDR pathway is activated HMEC cells after different treatments such as LPS-microparticles. In comparison to the H_2O_2-induced oxidative stress, neither 4-hr nor 24-hr treatment microparticles-based LPS treatments triggered noticeable Chk1 or RPA32 phosphorylation signal in HMEC cells (Fig. 33A-B).

4.3. Role of ATR DDR in CNS inflammation via oxidative stress

Next, I collaborated with Dr. Marriott lab to study the ATR DDR pathway in work with human glioblastoma cell lines U87-MG cells. Although CPT and ETO treatment

triggered robust Chk1 phosphorylation and RPA32 phosphorylation in U87-MG cells, H_2O_2 treatment (at 1 or 1.2mM concentrations for 3 or 4 hours) had almost no effect for the activation of the ATR-Chk1 DDR in U87-MG cells (Fig. 34B and 34D). However, our similar conditions of H_2O_2 triggered Chk1 phosphorylation and RPA32 phosphorylation in U2OS cells (Fig. 34A and 34C). On the other hand, ATM was activated by H_2O_2, CPT, and ETO in U87-MG cells similar to U2OS cells (Fig. 34). These observations suggest that U87-MG cells may be deficient in a specific factor that is needed for oxidative stress-induced ATR DDR. Alternatively, oxidative stress may lead to damage-dependent degradation of key DDR proteins that may explain the defective activation of ATR DDR by oxidative stress. The letter hypothesis is supported partially by the noticeable reduction of total Chk1 after longer treatment of hydrogen peroxide in U87-MG cells but not in U2OS cells (Fig. 34D).

To test the latter scenario directly, I treated cells with proteosome inhibitor MG-132 with the presence or absence of H_2O_2, and found that 5-hr treatment of MG-132 (1μM) can trigger robust Chk1 and RPA32 phosphorylation induced by H_2O_2 in U87-MG cells (Fig. 35B and 35D). However, MG-132 had almost no effect on H_2O_2-induced ATR DDR in U2OS cells and PANC1 cells (Fig. 35A and 35C).

4.4. Role of ATR DDR in nanoparticles-based cancer therapeutics

I also collaborated with Dr. Vivero-Escoto lab in Department of Chemistry to investigate the role of ATR DDR pathway in a novel target-specific nanoplatform-based cancer immunotherapy approach for the *in situ* differential co-delivery & release of Gem/cisPt where the Mesoporous Silica Nanoparticles (MSN) material showed higher target-specific tumor accumulation & stability in blood or tumor microenvironment. The

MSN-based combined drug therapy resulted in increased tumor inhibition with reduced off-target effect and improved the therapeutic performance of synergistic drug combination along with high drug content and reproducibility. My part in the study is to investigate whether the DDR pathway is activated by different treatments of GEM or cisplatin (cisPt) with or without MSN-nanoparticles. Our immunoblotting analysis showed that the ATR-Chk1 DDR pathway in KCM cells was triggered by treatment of MSN-(Gem+cisPt) but not by control MSN, MSN-Gem, MSN-cisPt, or MSN-Gem+MSN-cisPt (Fig. 36A-B**)**. Control experiment showed the activation of ATR DDR by Gem and Gem+cisPt (Fig. 36A-B**)**. We observed similar observations in HPAF-II cells (Fig. 36C-D), suggesting that the role of MSN-(Gem+cisPt) in triggering ATR DDR pathway activation is not cell line-specific effect. Consistent with our above observations, cell viability assays, immunofluorescence and immunohistochemical analyses in Dr. Vivero-Escoto lab also confirmed that MSN-(Gem-cisPt) has the most significant impact than in inducing cell cycle arrest and cell apoptosis in comparison to the monotherapy MSNs or the physical mixture of MSN-Gem and MSN-cisPt. Overall, these studies suggest that the activation of ATR DDR pathway by MSN-(Gem-cisPt) may be the most efficient treatment of cancer, which requires more future preclinical and clinical studies.

4.5. Discussion and future directions

Conserved functions of APE2 in genome and epigenome integrity in evolution

In the past decade or so, APE2 has been a rising vital player in the maintenance of genome and epigenome integrity. In particular, APE2 structure and function EEP (endonuclease/exonuclease/phosphatase) domain, PIP box and Zf-GRF motifs has been characterized in eight eukaryotic species including *Homo sapiens*, *Mus musculus*, *Xenopus*

laevis, *Ciona intestinalis*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and *Trypanosoma cruzi* (17, 36, 40, 42, 43, 93, 95, 144). The evolutionarily conserved function and mechanisms of APE2 in genome and epigenome integrity including various DNA repair pathways (BER, SSBR, and DSB repair), DDR pathways (ATR-Chk1 and p53-dependent), immunoglobulin class switch recombination and somatic hypermutation, as well as active DNA demethylation have been updated in our recent comprehensive review article in MUTREV (86). I would like to highlight one aspect of APE2 function: SSB end resection. Our lab has investigated the role and mechanism of APE2 in ATR DDR pathway in *Xenopus* and mammalian cells (17-19, 87), and we have proposed that APE2 is critical for the continuation stage of 3′-5′ SSB end resection (19, 85, 87). This new model of SSB end resection has been validated by several recent studies: **First**, yeast Apn2 (yeast homolog of human APE2/APEX2) does not directly remove rNMPs from DNA but acts as a highly versatile nuclease against a diverse set of 3′ blocked DNA ends to facilitate Top1-catalyzed rNMP repair (94, 145). Due to its nuclease function, Apn2 removes pTyr–DNA conjugates and further resects several nucleotides in the 3′-5′ direction to generate 3′-hydroxyl ends that are suitable substrates for DNA repair synthesis, which is consistent with our proposed APE2-mediated SSB end resection model. **Second**, human APE2 resolves endogenous DNA 3′-blocks derived from BRCA1 and BRCA2 deficiency to resect about 15 nt in the 3′-5′ direction (90). The reversal of 3′-blocked DNA lesions by human APE2 is critical to deal with BRCA-deficiency-derived vulnerabilities for genome stability (90). Future studies using other model systems will provide extra evidence showing the evolutionarily conserved function of APE2 in SSB end resection for genome integrity.

Distinct role and mechanism of APE2 in cancer etiology

From the cBiolPortal database analysis of cross-cancer alteration summary for APEX2 (human APE2), hundreds of missense, truncations and spliced mutations are found in APE2 especially its functional domains (i.e., EEP, PIP and Zf-GRF) (97, 146). Those mutations from cancer patients may affect APE2's catalytic functions as well as non-catalytic functions. For example, the R490H mutant within APE2 Zf-GRF was found in endometrium carcinoma patient (TCGA-BG-A0M4-01). Because the R490 reside is in the most conserved signature motif GRxF within Zf-GRF, we hypothesize that the R490H mutant may be deficient for PCNA interaction and ssDNA interaction as well as APE2's 3′-5′ exonuclease activity. Future studies will delineate which mutant of APE2 mutant is deficient for what functionalities. Consistent with abnormal expression and mutation of APE2 in cancer patients, it has been proposed that APE2 is a oncogene in lung cancer (98).

Targeting the function and mechanisms of APE2 in DDR and DNA repair pathways for cancer therapeutics

Above all, targeting the ATR-Chk1 DDR pathway has become a promising novel approach for pancreatic and other cancer entities. Thus, targeting APE2 function and mechanism in ATR DDR pathway represents a novel cancer therapy strategy. My study has initiated this direction and has characterized two potential small molecule regulators: Celastrol and NU7441. These APE2 regulators alone or in combination with other chemotherapy drugs or radiotherapy can be tested in future preclinical and clinical setting.

FIGURES AND LEGENDS

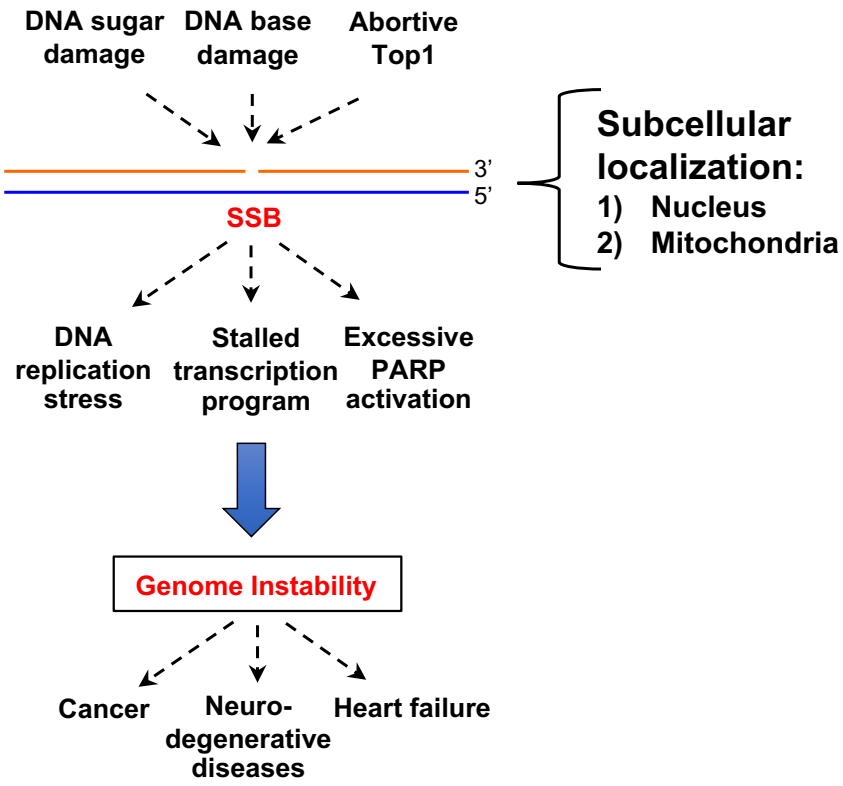

Figure 1. Generation and role of SSB in genome integrity. SSBs may be derived from DNA sugar damage, DNA base damage, and abortive Top1 activity, and are localized in nucleus and mitochondria. Unrepaired SSBs result in DNA replication stress, stalled transcription program, and excessive PARP activation, leading to genome instability and human diseases such as cancer, heart failure and neurodegenerative disorders.

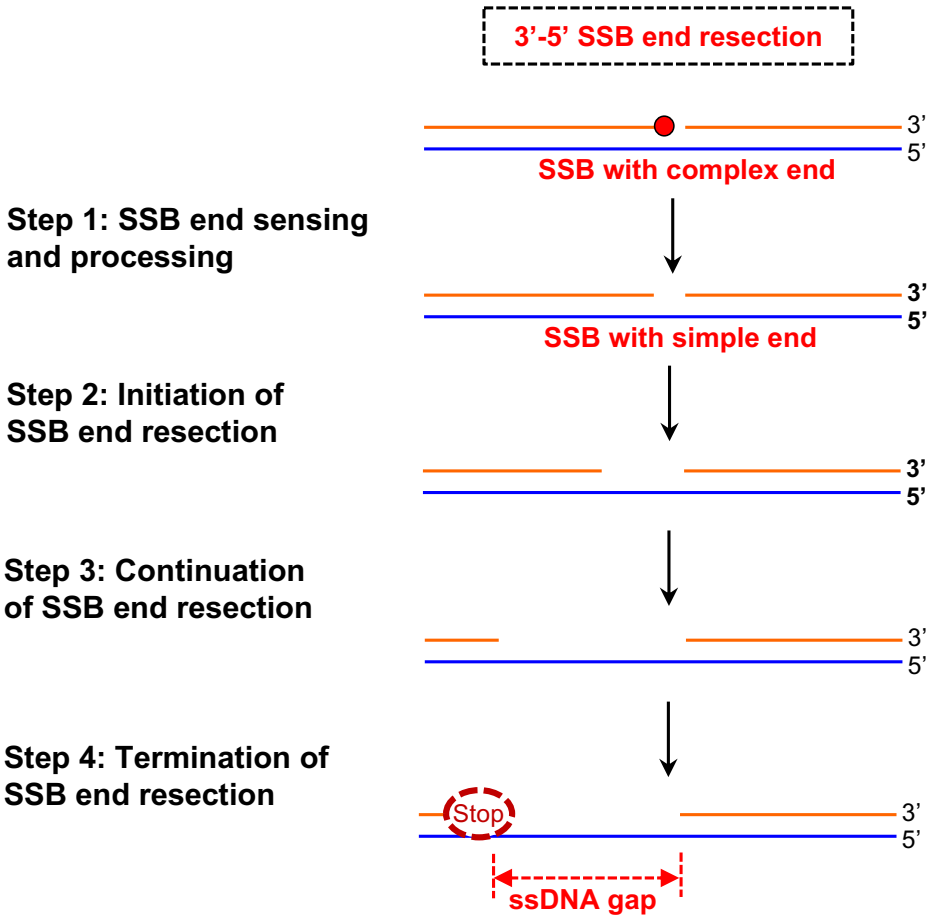

Figure 2. Four steps of SSB end resection: End sensing and processing, initiation, continuation, and termination of SSB end resection. 3'-5' SSB end resection steps are shown.

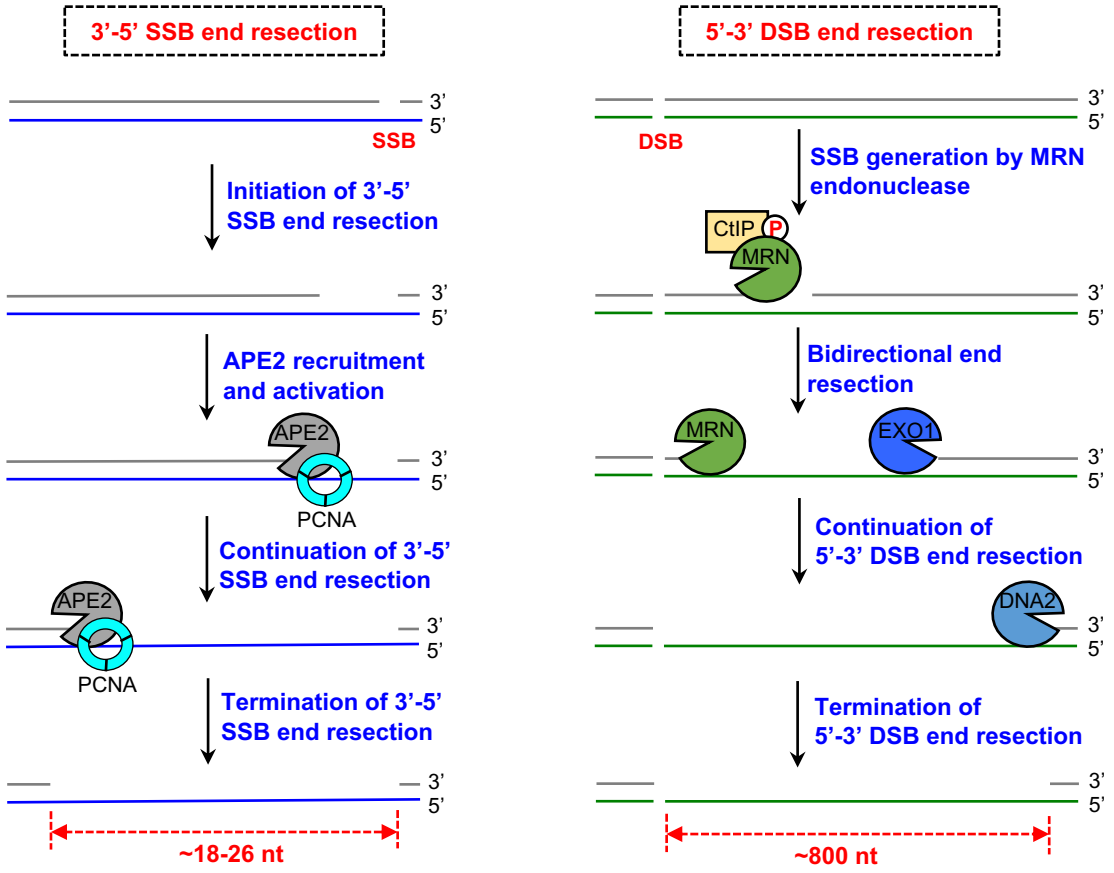

Figure 3. SSB end resection and DSB end resection. Left panel shows how SSB end is resected by APE2's 3'-5' exonuclease activity, generating ssDNA bound with RPA and recruiting ATR DDR protein complex. Right panel shows how DSB end is recognized by MRN complex and nicked by CtIP-mediated Mre11's endonuclease activity, followed by bidirectional end resection through MRN's 3'-5' exonuclease activity and EXO1's 5'-3' exonuclease activity. 5'-3' DSB end resection by EXO1 is further continued by DNA2 to generate a longer stretch of ssDNA.

