THE ROLE OF XRCC1 AND POLYMERASE BETA IN GENOME INTEGRITY IN *XENOPUS* EGG EXTRACTS

by

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ABSTRACT

STEVEN CUPELLO. The role of XRCC1 and Polymerase beta in genome integrity in *Xenopus* egg extracts. (Under the direction of DR. SHAN YAN)

Cells of organisms are constantly exposed to genomic insults such as oxidative stress from endogenous sources or environmental agents. Oxidative stress induces oxidative DNA damage such as apurinic/apyrimidinic (AP) sites, single-strand breaks (SSBs), and double-strand breaks (DSBs). Oxidative DNA damage is repaired primarily by base excision repair (BER) pathway as well as other DNA repair pathways. It is widely accepted that unrepaired oxidative DNA damage compromises DNA replication and transcription, leading to cancer and neurodegenerative disorders. As a cell-free biochemical model, *Xenopus laevis* egg extract has been utilized to investigate critical questions in the fields of DNA repair and DNA damage response pathways (Chapter 1). In my PhD thesis, I sought to elucidate how XRCC1 (Chapter 2) and Polymerase beta (Pol beta, Chapter 3), two protein crucial to the BER pathway, are involved in genomic integrity in *Xenopus* egg extracts. Here I report that when XRCC1 is depleted ATR-Chk1 signaling increases in *Xenopus* egg extracts following oxidative stress. When looking at the total amount of damage in the nucleus depletion of XRCC1 causes a statistically significant increase in the amount of DNA damage. XRCC1 is not, however, important for the repair of defined SSB plasmids in these extracts implying that oxidative DNA damage may be repaired in a BER independent manner. Despite this I show that XRCC1 can interact with APE2 *in-vitro* and plays a very minimal role for APE2's exonuclease activity. In addition, ATR-Chk1 signaling following oxidative stress was decreased when Pol beta was removed. However, we can't distinguish whether this Pol beta depletion phenotype is due to the absence of Pol beta, or co-depletion of integral proteins in the ATR-Chk1 pathway that interacts with Pol beta. When Pol beta is depleted, there is no significant increase in DNA damage following oxidative stress. Like

XRCC1, Pol beta is dispensable for the repair of a defined SSB plasmid in *Xenopus* egg extracts. Instead, Pol alpha may be involved in the defined SSB plasmid repair. Taken together, these findings suggest that XRCC1 and Pol beta play distinct roles in the maintenance of genomic integrity in *Xenopus* egg extracts.

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LIST OF ABBREVIATIONS

8oxoG: 8-oxo-7,8-dihydroguanine

9-1-1 Complex: Rad9, Hus1, and Rad1. DNA damage checkpoint sliding clamp

ANOVA: Analysis of Variance

AP site: Apurinic/Apyrimidinic site

APE1: Apurinic/Apyrimidinic Endonuclease

APE2: Apurinic/Apyrimidinic Endonuclease 2

APLF: Aprataxin and PNPK-like factor

APTX: Aprataxin

Apx: Aphidicolin

Arg: Arginine

AT70: DSB-mimic structure

ATM: Ataxia telangiectasia mutated

ATR: Ataxia telangiectasia and Rad3- related protein

ATRIP: Ataxia telangiectasia and Rad3- related- interacting protein

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

Chk1: Checkpoint Kinase 1

Chk2: Checkpoint Kinase 2

CIP: Calf Intestine Phosphotase

CtIP: C-terminal binding protein- interacting protein

Dbf4: DBF4 zinc finger

DDK: Dbf4-dependent kinase

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

DTT: Dithiothreitol

EcoRI: Restriction endonuclease that cleaves double stranded DNA at the sequence GTTAAC

ELB: Egg Lysis Buffer

FANC: Fanconi Anemia Complex

FEN1: Flap Endonuclease 1

FHA: Forkhead-Associated

FITC: Fluorescein Isothiocyanate

Gln: Glutamine

GST: Glutathione S-transferase

HaeIII: Restriction endonuclease that recognizes the sequence GGCC

HeLa cells: Immortal cell line used in scientific research. The oldest and most commonly used human cell line.

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

His: Histidine

His3: Histone 3

HSS: High Speed Supernatant

Hus1: HUS1 checkpoint clamp component

IACUC: Institutional Animal Care and Use Committee

IB: Interaction Buffer

ICL: Interstrand Cross Link

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

M13: Virus that infects the bacterium Escherichia coli

MCM: Minichromosome Maintenance

MMC: Mitomycin C

MMEJ: Microhomology-Mediated End Joining

MMR: Mismatch Repair

MMS: Methyl methanesulfonate

Mre11: MRE11 homolog, double strand break repair nuclease

MRN complex: Protein complex consisting of Mre11, Rad50 and Nbs1

Myc: Myc Proto-Oncogene Protein

Nbs1: Nijmegen breakage syndrome 1 (nibrin)

NEIL1: Nei Like DNA Glycosylase 1

NEIL2: Nei Like DNA Glycosylase 2

NER: Nucleotide Excision Repair

NHEJ: Non-Homologous End Joining

NIR: Nucleotide Incision Repair

NLS: Nuclear Localization Signal

NPE: Nucleoplasmic Extracts

Nt.BstNB1: Restriction enzyme that catalyzes a single strand nick 3' 4 bases beyond its GAGTC recognition sequence