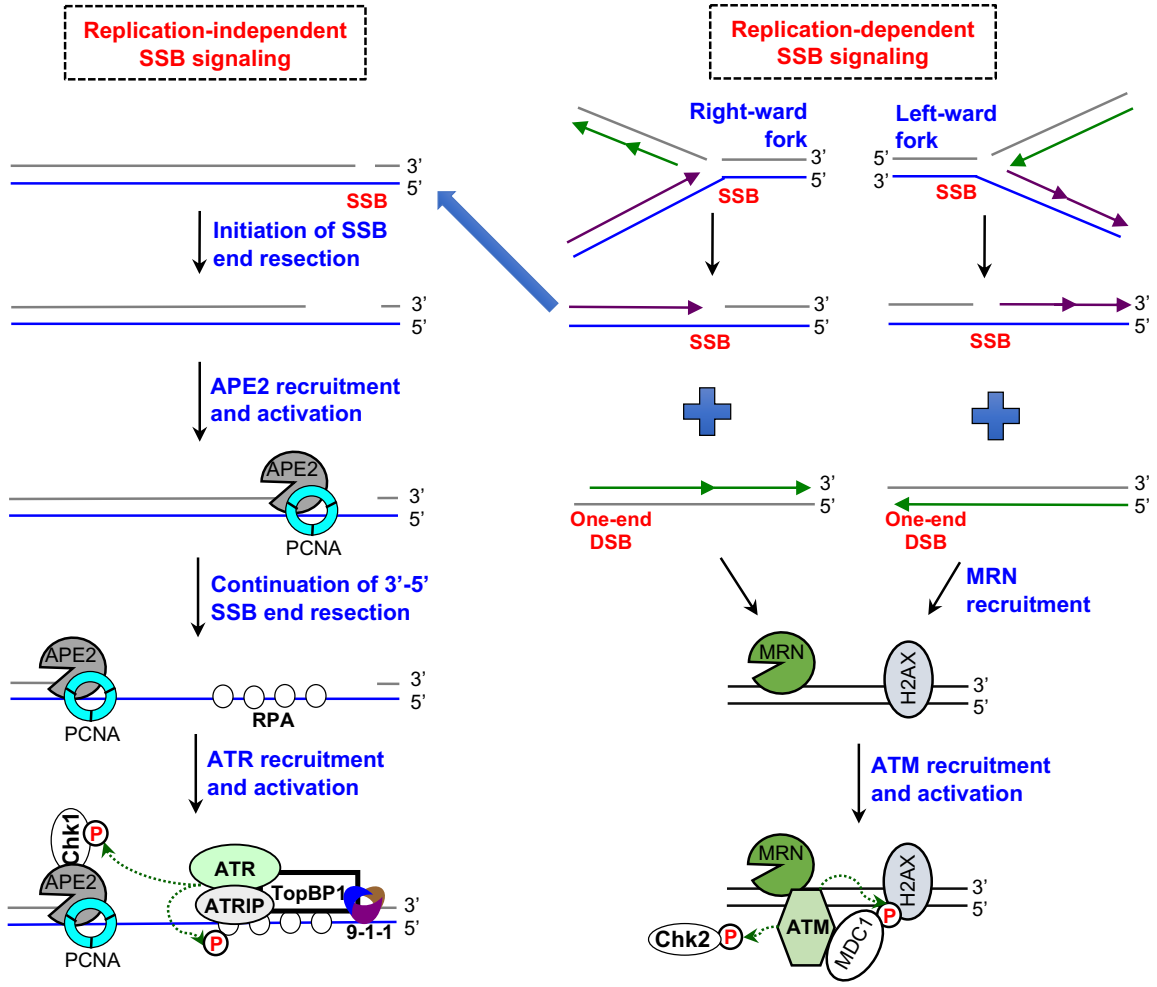

Figure 4. SSB signaling. Left panel demonstrates that APE2 is recruited to SSB sites and activated by two modes of PCNA interactions and ssDNA association. SSB end is resected by APE2 to generate ssDNA for assembly of ATR DDR protein complex including ATR, ATRIP, TopBP1, and the 9-1-1 complex. The SSB-induced ATR DDR pathway is replication-independent. Right panel shows replication-dependent generation of DSBs from unrepaired SSBs. The one-end DSB triggers MRN complex recruitment and ATM DDR activation including γ–H2AX and Chk2 phosphorylation

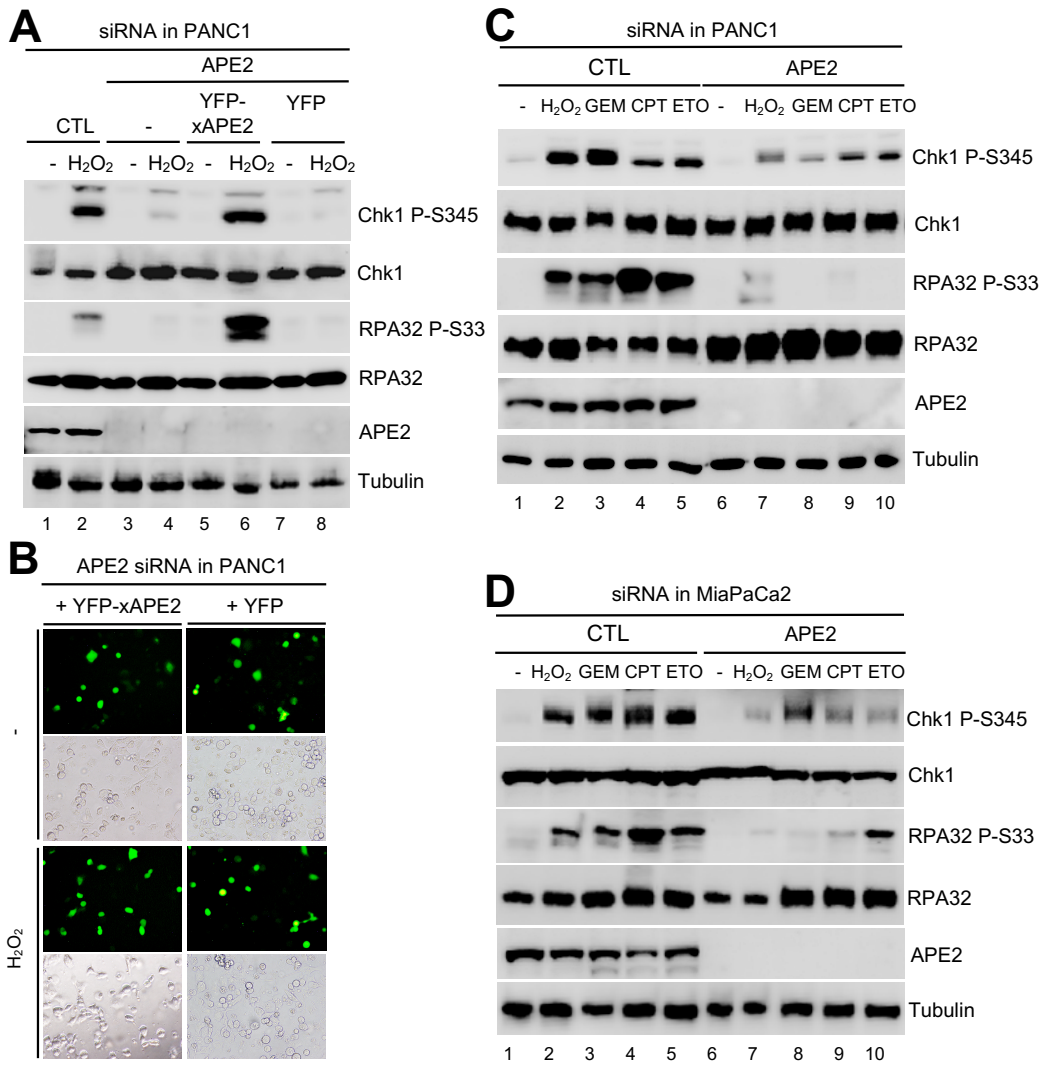

Figure 5. APE2 is important for the activation of the ATR-Chk1 DDR pathway in pancreatic cancer cells. (A) Transfecting YFP-xAPE2 but not YFP can rescue the Chk1 and RPA32 phosphorylation induced by H_2O_2 in APE2-KD PANC1 cells. PANC1 cells were treated with CTL siRNA or APE2 siRNA for 7 days. Plasmid expressing YFP-xAPE2 or YFP was transfected to APE2-KD MiaPaCa2 cells after 3 days of siRNA-mediated knockdown. After 4-hour treatment of H_2O_2 (1mM), total cell lysates were extracted and analyzed via immunoblotting as indicated. **(B)** Fluorescence microscopy analysis shows that YFP-xAPE2 or YFP was expressed similarly in APE2-KD PANC1 cells with or without treatment of H_2O_2. **(C-D)** The ATR DDR signaling in cell lysates of PANC1 cells (**C**) or MiaPaCa2 cells (**D**) with control (CTL) or APE2 siRNAs after treatment of various DNA damaging conditions was examined via immunoblotting analysis as indicated. Cells were treated with H_2O_2 (1mM), GEM (50μM), CPT (5μM), or ETO (50μM) for 4 hrs, respectively.

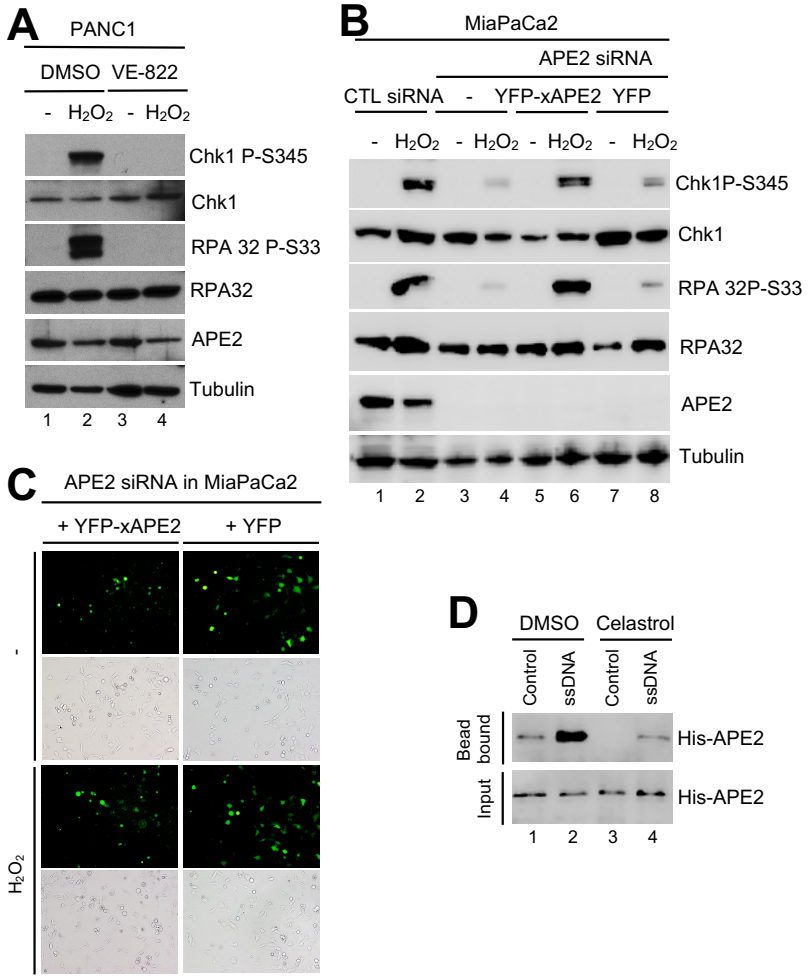

Figure 6. Important function of APE2 in ATR-Chk1 DDR pathway in pancreatic cancer cells. (A) After 1-hour pretreatment of VE-822 (5μM), PANC1 cells were added with or without H_2O_2 (1mM) for 4 hours. Cell lysates were examined via immunoblotting analysis as indicated. **(B)** MiaPaCa2 cells were treated with CTL siRNA or APE2 siRNA for 7 days. Plasmid expressing YFP-xAPE2 or YFP was transfected to APE2-KD MiaPaCa2 cells after 3 days of siRNA-mediated knockdown. After 4-hour treatment of H_2O_2 (1mM), total cell lysates were extracted and analyzed via immunoblotting as indicated. **(C)** Fluorescence microscopy analysis shows that the YFP-xAPE2 and YFP was expressed similarly in APE2-KD MiaPaCa2 cells. **(D)** The binding of His-tagged human APE2 recombinant protein (100ng/μl) to beads coupled with ssDNA was compromised by the addition of Celastrol (0.1mM).

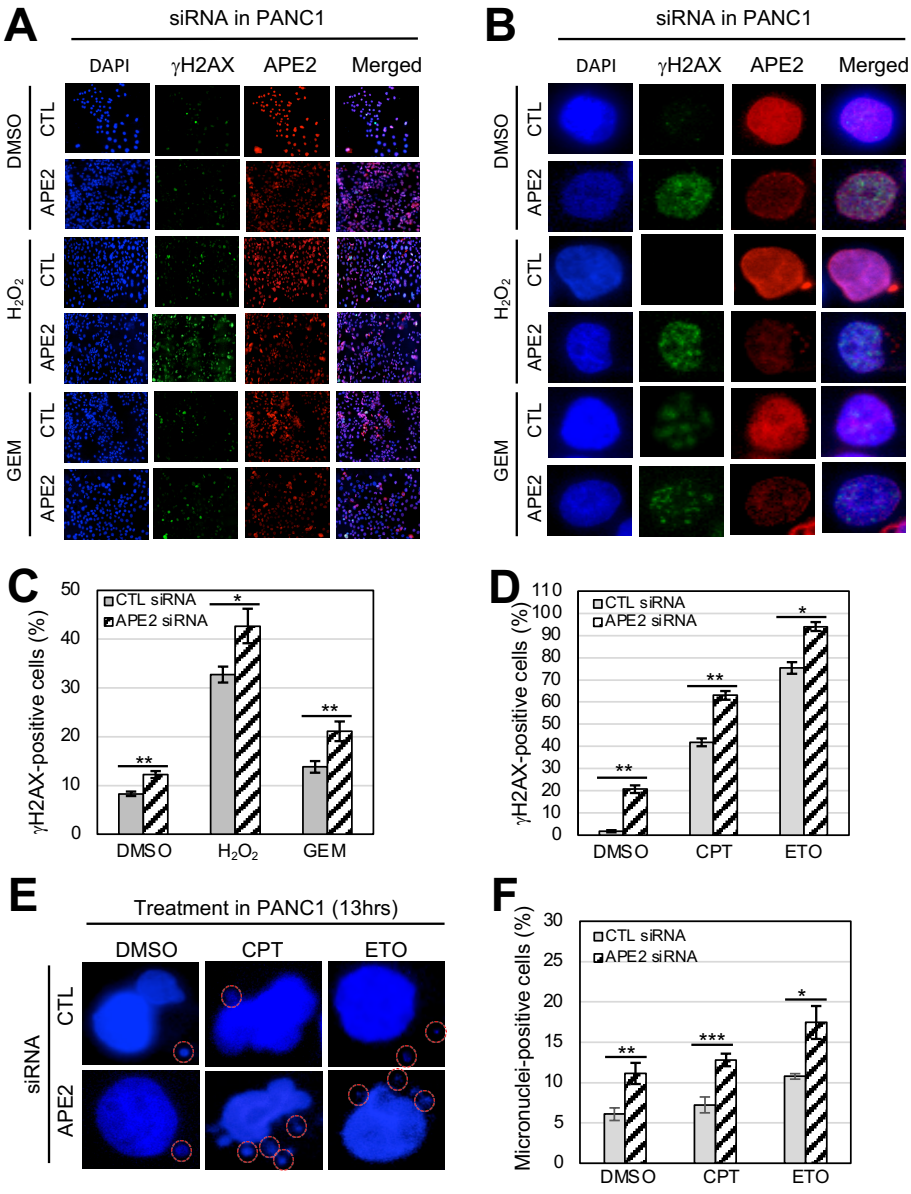

Figure 7. APE2-KD induces substantially more γH2AX foci and micronuclei under normal or stress conditions in PANC1 cells. (A-B) Immunofluorescence microscopy analysis shows γH2AX and APE2 foci after DMSO or treatment of H_2O_2 (1mM for 5 hrs) or GEM (100μM for 5 hrs) in PANC1 cells with CTL or APE2 siRNA in a slide view (**A**) or in a single cell view (**B**). **(C-D)** Percentage of γH2AX-positive PANC1 cells after treatment of H_2O_2/GEM (**C**) or CPT/ETO (**D**). **e** Microscopy analysis shows micronuclei (circled with red) after DAPI staining in PANC1 cells after treatment of CPT (1μM for 13 hrs) or ETO (20μM for 13 hrs) with CTL or APE2 siRNA. **f** Percentage of micronuclei-positive PANC1 cells after treatment of CPT or ETO with CTL or APE2 siRNA was quantified. **(C-D), (F)** * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$; n=3.

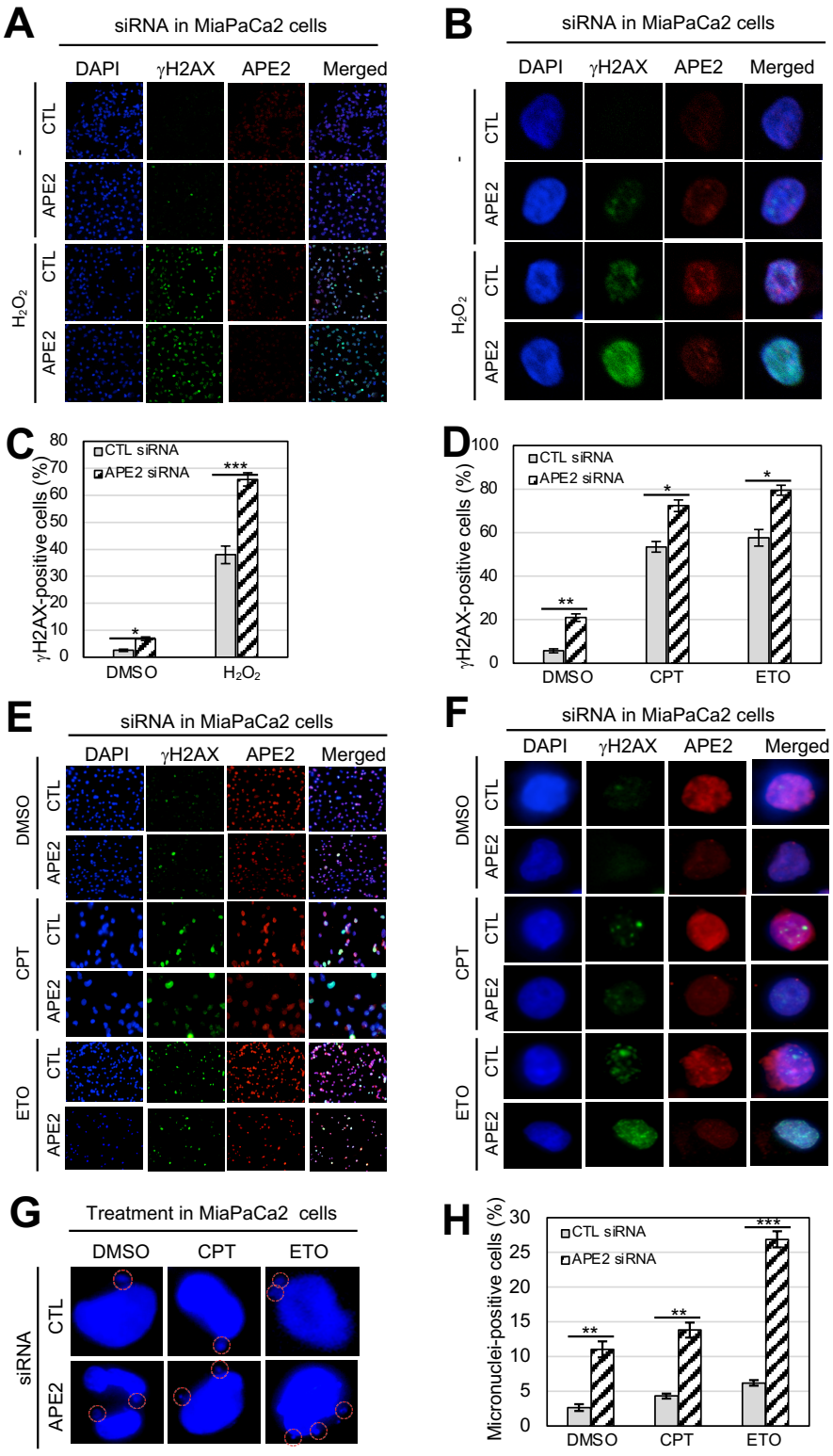
A
siRNA in MiaPaCa2 cells
DAPI
γH2AX
APE2
Merged
-
CTL
APE2
H2O2
CTL
APE2
B
siRNA in MiaPaCa2 cells
DAPI
γH2AX
APE2
Merged
C
γH2AX-positive cells (%)
CTL siRNA
APE2 siRNA
DMSO
H2O2
D
γH2AX-positive cells (%)
CTL siRNA
APE2 siRNA
DMSO
CPT
ETO
E
siRNA in MiaPaCa2 cells
DAPI
γH2AX
APE2
Merged
DMSO
CPT
ETO
CTL
APE2
F
siRNA in MiaPaCa2 cells
DAPI
γH2AX
APE2
Merged
G
Treatment in MiaPaCa2 cells
DMSO
CPT
ETO
siRNA
CTL
APE2
H
Micronuclei-positive cells (%)
CTL siRNA
APE2 siRNA
DMSO
CPT
ETO

Figure 8. APE2-KD induces substantially more γH2AX and micronuclei in MiaPaCa2 cells.

(A-B, E-F) Immunofluorescence analysis was performed in MiaPaCa2 cells after CTL siRNA or APE2 siRNA with or without treatment of H_2O_2 (625μM for 5 hrs), CPT (5μM for 5 hrs) or ETO (50μM for 5 hrs). DAPI, γH2AX, APE2, and merged images from presentative cells were shown in a slide view (A and E) and a single cell view (B and F). **(C-D)** Percentage of γH2AX-positive cells from (A) or (E) was quantified in (C) and (D), respectively. **(G)** Micronuclei formation of MiaPaCa2 after CTL siRNA or APE2 siRNA with or without 5-hour treatment of CPT (5μM) or ETO (50μM) was examined via microscopy analysis. Red-dotted circles indicated the micronuclei.

(**H)** Percentage of micronuclei-positive MiaPaCa2 cells after treatment of CPT or ETO with CTL or APE2 siRNA from (G) was quantified. **(C, D, H)** * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$.

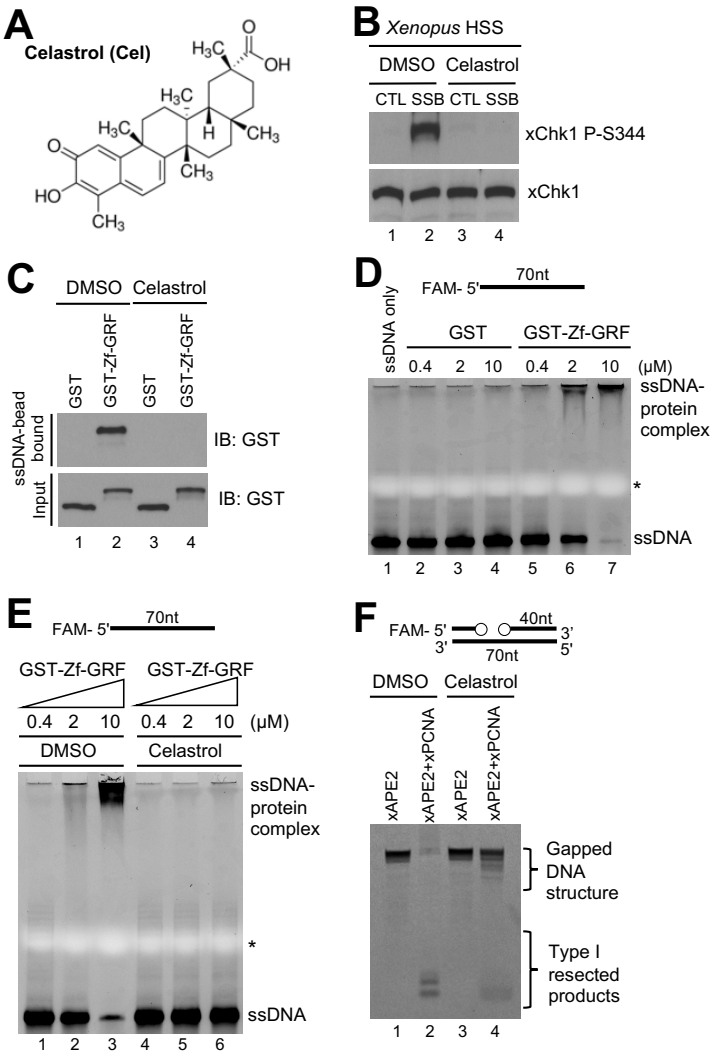

Figure 9. A small molecule inhibitor Celastrol impairs APE2 function in the SSB-induced ATR DDR pathway in the *Xenopus* system. (A) Chemical structure of a small molecule inhibitor compound Celastrol. **(B)** Celastrol (1mM) compromises Chk1 phosphorylation induced by SSB plasmid but not CTL plasmid in the *Xenopus* HSS system via immunoblotting analysis. **(C)** The binding of recombinant GST-Zf-GRF but not GST to Dynabead coupled with ssDNA was impaired by Celastrol in GST-pulldown assays. **(D)** EMSA assay shows that the binding of GST-Zf-GRF but not GST can form ssDNA (70nt)-protein complex. **(E)** The binding of GST-Zf-GRF to ssDNA was impaired by Celastrol in EMSA assays. **(F)** The PCNA-stimulated 3′-5′ exonuclease activity of *Xenopus* APE2 *in vitro* was inhibited by Celastrol in TBE-Urea gel electrophoresis.

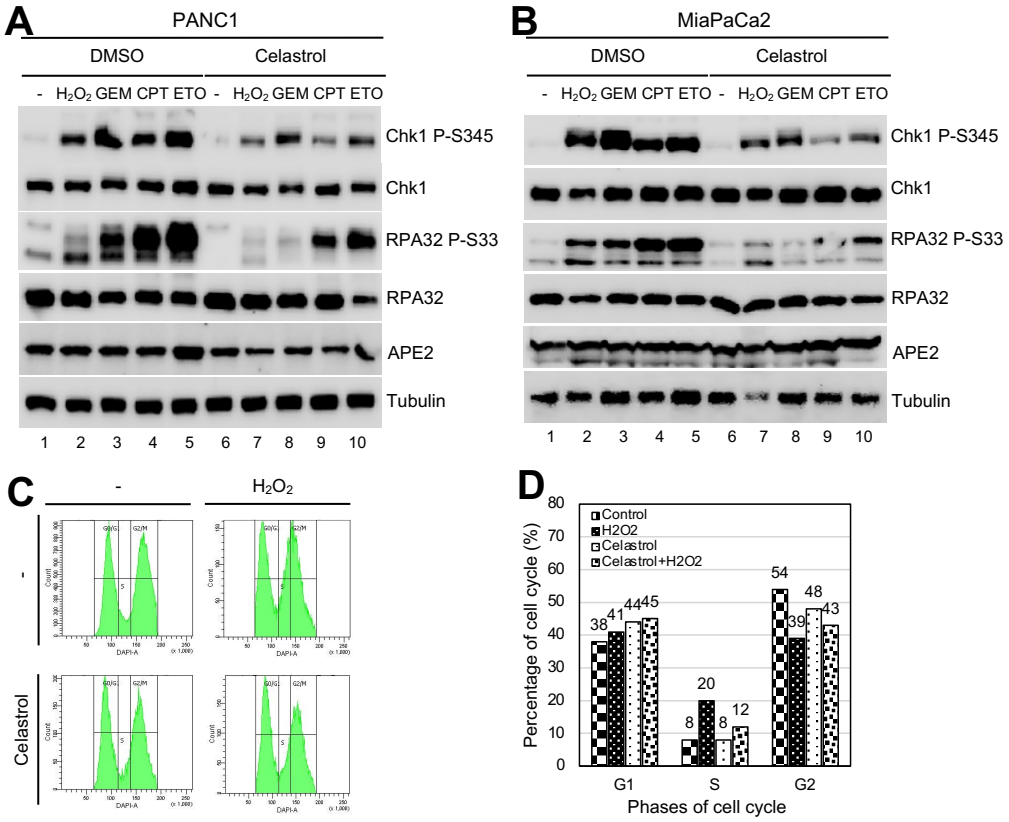

Figure 10. Celastrol compromises the ATR-Chk1 DDR pathway activation in pancreatic cancer cells. (A-B) PANC1 (**A**) or MiaPaCa2 (**B**) cells were pretreated with Celastrol (2.5μM) for 1 hr followed by 4-hour treatment of H_2O_2 (1mM), GEM (50μM), CPT (5μM), or ETO (50μM), respectively. Cell lysates were then extracted and examined via immunoblotting analysis as indicated. **(C)** Cell cycle profiling of PANC1 cells was examined via FACS analysis after 1-hour treatment of Celastrol (5μM) followed by 4-hour treatment of H_2O_2 (1mM). (**D)** Quantification of FACS analysis in (**C**) shows percentage of cell cycle phases.

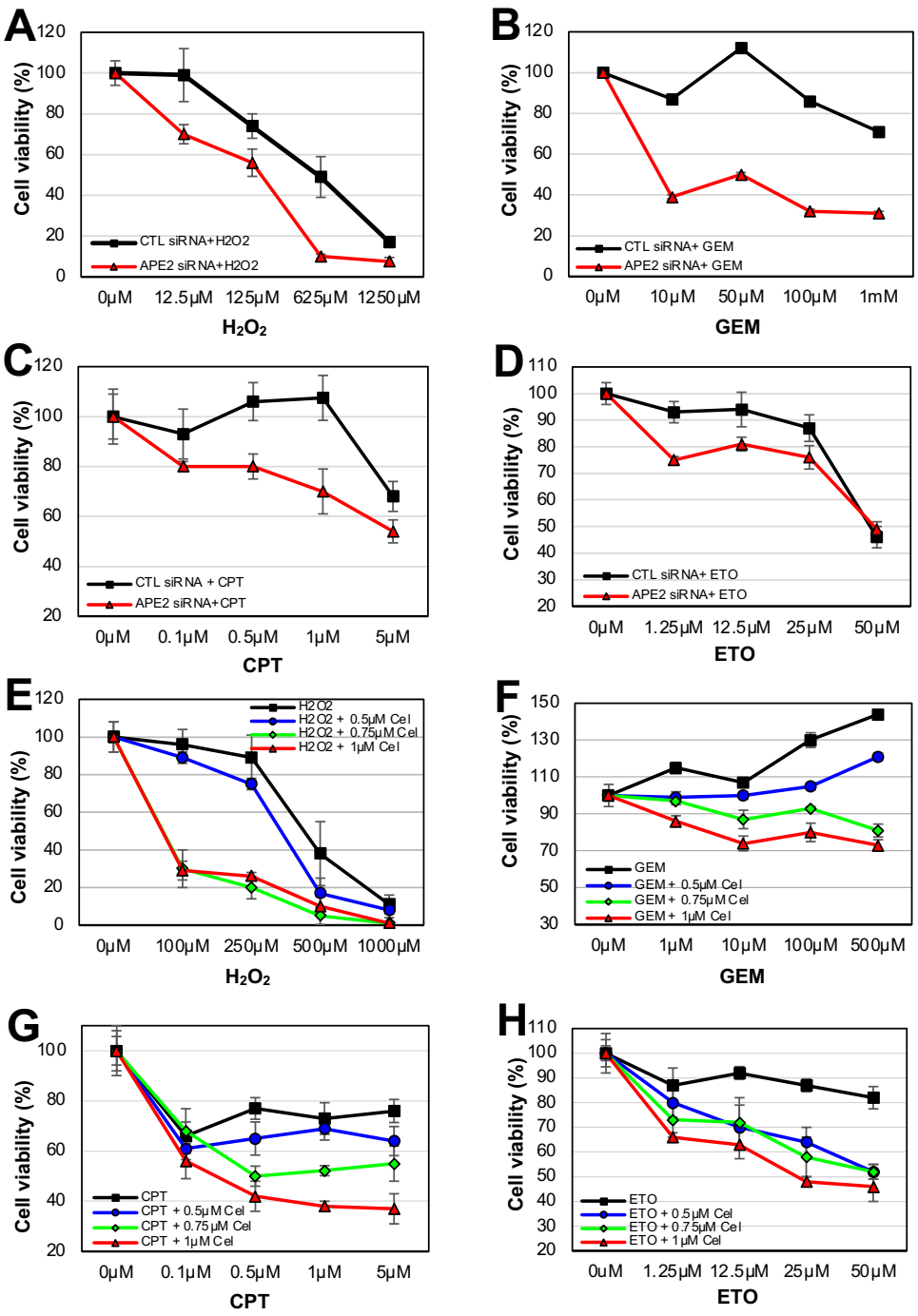

Figure 11. APE2 suppression or Celastrol sensitizes PANC1 cells to chemotherapy drugs. (A-D) Cell viability assays show that APE2-KD PANC1 cells are more vulnerable to stress conditions than Control (CTL) siRNA transfected cells. **(E-H)** Cell viability assays demonstrate that Celastrol (0.5μM, 0.75μM, or 1μM) sensitizes PANC1 cells to H_2O_2 (**E**), GEM (**F**), CPT (**G**), or ETO (**H**).

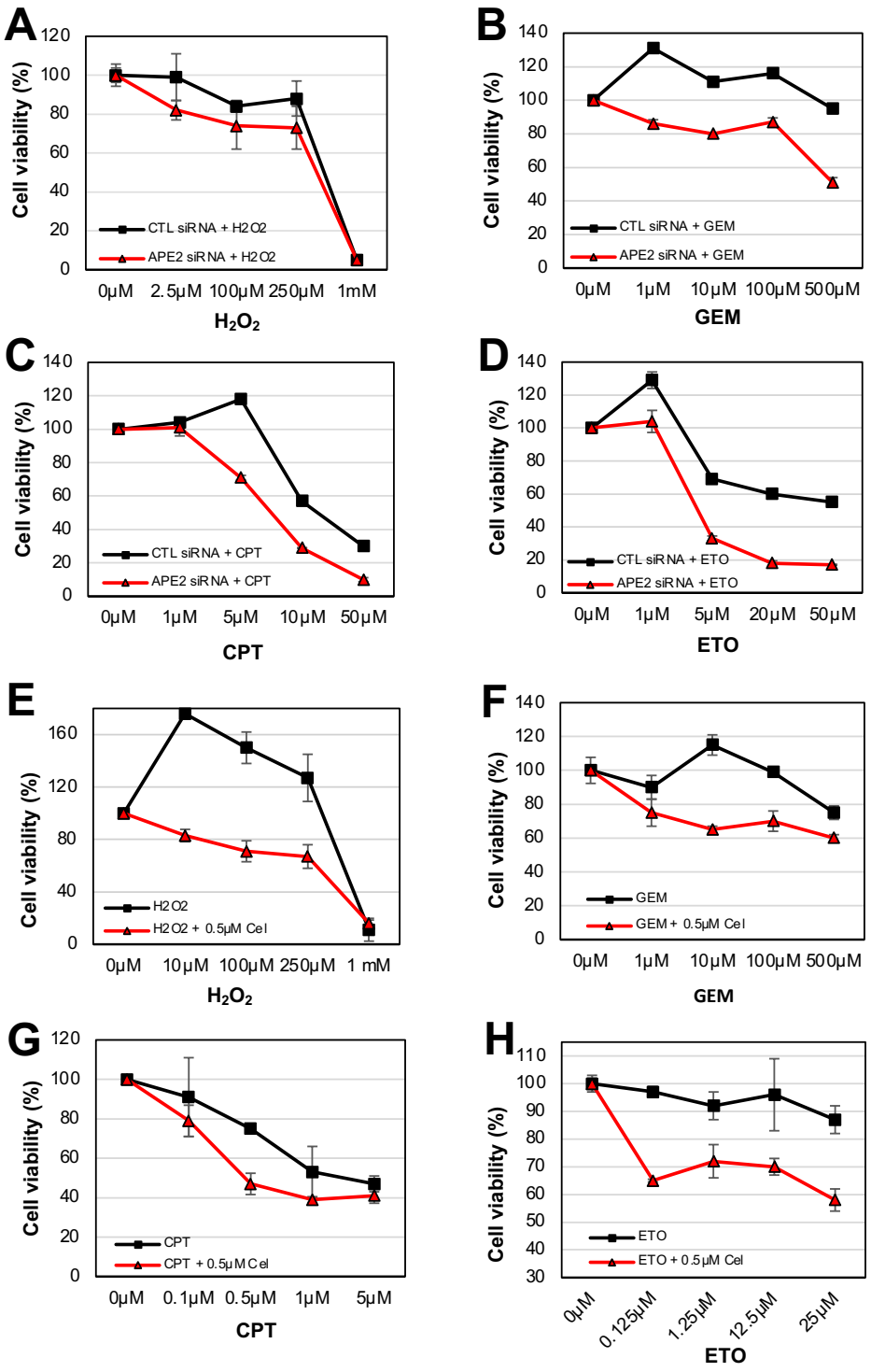

Figure 12. APE2-KD or Celastrol sensitized MiaPaCa2 cells to chemotherapy drugs. (**A-D)** Cell viability assays show that APE2-KD MiaPaCa2 cells were more vulnerable to stress conditions (H_2O_2, GEM, CPT, or ETO) compared to CTL siRNA transfected cells. **(E-H)** Cell viability assays demonstrate that Celastrol sensitized MiaPaCa2 cells to H_2O_2, GEM, CPT, and ETO.

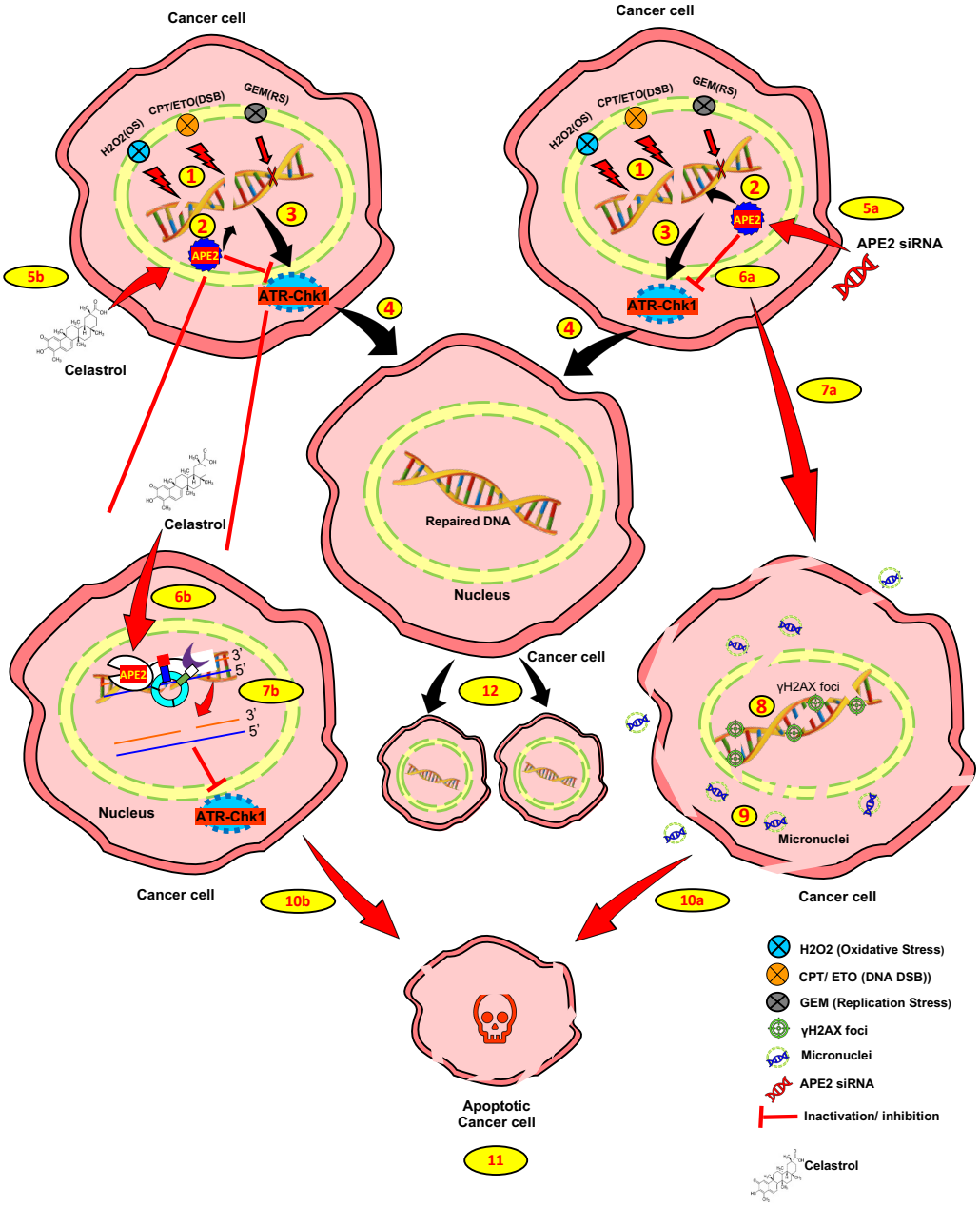

Figure 13. A cellular illustration of APE2 protecting cancer cells in response to DNA damage/ stress conditions. A hypothetical cellular model illustrating the mechanism of APE2 in protecting cancer cells in response to OS/DSB/RS stress conditions. (**1**) H_2O_2, GEM & CPT, ETO induce OS, RS & DSB damage conditions in cancer cells**. (2,3**) On the damage sites (SSBs, DSBs, Replication Fork obstruction) the recruitment of PCNA followed by APE2, Chk-1 start up the ssDNA resection in 3'-5' direction where RPA binds and coats the ssDNA stretch and make the basement for the recruiting of repair protein complex comprising of ATR, ATRIP, TopBP1 and 9-1-1 complex. Next, ATR bound with ATRIP activates the Chk1 on APE2, which afterwards activate the 9-1-1 complex that in a positive feedback mechanism will trigger the ATR-Chk1 DDR pathway[53]. (**4,12**) Damaged

DNA of cancer cells are repaired via ATR-Chk1 DDR pathway which can further replicate its DNA and go through cell division cycles. **(5a,6a)** siRNA mediated KD of *APE2* results in inactivation of ATR-Chk1 DDR pathway. (**7a,8,9)** *APE2* downregulation induces more prominent γH2AX foci (DSB damage) & Micronuclei formation (Chromosomal DNA damage). (**5b,6b,7b)** Celastrol inhibits the binding of APE2 Zf-GRF motif to ssDNA thus making a defective APE2 Zf-GRF-ssDNA interaction which afterwards leads to a defective SSB end resection, hence inactivating ATR-Chk1 DDR pathway. (**10a,10b,11)** Persistent damages to DNA, nuclei, membranes etc. of the cancer cells make the cells go through cell death/ apoptotic pathways and culminate into cell death.

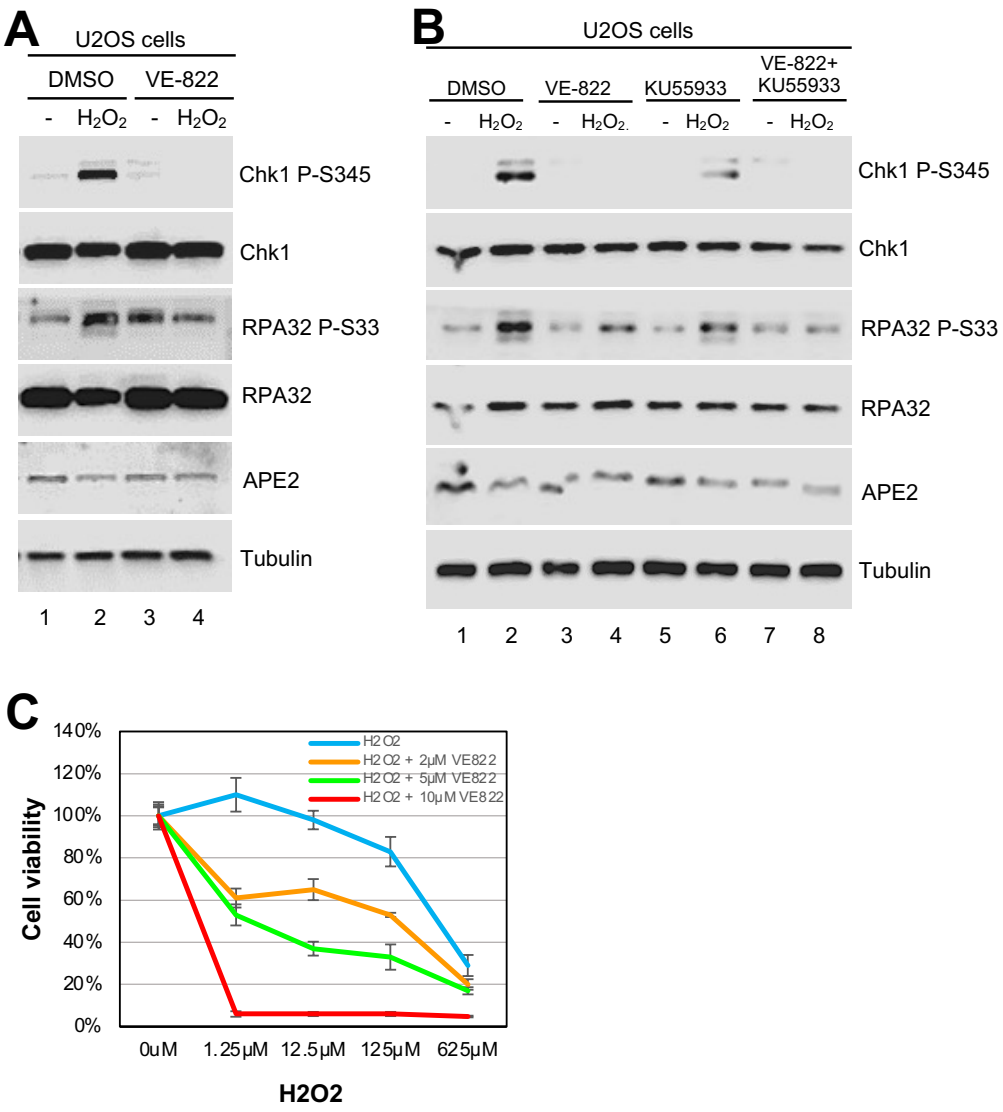

Figure 14. Oxidative stress triggers ATR-Chk1 DDR & VE-822 but not KU55933 compromises ATR-Chk1 DDR activation in U2OS cells & sensitizes U2OS cells to Oxidative stress conditions. (A) After 1-hour pretreatment of VE-822 (5μM), U2OS cells were added with or without H_2O_2 (1mM) for 4 hours. **(B)** After 1-hour pretreatment of VE-822 (5μM), KU55933 (5μM) or both treatments U2OS cells were added with or without H_2O_2 (1mM) for 4 hours. Cell lysates were examined via immunoblotting analysis as indicated. (**C**) Cell viability assay demonstrate that VE-822 sensitizes U2OS cells to H_2O_2 in a dose-dependent manner.

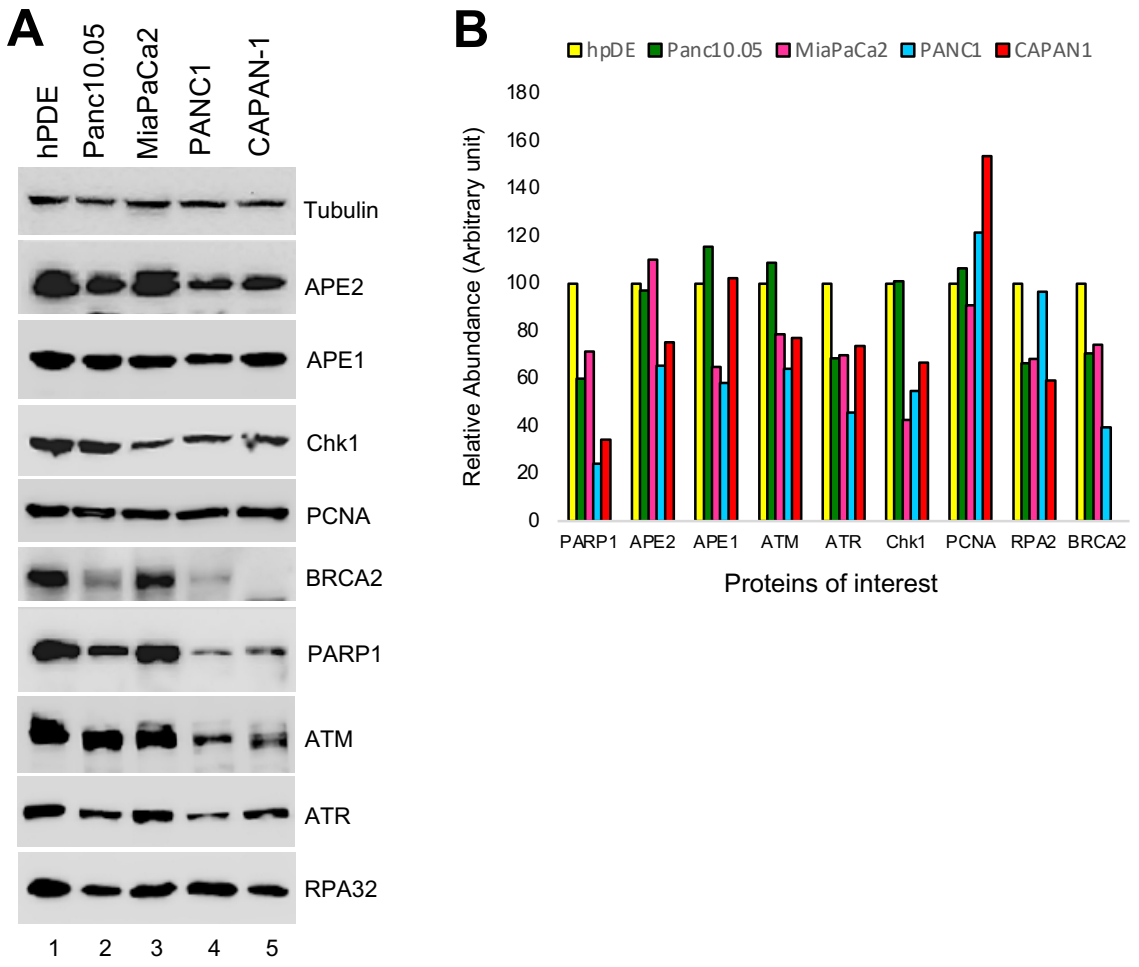

Figure 15. APE2 & other DDR protein abundance in Pancreatic cells of different genetic background, mutation & transformation status. (A) Total cell lysates were extracted from noncancerous, hPDE cells; BRCA2 WT cancerous; Panc10.05, MiaPaCa2 & PANC1 cells; BRCA2 mutant/deficient cancerous, CAPAN-1 cells and analyzed via immunoblotting as indicated. (**B**) Quantification of the western blot presented in (**A**). Signal was measured using BIORAD ImageLab Software, normalized to hpDE cell lystaes' loading control (Tubulin).

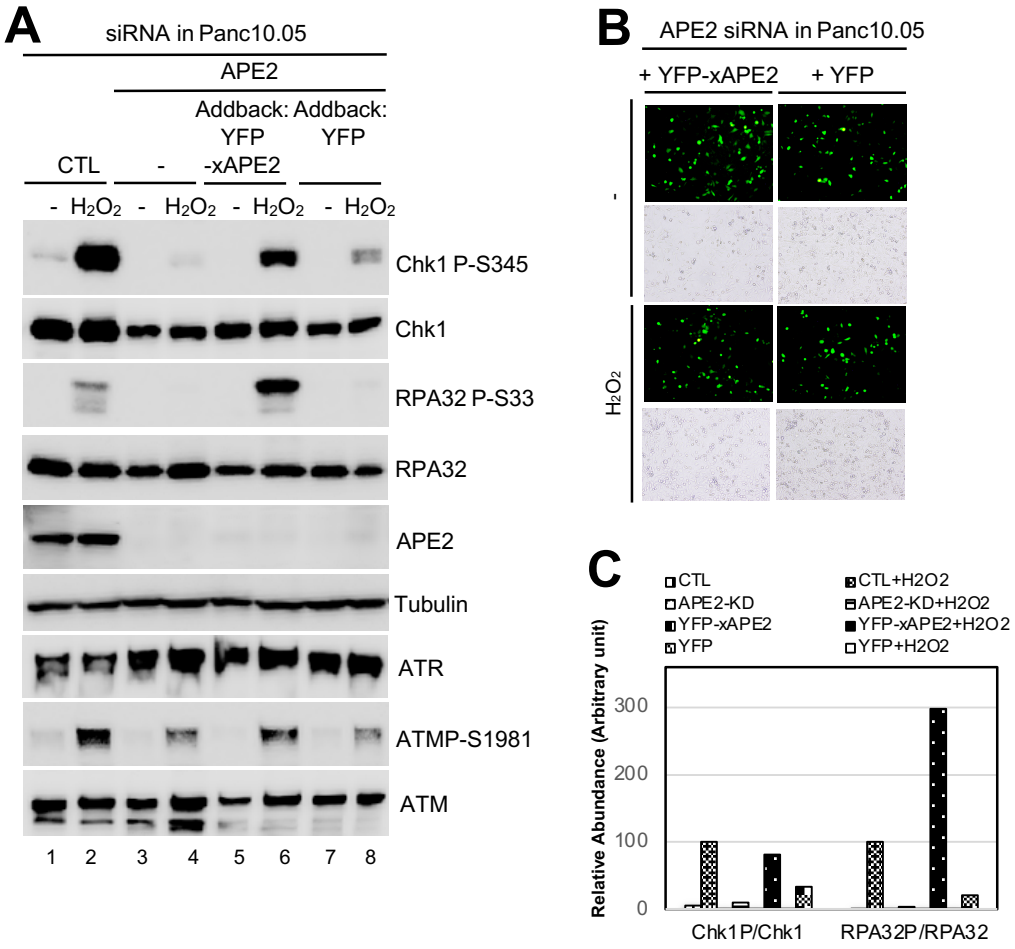

Figure 16. Important function of APE2 in ATR-Chk1 DDR pathway in Panc10.05 cells. (A) Panc10.05 cells were treated with CTL siRNA or APE2 siRNA for 7 days. Plasmid expressing YFP-xAPE2 or YFP was transfected to APE2-KD Panc10.05 cells after 3 days of siRNA-mediated knockdown. After 4-hour treatment of H_2O_2 (1mM), total cell lysates were extracted and analyzed via immunoblotting as indicated. **(B)** Fluorescence microscopy analysis shows that the YFP-xAPE2 and YFP was expressed similarly in APE2-KD MiaPaCa2 cells. **(C)** Quantification of the western blot presented in (**A**). Signal was measured using BIORAD ImageLab Software, normalized to loading control (Tubulin).

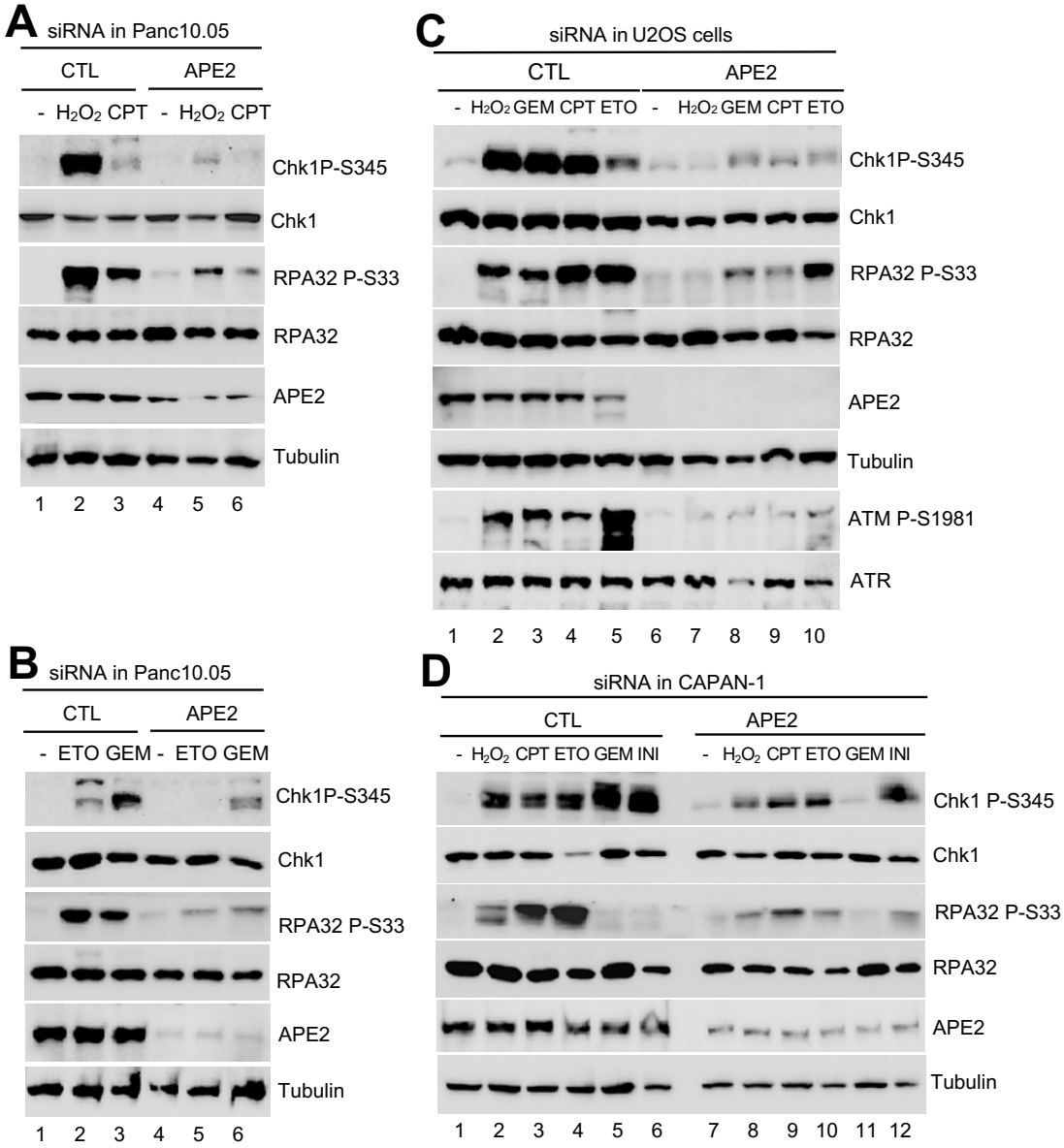

Figure 17. APE2 is important for the activation of the ATR-Chk1 DDR pathway in other cancer cells/types having different genetic/morphological background, mutation & transformation status. The ATR DDR signaling in cell lysates of Panc10.05 (**A-B**) or U2OS (**C**) or CAPAN-1 cells (**D**) with control (CTL) or APE2 siRNAs after treatment of various DNA damaging conditions was examined via immunoblotting analysis as indicated. Cells were treated with H_2O_2 (1mM), GEM (50μM), CPT (5μM), ETO (50μM) or INI (100μM) for 4 hrs, respectively.

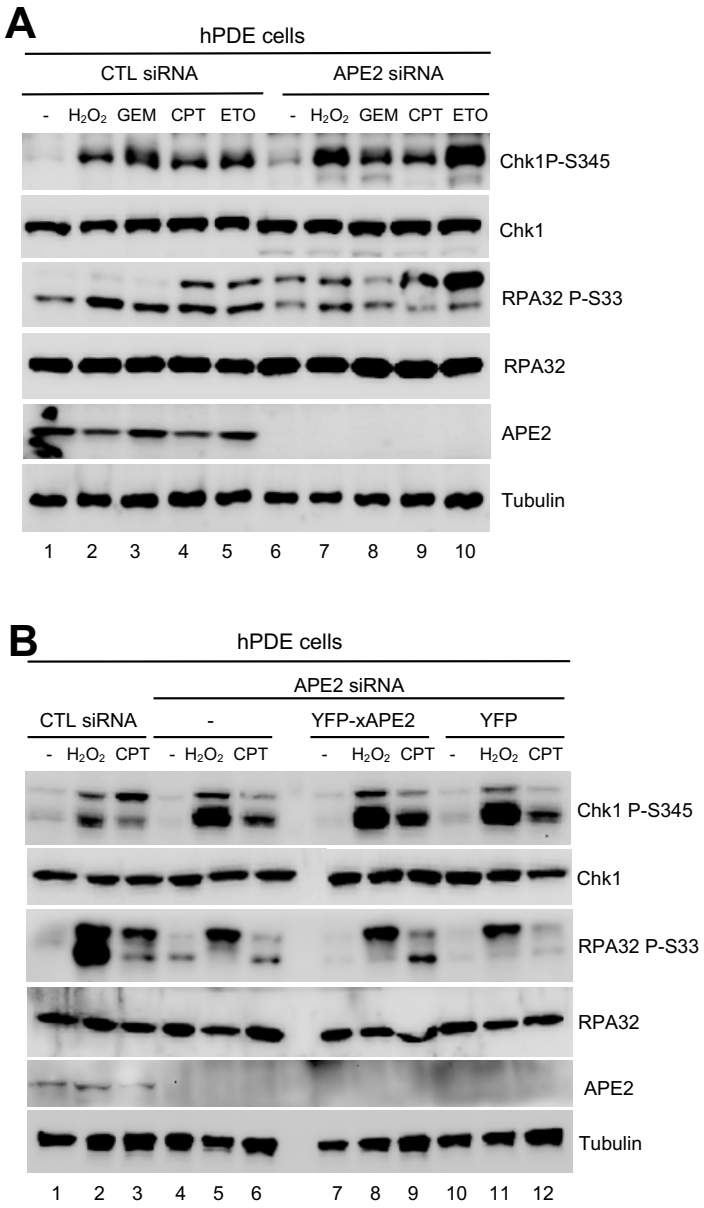

Figure 18. APE2 is not required for the activation of the ATR-Chk1 DDR pathway in pancreatic normal hPDE cells. (A) The ATR DDR signaling in cell lysates of hPDE cells with control (CTL) or APE2 siRNAs after treatment of various DNA damaging condition was examined via immunoblotting analysis as indicated. Cells were treated with H_2O_2 (1mM), GEM (50μM), CPT (5μM), or ETO (50μM) for 4 hrs, respectively. **(B)** Transfection of YFP-xAPE2 & YFP can't change the phenotype of the Chk1 and RPA32 phosphorylation induced by H_2O_2 in APE2-KD hPDE cells. hPDE cells were treated with CTL siRNA or APE2 siRNA for 7 days. Plasmid expressing YFP-xAPE2 or YFP was transfected to APE2-KD hPDE cells after 3 days of siRNA-mediated knockdown. After 4-hour treatment of H_2O_2 (1mM) & CPT (5μM), total cell lysates were extracted and analyzed via immunoblotting as indicated**.**

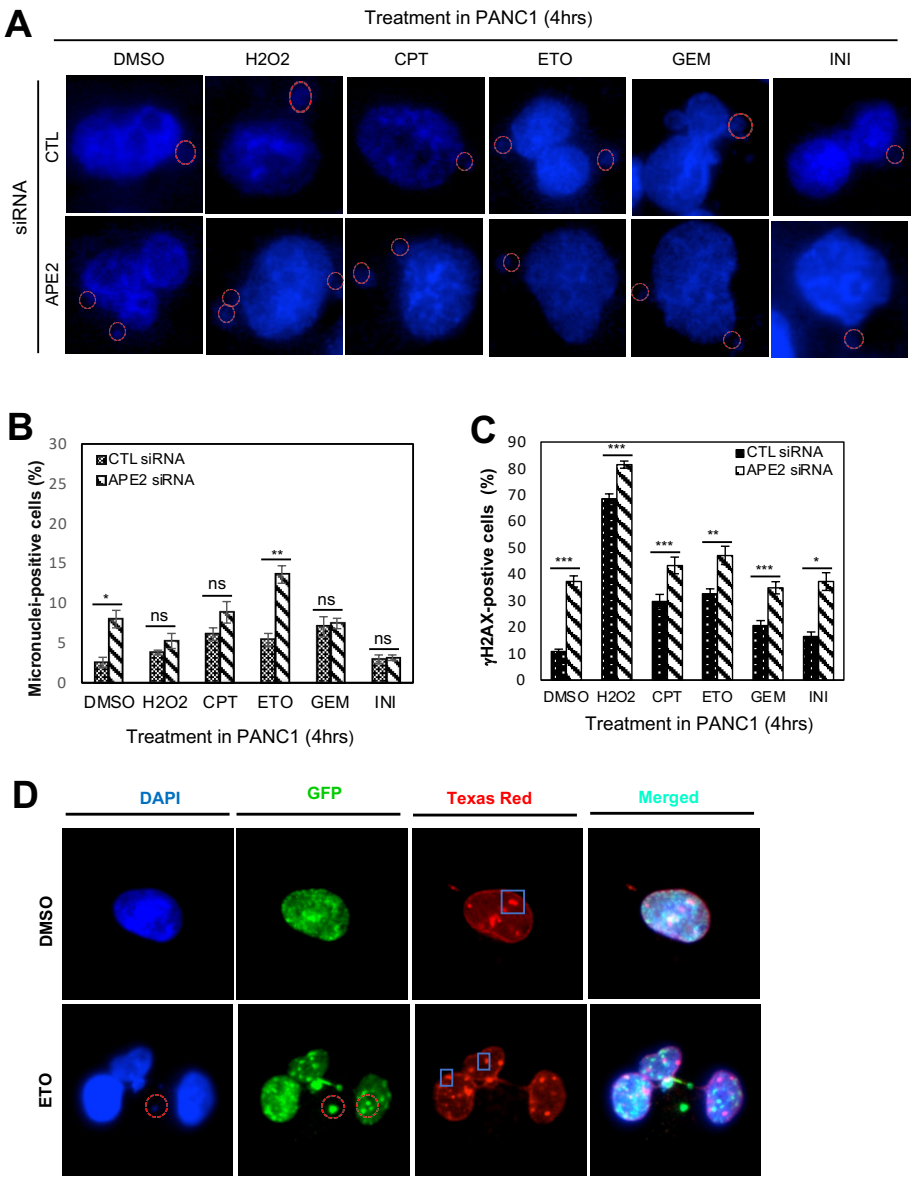

Figure 19. APE2-KD induces substantially more γH2AX foci and micronuclei under normal or stress conditions in PANC1 cells & γH2AX, APE2 foci & Micronuclei staining in mammalian cells via Leica-LASX microscope. (A) Microscopy analysis shows micronuclei (circled with red) after DAPI staining in PANC1 cells after treatment of H_2O_2 (1mM), GEM (100μM), CPT (10μM), ETO (100μM) or INI (25μM) for 4 hrs with CTL or APE2 siRNA. **(B)** Percentage of micronuclei-positive PANC1 cells from experimental setup **(A)** was quantified. **(C)** Percentage of γH2AX-positive PANC1 cells after treatment of H_2O_2 (1mM), GEM (100μM), CPT (10μM), ETO (100μM) or INI (25μM) for 4 hrs with CTL or APE2 siRNA was quantified. **(B), (C)** * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$; ns indicates $p>0.05$, n=2. (**D**) MiaPaCa2 cells after treatment of DMSO or ETO (100μM) for 4 hrs imaged via Leica-LASX microscope, γH2AX foci (boxed in blue) & APE2 foci (circled in red) & Micronuclei (circled in red) are pointed out in the image.

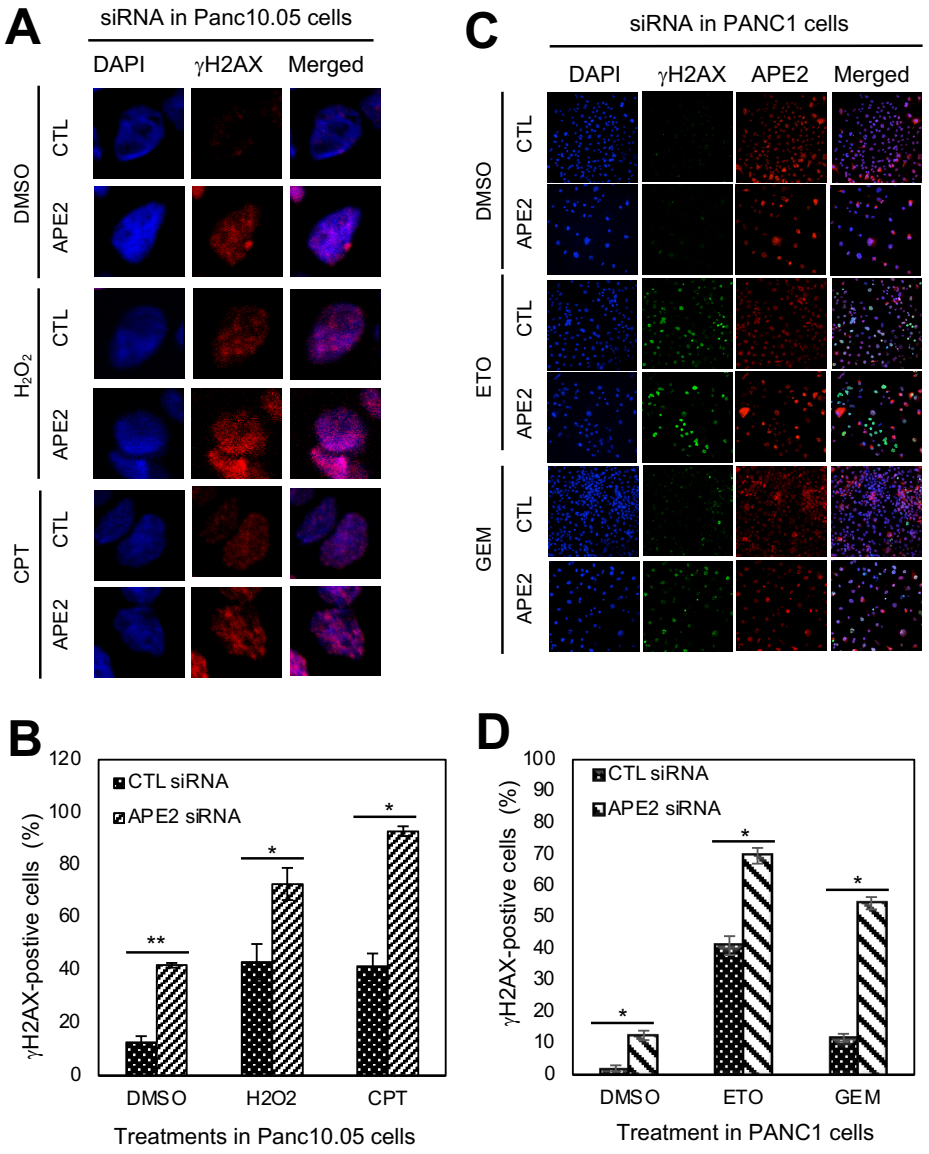

Figure 20. APE2-KD induces substantially more γH2AX foci and micronuclei under normal or stress conditions in pancreatic cancer cells. (A, C) Immunofluorescence microscopy analysis shows γH2AX and APE2 foci after DMSO or treatment of H_2O_2 (1.25 mM for 4 hrs) or CPT (5μM for 4 hrs) in Panc10.05 cells **(A)** or ETO (100μM for 4 hrs) or GEM (100μM for 4 hrs) in PANC1 cells **(C)** with CTL or APE2 siRNA in a single cell view (A) or in a slide view **(C)**. **(B, D)** Percentage of γH2AX-positive cells from **(A)** or **(C)** was quantified in **(B)** and **(D),** respectively **(B)** & **(D)** * indicates $p<0.05$; ** indicates $p<0.01$; ns indicates $p>0.05$, n=2.

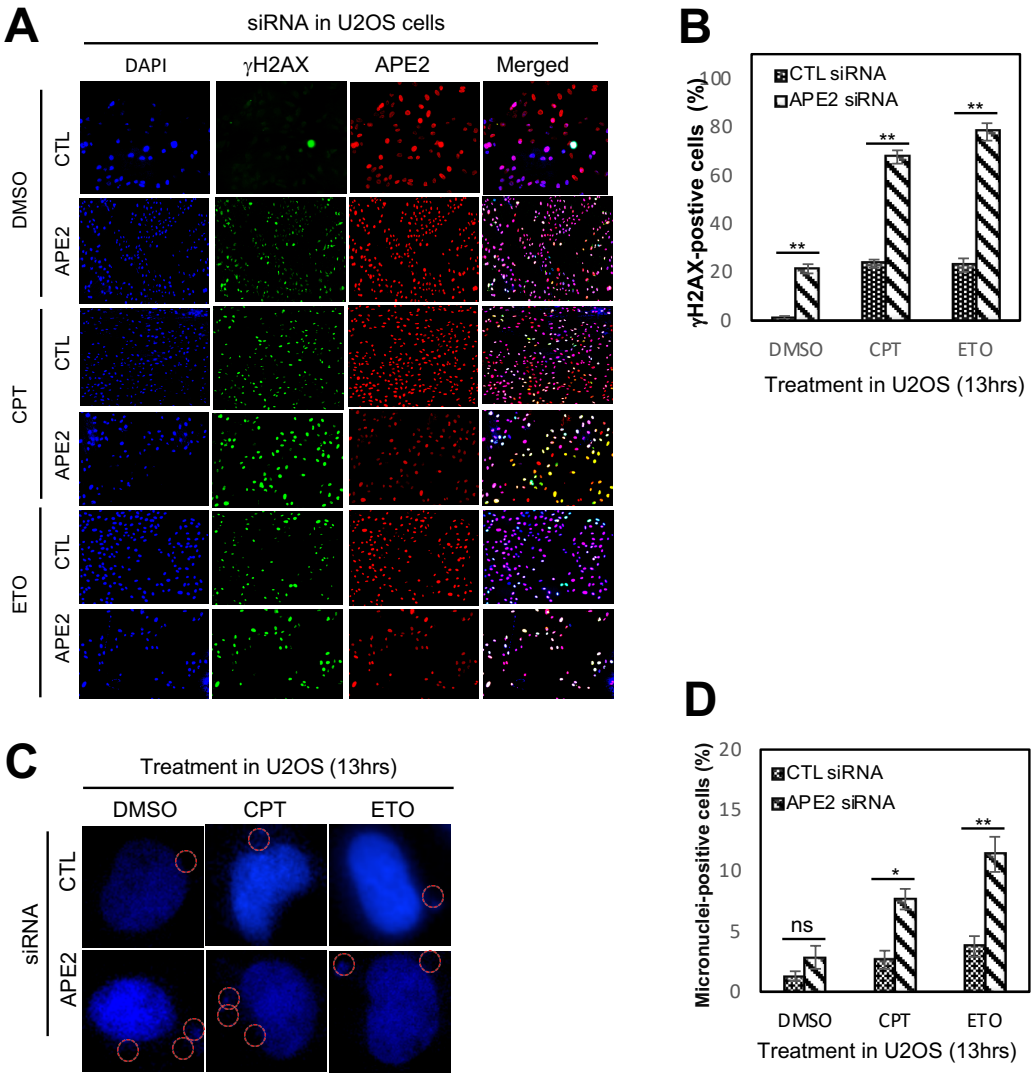

Figure 21. APE2-KD induces substantially more γH2AX foci and micronuclei under normal or stress conditions in U2OS cells. (A) Immunofluorescence microscopy analysis shows γH2AX and APE2 foci after DMSO or treatment of CPT (1μM for 13 hrs) or ETO (20μM for 13 hrs) in U2OS cells with CTL or APE2 siRNA in a slide view. **(B)** Percentage of γH2AX-positive U2OS cells after treatment of CPT/ETO was quantified. **(C)** Microscopy analysis shows micronuclei (circled with red) after DAPI staining in U2OS cells after treatment of CPT (1μM for 13 hrs) or ETO (20μM for 13 hrs) with CTL or APE2 siRNA. **(D)** Percentage of micronuclei-positive PANC1 cells after treatment of CPT or ETO with CTL or APE2 siRNA was quantified. **(B)** & **(D)** * indicates $p<0.05$; ** indicates $p<0.01$; ns indicates $p>0.05$, $n=3$.

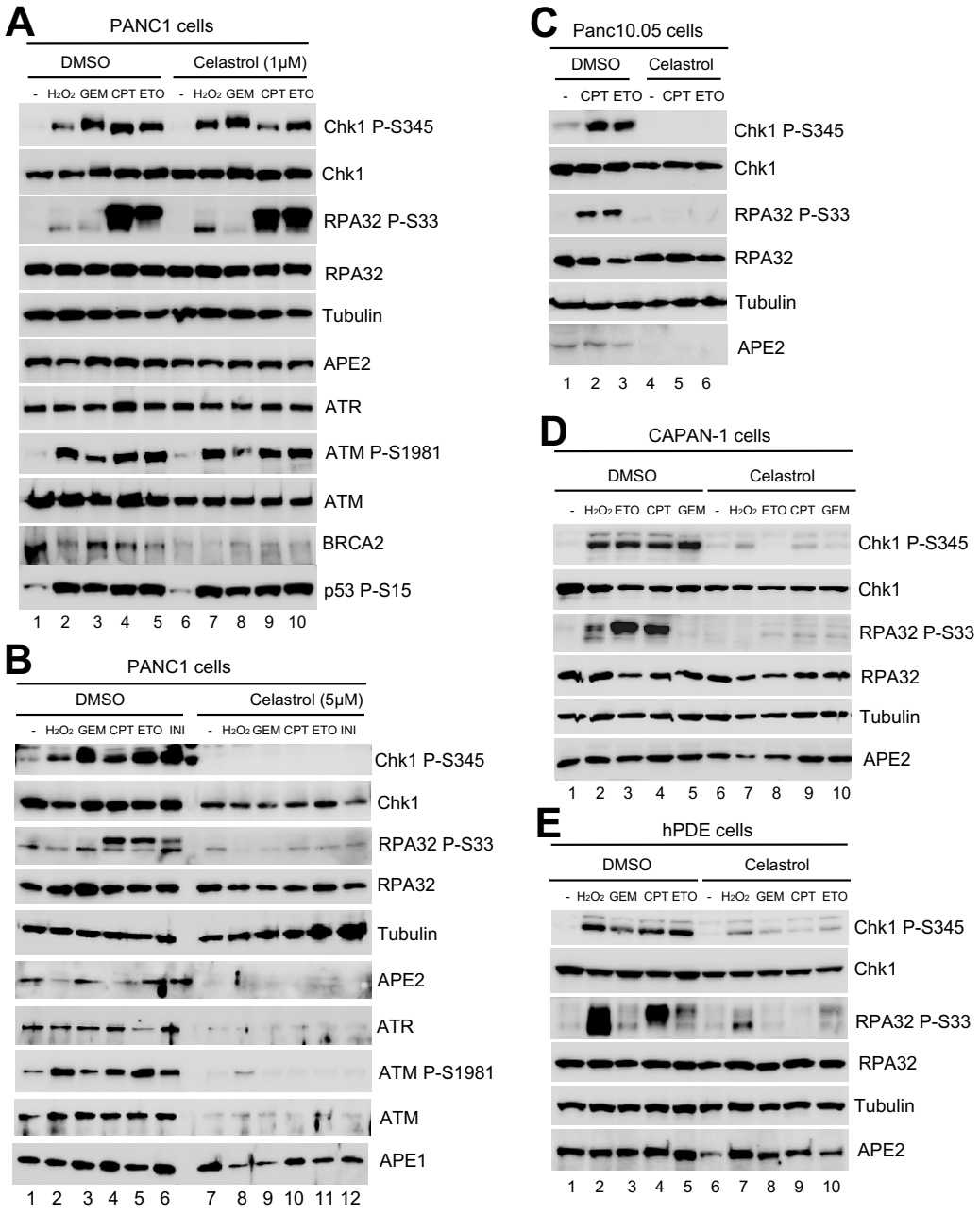

Figure 22. Celastrol compromises the ATR-Chk1 DDR pathway activation & reduce the abundance of APE2 in a dose-dependent manner in cancer cells/types having different genetic/morphological background, mutation & transformation status. (**A-B**) PANC1 cells were pretreated with Celastrol (1μM) (**A**) or Celastrol (5μM) (**B**) for 1 hr followed by 2-hour (**A**) or 4-hour (**B**) treatment of H_2O_2 (1mM), GEM (50μM), CPT (10μM), or ETO (50μM), respectively. (**C-E**) Panc10.05 (**C**) or CAPAN-1 (**D**) or hPDE (**E**) cells were pretreated with Celastrol (5μM) (**C**) or Celastrol (2.5μM) (**D-E**) for 1 hr followed by 4-hour treatment of H_2O_2 (1mM), GEM (50μM), CPT (10μM), or ETO (50μM), respectively. (**A-E**) Cell lysates were then extracted and examined via immunoblotting analysis as indicated.

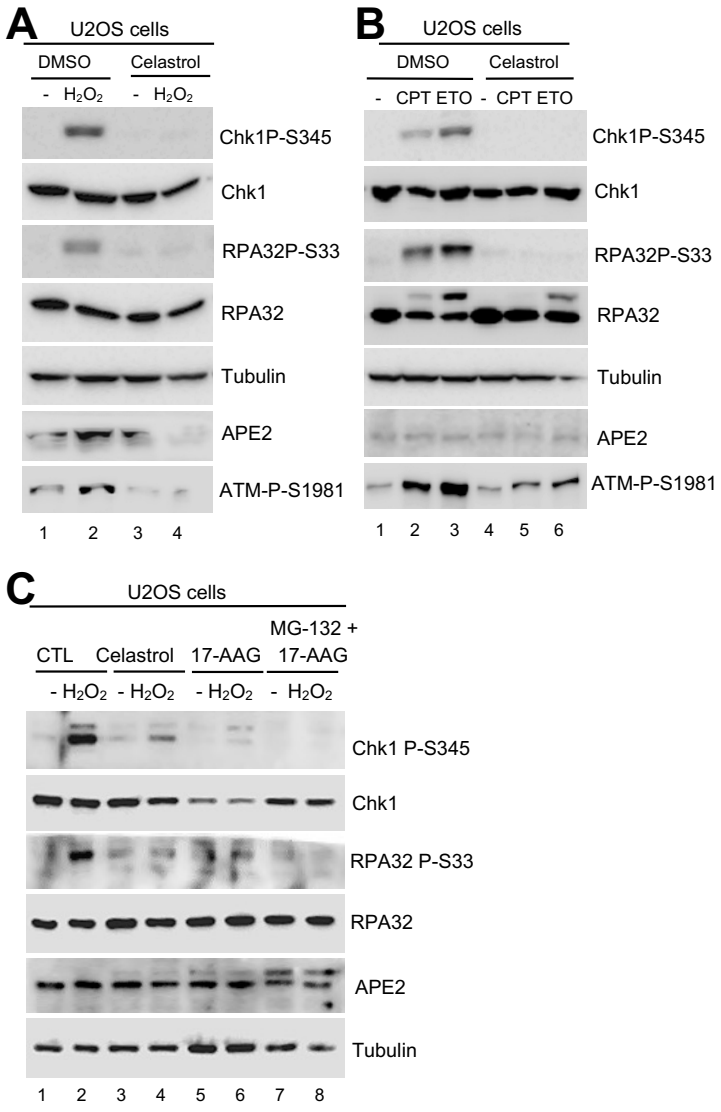

Figure 23. Celastrol compromises the ATR-Chk1 DDR pathway activation & does it differently than Hsp90 inhibitor, 17-AAG in osteosarcoma U2OS cells. (A-B) U2OS cells were pretreated with Celastrol (5μM) for 1 hr followed by 4-hour treatment of H_2O_2 (1mM) **(A),** CPT (5μM), or ETO (50μM), respectively **(B).** (**C**) U2OS cells were pretreated with/without Celastrol (2μM), 17-AAG (2μM) for 24 hrs & MG-132 (1μM) for 25 hrs followed by 4-hour treatment of CPT (1μM). (**A-C**) Cell lysates were then extracted and examined via immunoblotting analysis as indicated.

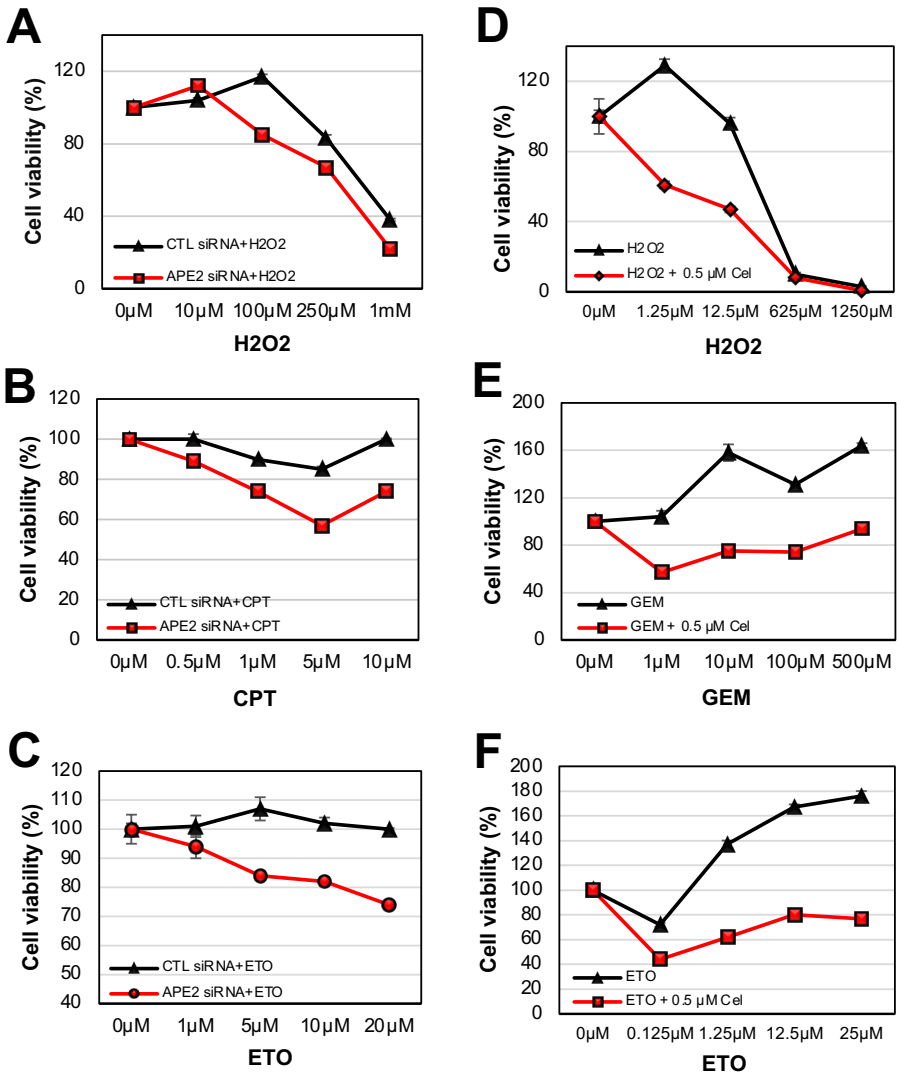

Figure 24. APE2 suppression or Celastrol sensitizes Panc10.05 cells to chemotherapy drugs. (A-C) Cell viability assays show that APE2-KD Panc10.05 cells are more vulnerable to stress conditions H_2O_2 **(A),** CPT **(B),** or ETO **(C)** than Control (CTL) siRNA transfected cells. **(D-F)** Cell viability assays demonstrate that Celastrol (0.5μM) sensitizes CAPAN-1 cells to H_2O_2 **(D),** GEM **(E),** or ETO **(F).**

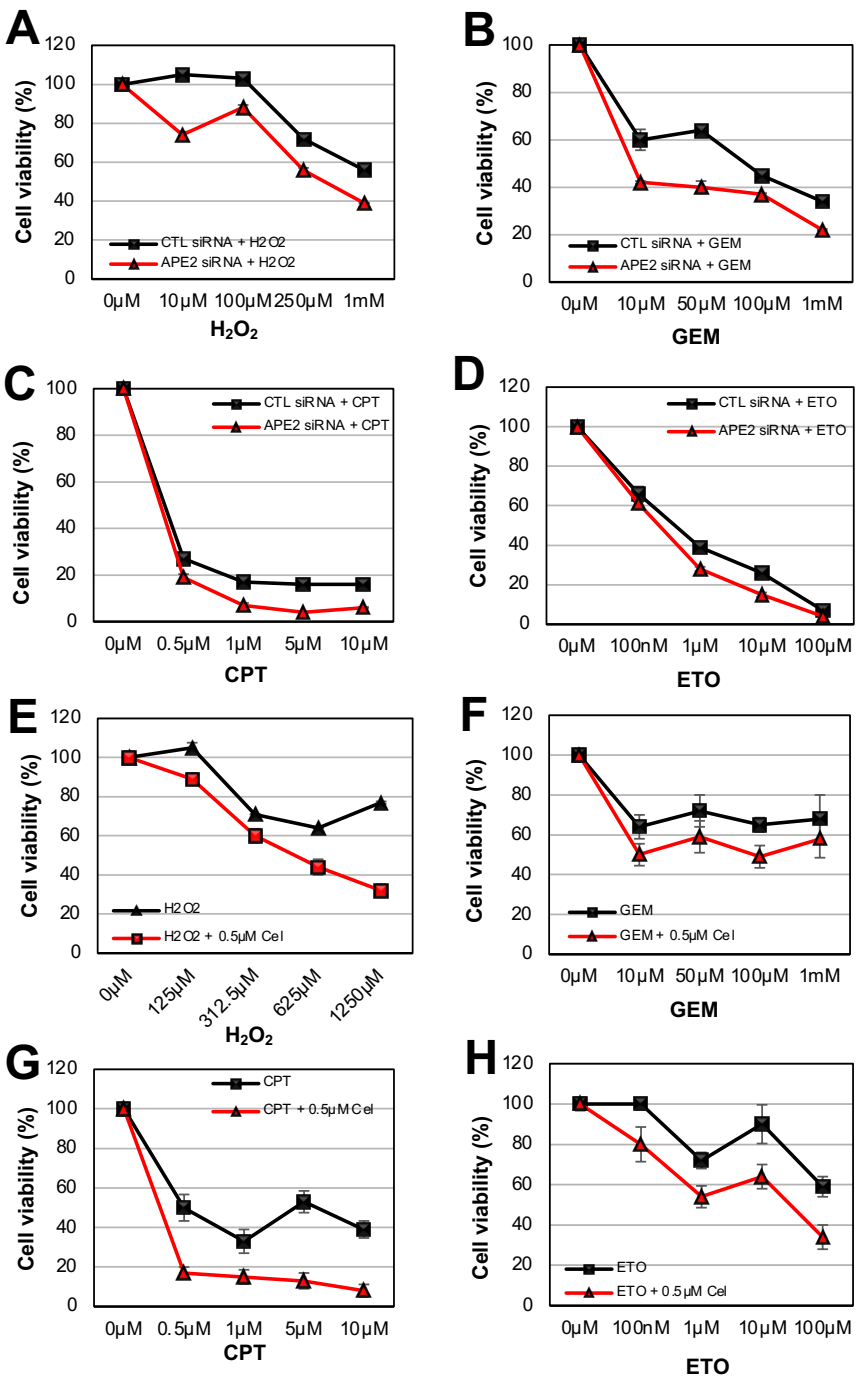

Figure 25. APE2 suppression or Celastrol sensitizes CAPAN-1 cells to chemotherapy drugs. (A-D) Cell viability assays show that APE2-KD CAPAN-1 cells are more vulnerable to stress conditions than Control (CTL) siRNA transfected cells. **(E-H)** Cell viability assays demonstrate that Celastrol (0.5μM) sensitizes CAPAN-1 cells to H_2O_2 **(E),** GEM **(F),** CPT **(G),** or ETO **(H).**

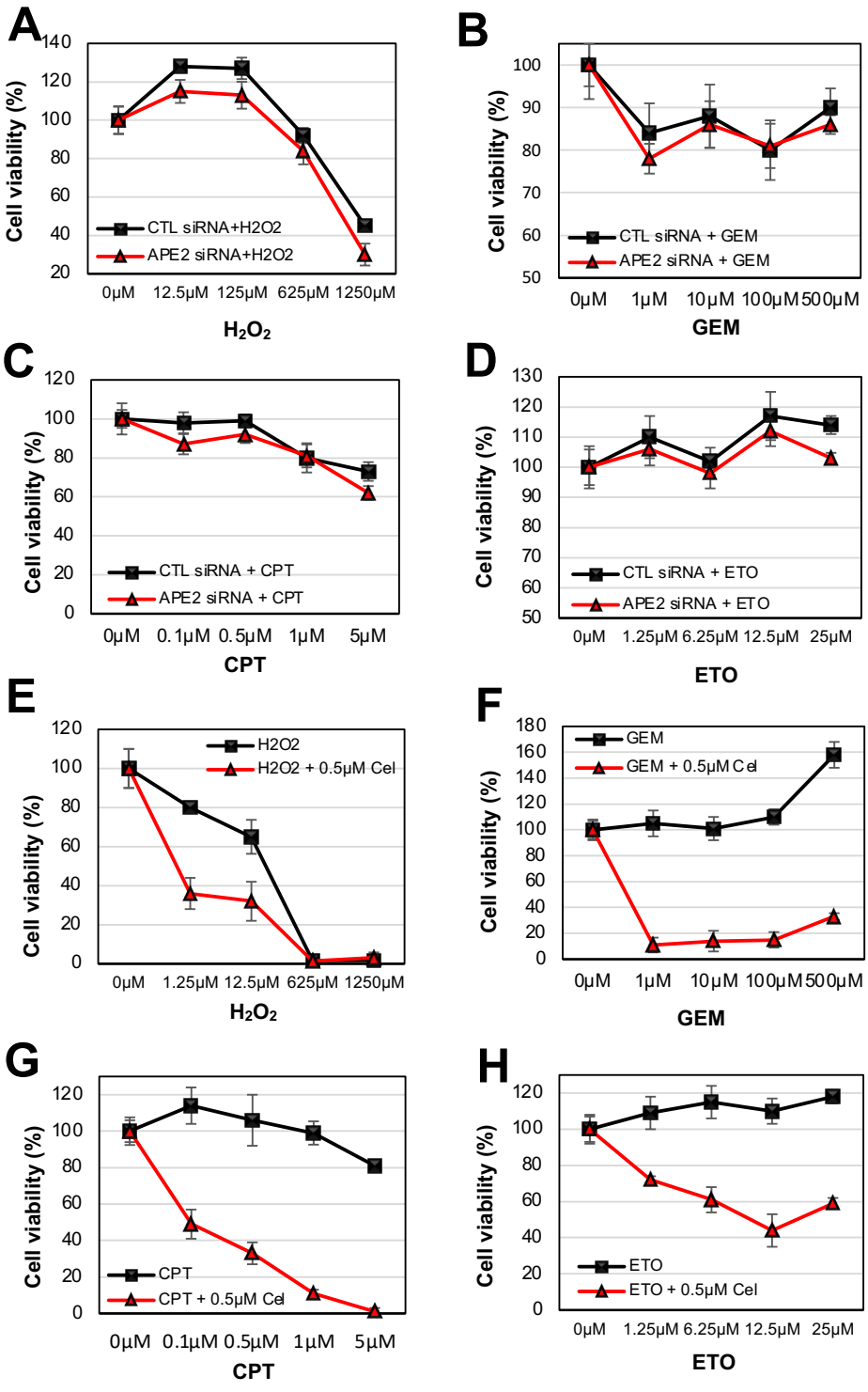

Figure 26. APE2 suppression doesn't sensitize hPDE cells in stressful conditions but Celastrol sensitizes hPDE cells to chemotherapy drugs. (A-D) Cell viability assays show that APE2-KD hPDE cells are not sensitive to stress conditions than Control (CTL) siRNA transfected cells. **(E-H)** Cell viability assays demonstrate that Celastrol (0.5μM) sensitizes hPDE cells to H_2O_2 **(E)**, GEM **(F),** CPT **(G),** or ETO **(H).**

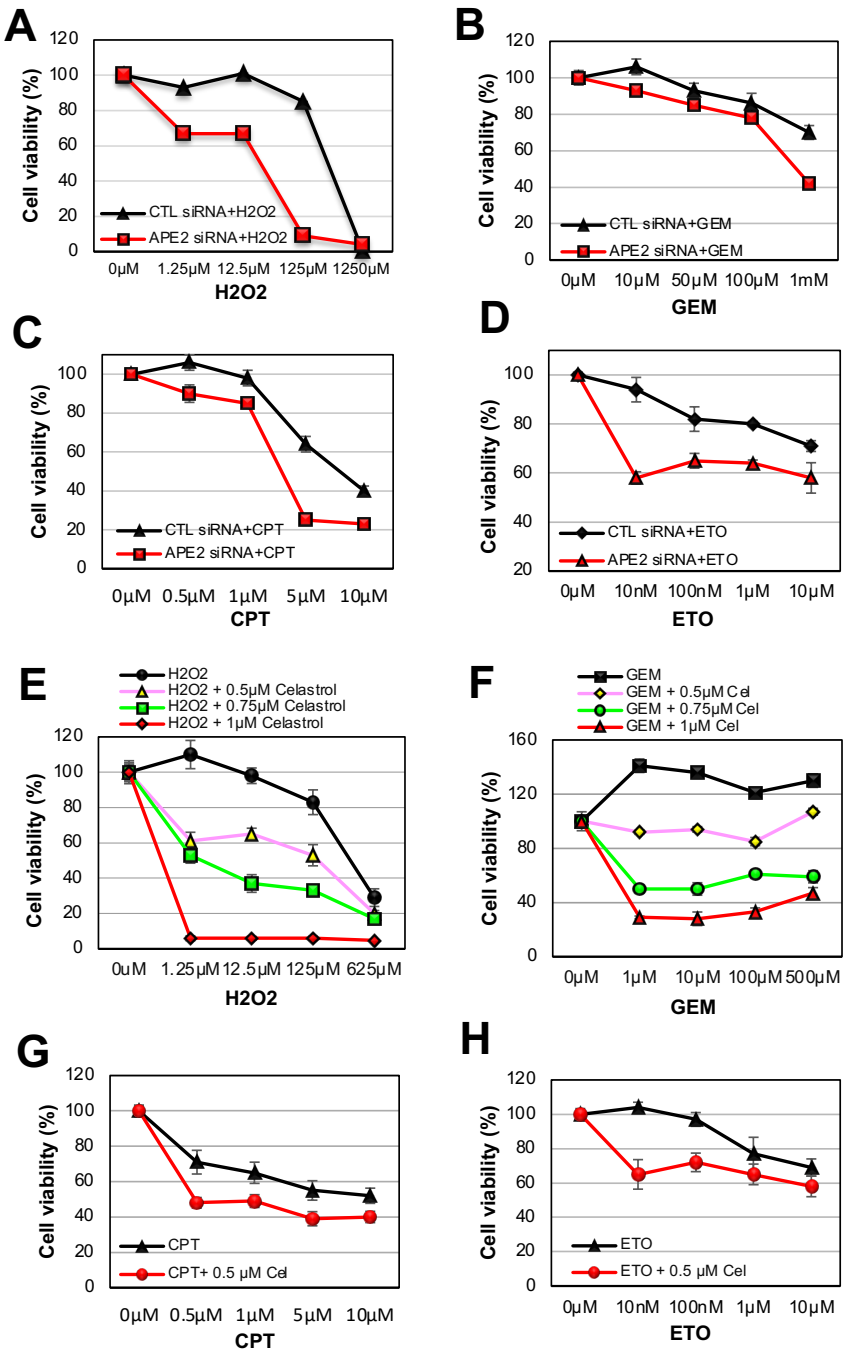

Figure 27. APE2 suppression or Celastrol sensitizes U2OS cells to chemotherapy drugs. (A-D) Cell viability assays show that APE2-KD PANC1 cells are more vulnerable to stress conditions than Control (CTL) siRNA transfected cells. **(E-H)** Cell viability assays demonstrate that Celastrol (0.5μM, 0.75μM, or 1μM) sensitizes U2OS cells to H_2O_2 **(E),** GEM **(F),** CPT **(G),** or ETO **(H).**

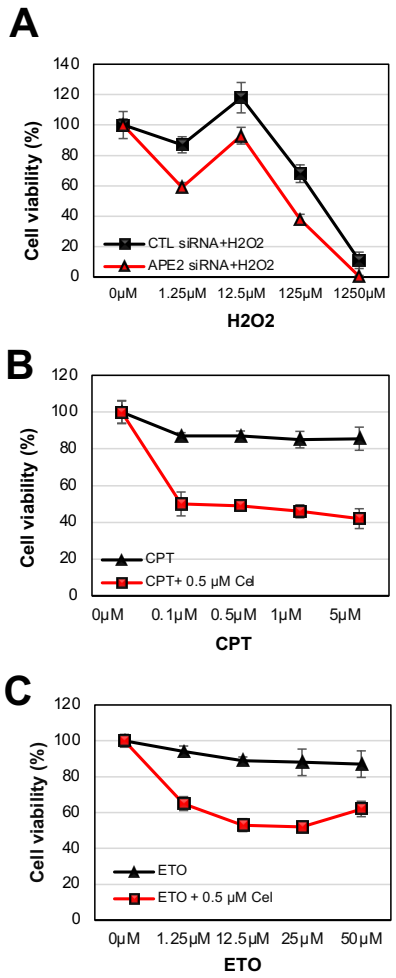

Figure 28. APE2 suppression or Celastrol sensitizes U87-MG cells to chemotherapy drugs. (A) Cell viability assays show that APE2-KD U87-MG cells are more vulnerable to oxidative stress conditions (H_2O_2) than Control (CTL) siRNA transfected cells. **(B-C)** Cell viability assays demonstrate that Celastrol (0.5μM) sensitizes U87-MG cells to CPT **(B)** or ETO **(C).**

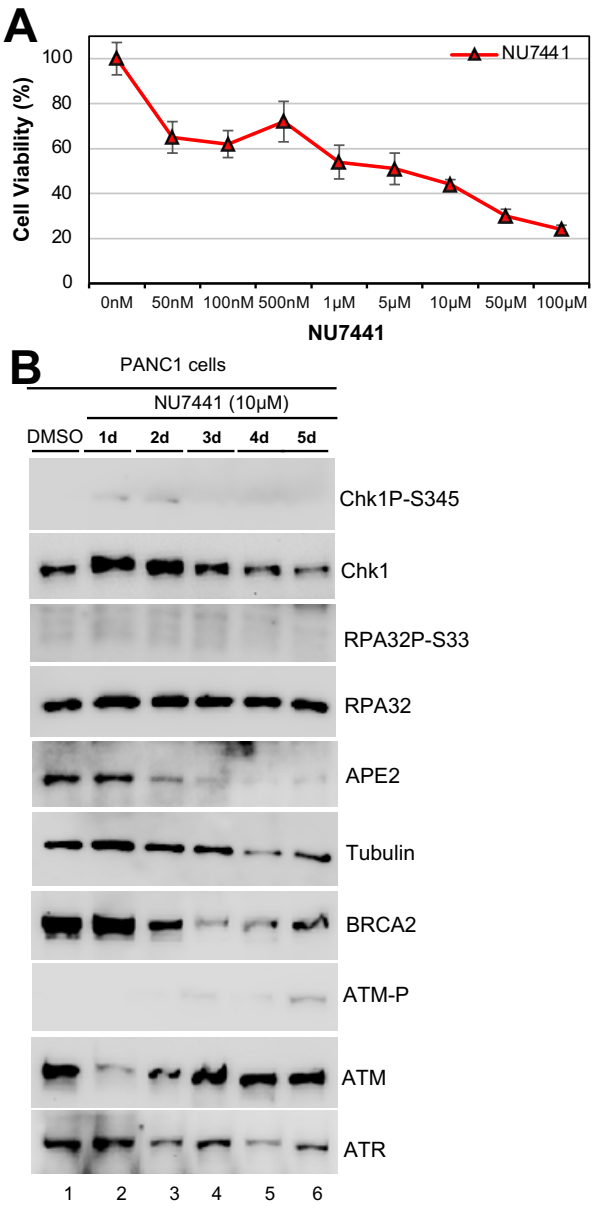

Figure 29. Determination of the optimum treatment conditions & IC50 value of NU7441, effective in reducing APE2 abundance & PANC1 cell viability. (A) Cell viability assay demonstrate that NU7441 (5-10μM) is the IC50 value for PANC1 cells. **(B)** PANC1 cells were pretreated with NU7441 (10μM) for 5 timepoints such as 1 day, 2 days, 3 days, 4 days & 5 days. Cell lysates were then extracted and examined for ATR DDR signaling via immunoblotting analysis as indicated.

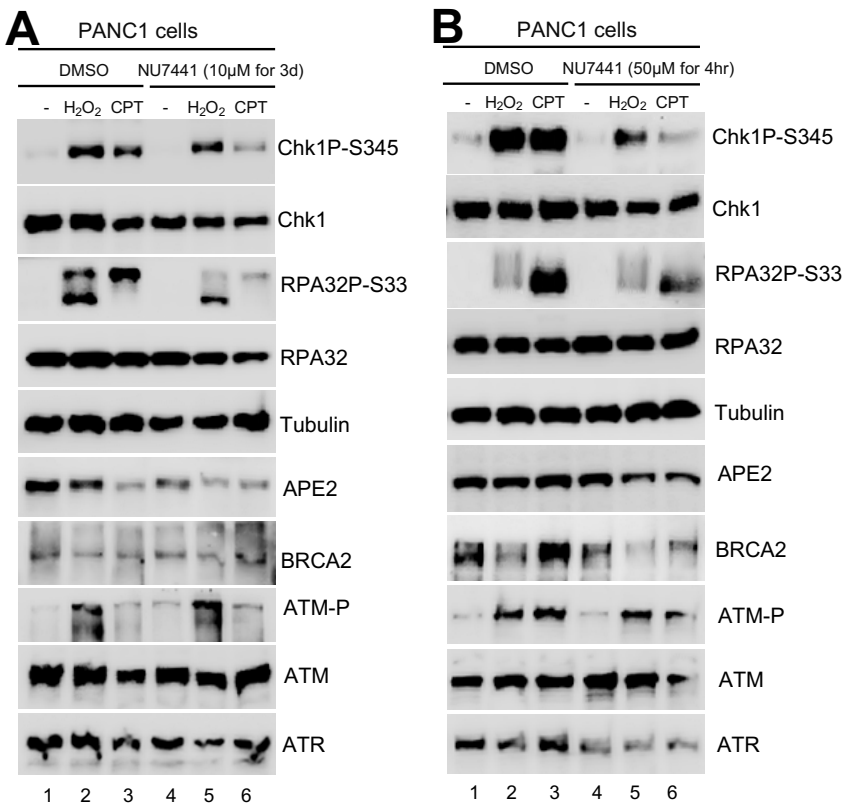

Figure 30. NU7441 compromises the ATR-Chk1 DDR pathway activation in PANC1 cells in dose-dependent & time-dependent manner. (A-B) PANC1 cells were pretreated with NU7441 (10μM) for 3 days **(A)** or NU7441 (50μM) for 4 hours **(B)** followed by 4-hour treatment of H_2O_2 (1mM) & CPT (10μM) respectively. Cell lysates were then extracted and examined via immunoblotting analysis as indicated.

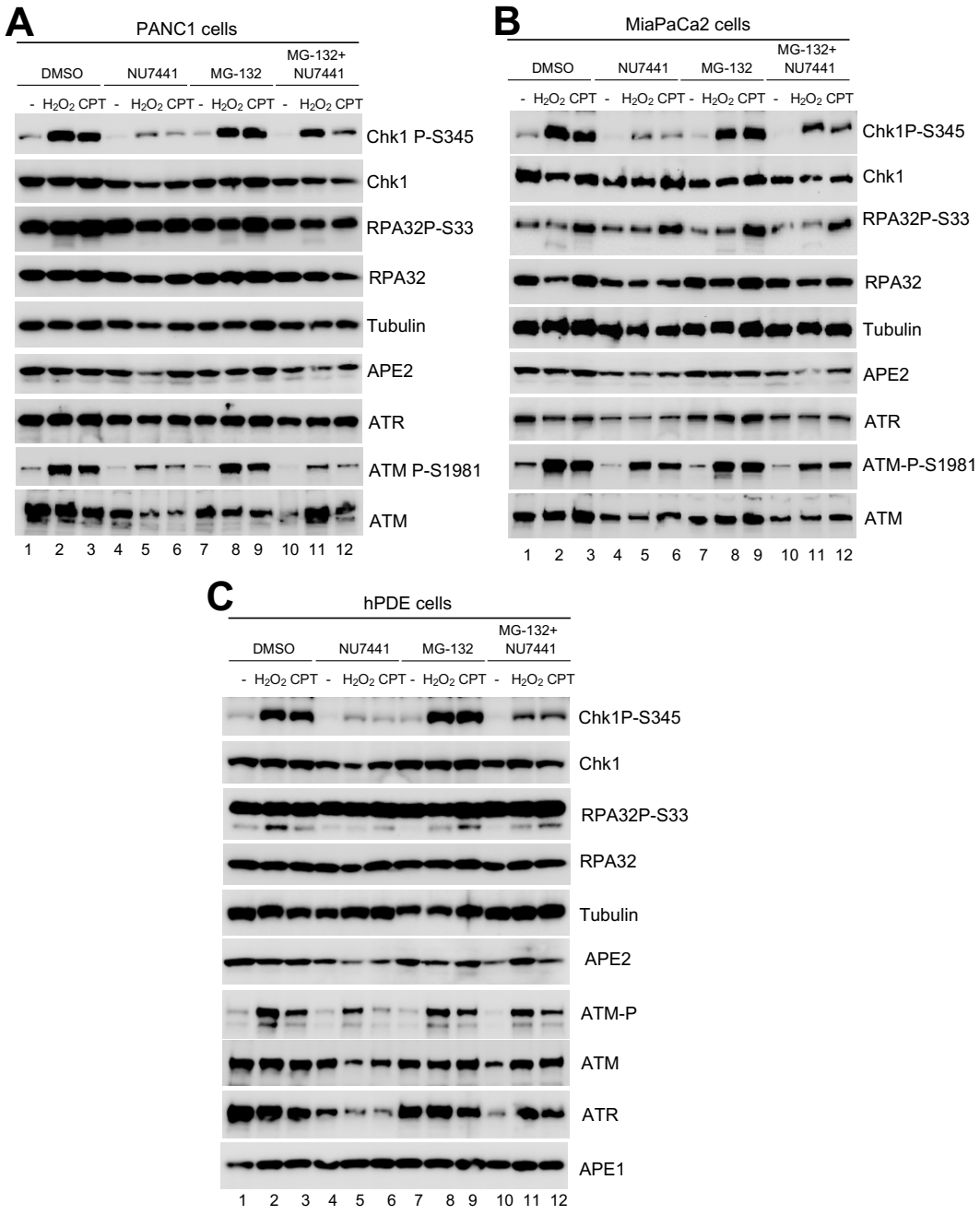

Figure 31. Deficiency of the ATR-Chk1 DDR in NU7441 treated pancreatic cancer and normal cells can be rescued by MG-132. (A-C) PANC1 (**A**), MiaPaCa2 (**B**) & hPDE (**C**) cells were pretreated with MG-132 (100nM) & NU7441 (20μM) for 3 days followed by 4-hour treatment of H_2O_2 (1mM) & CPT (10μM) respectively. Cell lysates were then extracted and examined for ATR DDR signaling via immunoblotting analysis as indicated.

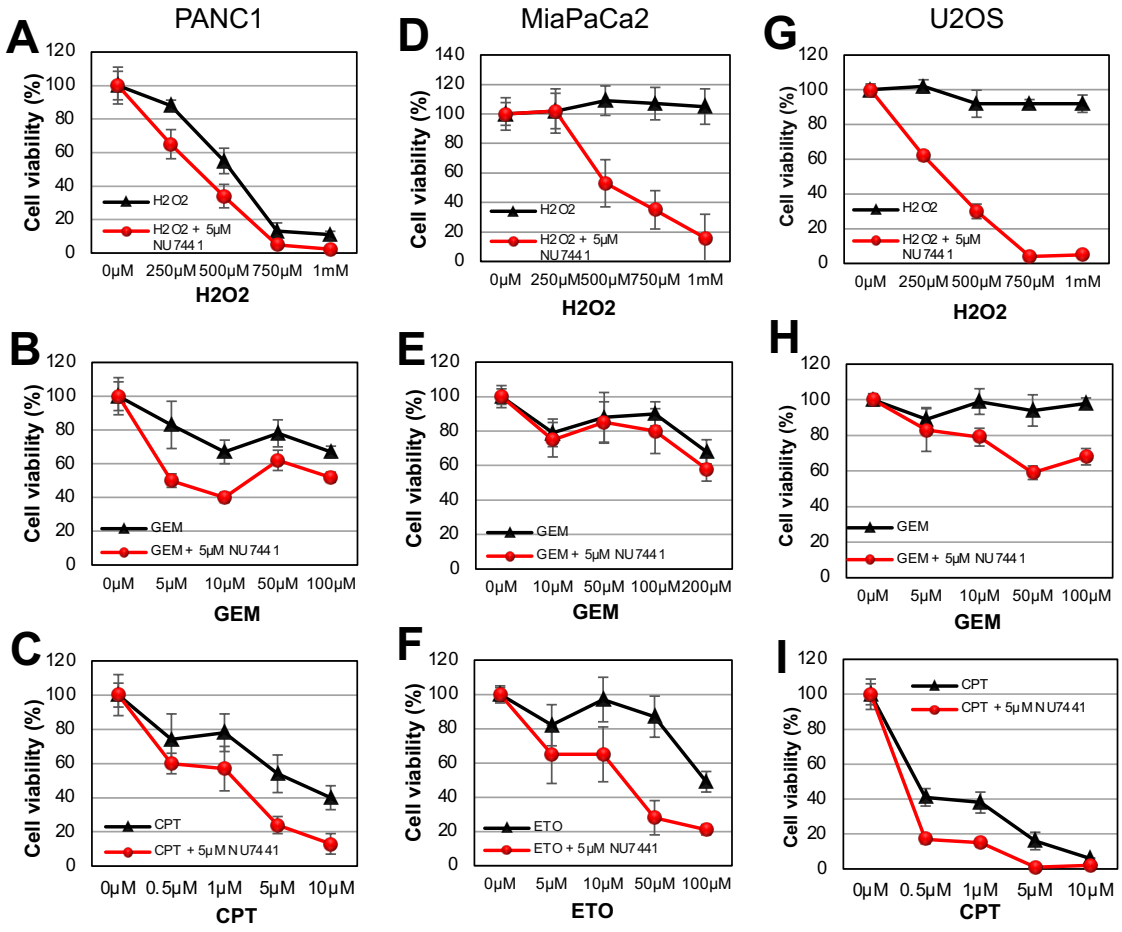

Figure 32. NU7441 sensitizes cancer cells to chemotherapy drugs. (A-I) Cell viability assays demonstrate that NU7441 (5μM) sensitizes PANC1 (**A-C**), MiaPaCa2 (**D-F**) & U2OS (**G-I**) cells to H_2O_2 **(A, D, G),** GEM **(B, E, H),** CPT (**C, I**) or ETO **(F).**

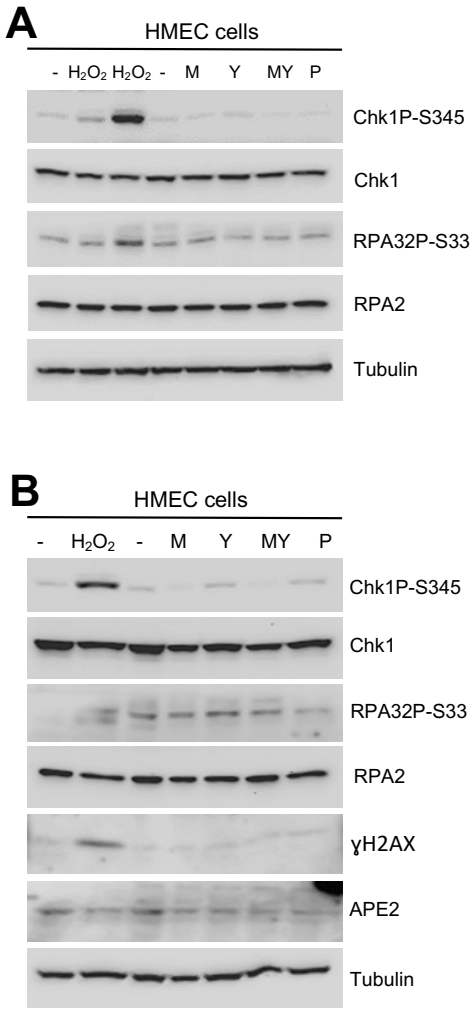

Figure 33. Microparticles treatment in HMEC cells doesn't have any effect in ATR DDR signaling pathway. **(A-B)** HMEC cells were treated with Microparticles for 6 hrs **(A)** or 24 hrs **(B)** and the performance was evaluated in H_2O_2; Microparticles, M; Negative control Rho-kinase pathway inhibitor, P; Rho-kinase pathway inhibitor Y or combination of M+Y treatments. Cell lysates were then extracted and ATR DDR signaling was examined via immunoblotting analysis as indicated.

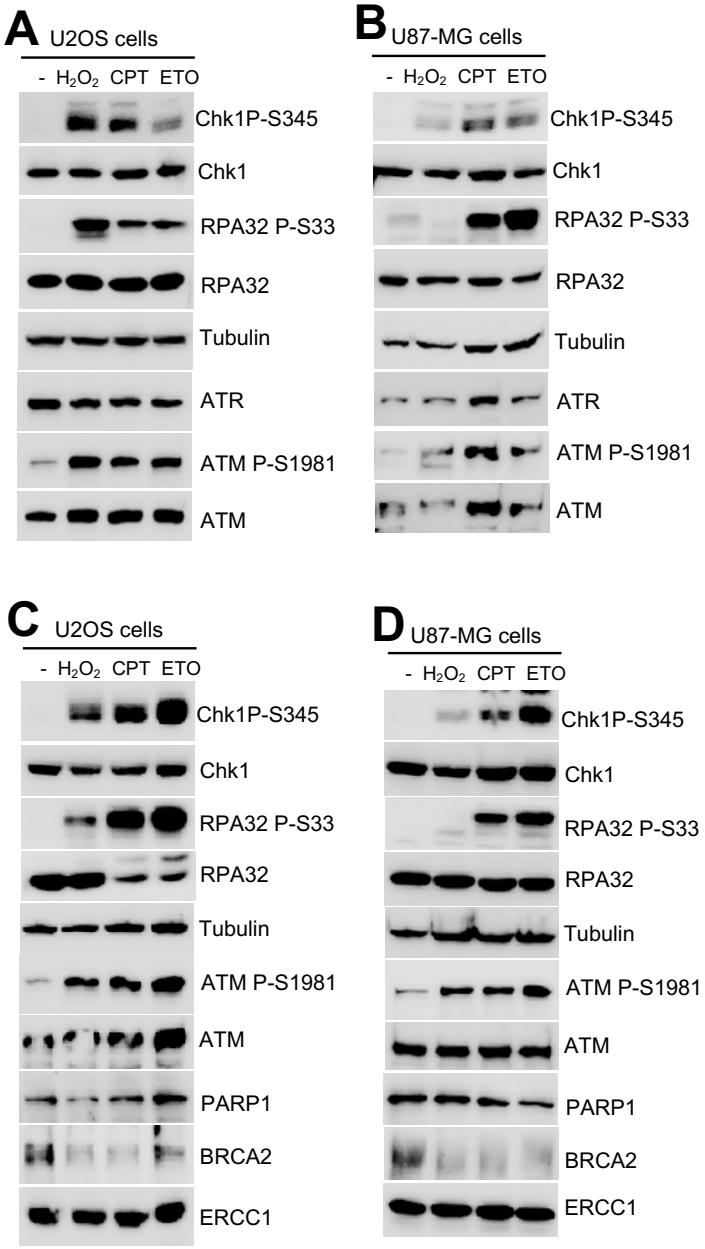

Figure 34. ATR-Chk1 DDR activation is compromised in U87-MG compared to U2OS in oxidative stress conditions (H_2O_2). (A-D) U2OS **(A, C)** & U87-MG **(B, D)** cells were incubated with 3-hour treatment of H_2O_2 (1mM), CPT (10μM), or ETO (100μM), respectively (**A-B**) and with 4-hour treatment of H_2O_2 (1.2 mM), CPT (5μM), or ETO (50μM), respectively (**C-D**). Cell lysates were then extracted and ATR-DDR signaling was examined via immunoblotting analysis as indicated.

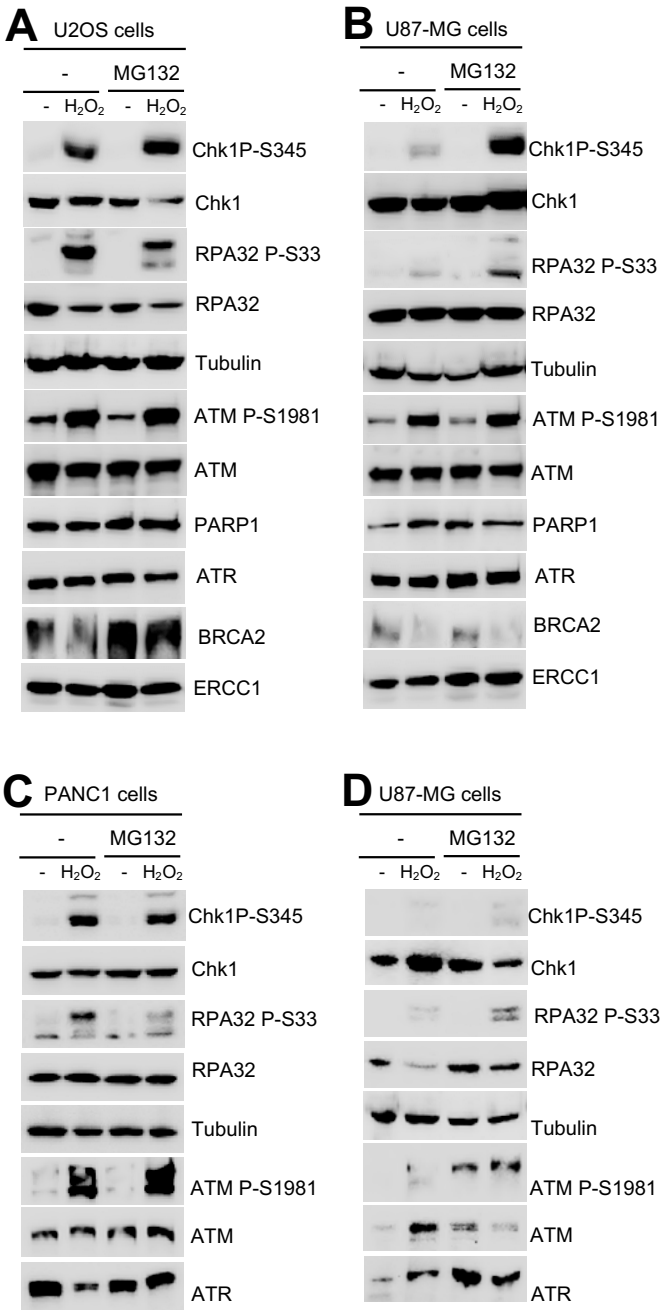

Figure 35. Deficiency of ATR-Chk1 DDR in U87-MG cells can be rescued by MG-132 in oxidative stress conditions. (A-D) U2OS **(A),** PANC1 (**C**) & U87-MG **(B, D)** cells were pretreated with MG-132 (1μM) for 5 hrs (**A, B**) or for 4 hrs (**C, D**) followed by 4-hour treatment of H_2O_2 (1.2 mM) (**A, B**) or 3-hour treatment of H_2O_2 (1 mM) (**C, D**). Cell lysates were then extracted and examined for ATR DDR signaling via immunoblotting analysis as indicated.

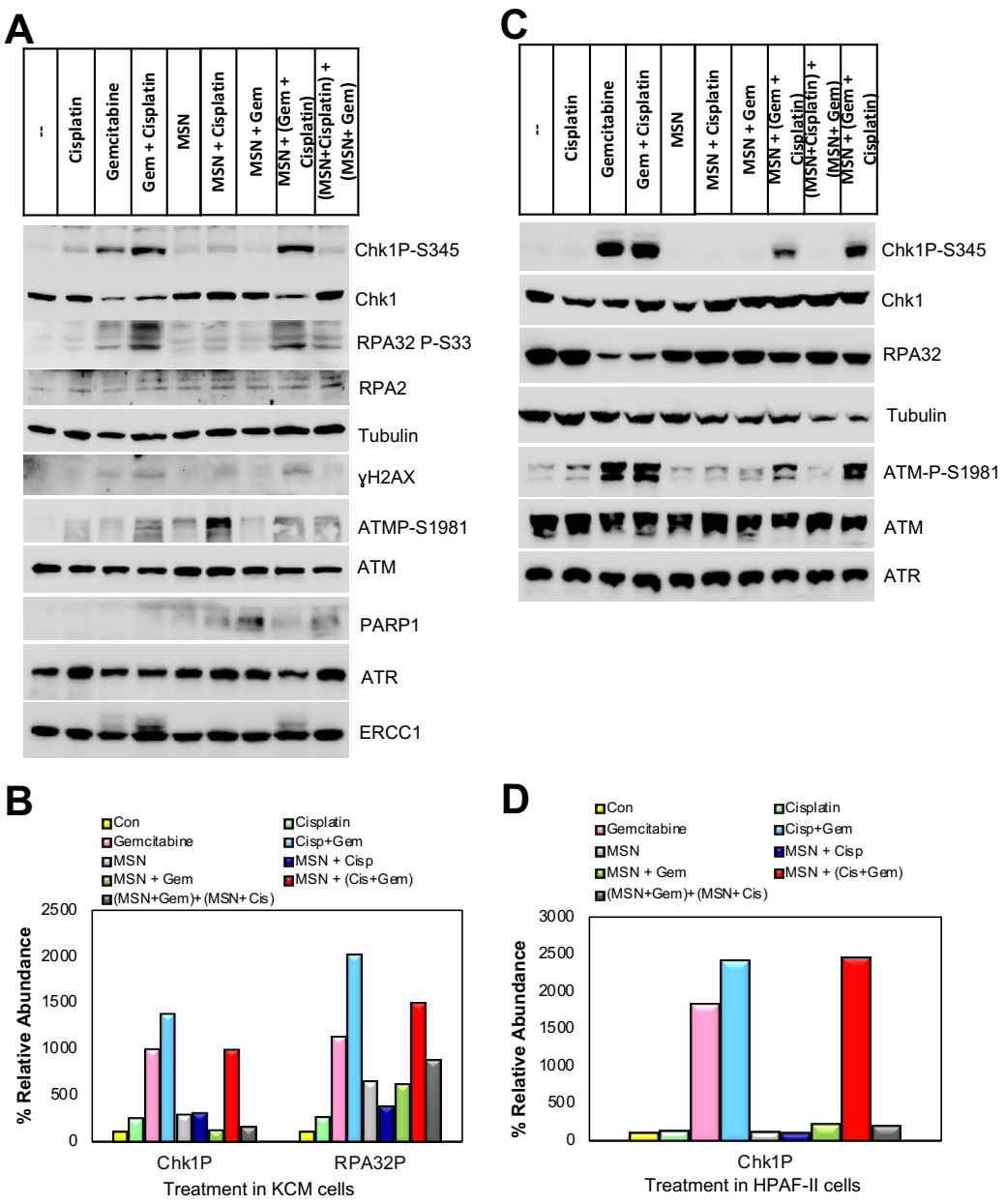

Figure 36. Gem-cisPt-MSNs activates the ATR-Chk1 DDR pathway activation compared to the physical mixture (Gem-MSNs plus cisPt-MSNs) in KCM & HPAF-II cells. (A, C) KCM (**A**) & HPAF-II (**C**) cells were treated with MSNs (20μg/μl) (**A**) or MSNs (10μg/μl) (**C**) for 24 hrs and the performance was evaluated in free MSNs, free drug, monotherapies of MSNs (cisPt-MSNs or Gem-MSNs) or in the physical mixture treatment conditions. Cell lysates were then extracted and ATR DDR signaling was examined via immunoblotting analysis as indicated. **(B, D)** Quantification of the western blot presented in **(A & C).** Chk1P-S33 (**B, D**) & RPA32P-S33 (**B**) signal was measured using BIORAD ImageLab Software, normalized to loading control (Tubulin).

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APPENDIX

Awards, Publications, and Presentations

Awards:

1) Spring 2021: Research Assistant (RA), $11,000 for stipend, $1,308.40 for medical insurance, $1,084.25 for tuition, and $870.15 for academic fees. The RA position is supported by the NIH/NCI grant.

2) Fall 2020: Research Assistant (RA), $11,000 for stipend, $1,308.40 for medical insurance, $1,084.25 for tuition, and $870.15 for academic fees. The RA position is supported by the NIH/NCI grant.

3) Summer 2020: Graduate School Summer Fellowship (GSSF), $6,000 for stipend, Graduate School at UNC Charlotte

4) Summer 2019: Graduate School Summer Fellowship (GSSF), $6,000 for stipend, Graduate School at UNC Charlotte

5) Fall 2016-Fall 2020: Graduate Assistant Support Plan (GASP) awards covering tuition, Graduate School at UNC Charlotte

Publications:

1) **Hossain MA**, Lin Y, Driscoll G, Tsuchimoto D, Nakabeppu Y, Yan S. **2021**. APE2 is a critical regulator of the DNA damage response to maintain genome integrity in pancreatic cancer cells. [In peer-review at *Oncogene* since March 8, 2021]

2) Tarannum M, **Hossain MA**, Holmes B, Yan S, Mukherjee P, and Vivero-Escoto J. **2021**. Advanced nanoengineered approach for target-specific, spatiotemporal, and ratiometric delivery of Gemcitabine-cisplatin combination for improved therapeutic outcome in pancreatic cancer. [Under review at *ACS Nano* since March 26, 2021]

3) Lin Y, Raj J, Li J, Ha A, **Hossain MA**, Richardson C, Mukherjee P, Yan S. **2020**. APE1 senses DNA single-strand breaks for repair and signaling. *Nucleic Acids Research*. 48(4):1925-1940 (PMCID: PMC7038996; PMID: 31828326) DOI: https://doi.org/10.1093/nar/gkz1175 .

   Commented and recommended by **Maiorano D**: F1000Prime Recommendation of [Lin Y et al., *Nucleic Acids Res* 2020 48(4):1925-1940]. In F1000Prime, 03 Jan 2020; DOI: 10.3410/f.737076253.793568982

4) **Hossain MA**, Lin Y, Yan S. **2018**. SSB end resection in genome stability: mechanism and regulation by APE2. *International Journal of Molecular Sciences*. 19 (8): 2389. (PMCID: PMC6122073; PMID:30110897) DOI:

https://doi.org/10.3390/ijms19082389

5) Lin Y, Bai L, Cupello S, **Hossain MA**, Deem B, McLeod M, Raj J, Yan S. **2018**. APE2 promotes DNA damage response pathway from a single-strand break. *Nucleic Acids Research*. 46 (5): 2479-2494. (PMCID: PMC5861430; PMID: 29361157) DOI: https://doi.org/10.1093/nar/gky020

Presentations:

1) **January 2019:** Departmental Seminar titled "Mechanisms of APE2 in DNA damage response in mammalian cells", Department of Biological Sciences, UNC Charlotte.

2) **May 2017:** Poster presentation titled "Distinct role of APE2 in genome integrity in pancreatic cancer cells" at 2017 Poster Competition for Graduate Students at the Center for Biomedical Engineering and Science (CBES), UNC Charlotte.

3) **April 2017:** Poster presentation entitled "Distinct roles of APE2 in genome integrity in pancreatic cancer cells" in the 4th Annual Biological Sciences Symposium, UNC Charlotte.