NTD: N-Terminal Domain

NTH1: Nth Like DNA Glycosylase 1

OGG1: 8-Oxoguanine DNA Glycosylase

p53: Tumor protein p53

PAR: Poly(ADP-ribose)

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

Pf1MI: Restriction endonuclease that recognizes the sequence CCAN∨NNN^NTGG

pGEX-4T1: Protein production plasmid with Ampicillin resistance

PIP: PCNA interacting peptide

PNK: Polynucleotide kinase

PNKP: Polynucleotide Kinase 3'-Phosphatase

PNPK- Polynucleotide Kinase Phosphotase

Pol α: DNA Polymerase Alpha

Pol β: DNA Polymerase Beta

Pol δ: DNA Polymerase Delta

Pol ε: DNA Polymerase Epsilon

Pol ζ: DNA Polymerase Zeta

Pol η: DNA Polymerase Eta

Pol ι: DNA Polymerase Iota

Pol κ: DNA Polymerase Kappa

pS: Mutant pUC19 that has one Nt.BstNB1 recognition site

pUC19: Plasmid that has 4 Nt.BstNB1 recognition sites

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

REV1: REV1 DNA directed polymerase

RIR: Rev1- interacting region

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

RPA: Replication Protein A

SbfI-HF: Restriction enzyme that cuts CC∨TGCA^GG

S-CDK: S-phase cyclin-dependent kinase

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylimide Gel Electrophoresis

Ser: Serine

Sp1: Sp1 Transcription Factor

Spo-11: SPO11 Initiator of Meiotic Double Stranded Breaks

SSB: Single Strand Break

SSBR: Single Strand Break Repair

ssDNA: Single Stranded DNA

SUMO: Small Ubiquitin-like Modifier

SV40: Simian Virus 40

SYBR Gold: Nucleic Acid fluorescent stain

TBE: Tris/Borate/EDTA

TBST: Tris Buffer Saline with Tween 20

TE: Tris EDTA buffer

Thr: Threonine

TLS: Translesion Synthesis

TMZ: Temozolomide

TNT SP6: High-Yield Wheat Germ Protein Expression System

TopBP1: DNA topoisomerase 2-binding protein 1

Trp: Tryptophan

UNG2: Uracil DNA Glycosylase 2

UV: Ultraviolet

VE822: Berzosertib; ATR inhibitor

XB: Xenopus Buffer

XL1: XRCC1 Linker 1 region

XL2: XRCC1 Linker Region 2

XRCC1: X-Ray Cross Complimenting Protein 1

Zf-GRF: Zinc finger (Glycine, Arginine, Phenylalanine)

γ-H2AX: Phosphorylated H2A Histone Family Member X

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CHAPTER 1: INTRODUCTION

The genomes of all cells are exposed to a variety of insults from endogenous and exogenous sources (1, 2), leading to DNA replication stress, double-strand breaks (DSBs), inter-strand crosslinks (ICLs), and oxidative stress (**Figure 1**). To sense and signal DNA damage and replication stress, the DDR pathways including ATR-Chk1 and ATM-Chk2-mediated signaling cascades are triggered to coordinate DNA repair with cell cycle progression. Defective DDR pathways have been implicated with cancer development and neurodegenerative disorders (3). In addition, cells develop a tolerance to DNA damaging agents, possibly through the translesion DNA synthesis (TLS) pathway that includes Y-family DNA polymerases (REV1, Pol η, Pol κ and Pol ι) and a B-family DNA polymerase Pol ζ (4). When DNA lesions cannot be replicated by replicative DNA polymerases (Pol δ/ε), they can be bypassed by TLS polymerases, leading to mutagenesis as a tradeoff of survival (5). Although we have acquired a better understanding of DDR pathways in the last 20 years or so (2), the study of DDR pathways remains an intense topic of investigation, and it is a critical outstanding question of how TLS polymerases and DDR pathways regulate each other in cellular responses to DNA damage or replication stress.

A soluble cell-free extract system from stage 6 *Xenopus laevis* oocytes was first used to investigate the DNA replication of simian virus 40 (SV40) in 1976 (6). Assembly of SV40 chromatin was reported in a cell-free *Xenopus* egg extract system (7). Since then, *Xenopus* egg extracts have been utilized for studies in DNA metabolism and cellular signaling pathways including DNA replication, DNA repair, and DNA damage response

(DDR) (**Figure 2**) (8-12). This work attempts to better understand how/if the Base Excision Repair (BER) interplays with the DDR pathway to promote genome integrity.

1.1 The DNA Damage Response

The DDR pathways include the ATR-Chk1 and ATM-Chk2 checkpoint signaling cascades, coordinating DNA repair with cell cycle progression and apoptosis/senescence (2, 13-15). Defects in DDR pathways lead to genomic instability, which is a hallmark of cancer (16). A better understanding of the DDR pathways has increased our understanding of cancer development and led to new approaches for cancer therapy.

ATR can be activated by primed single-stranded DNA (ssDNA) from the functional uncoupling of MCM (minichromosome maintenance) helicase and DNA polymerase activities in response to stalled DNA replication forks (2, 13, 17). The 5'-3' end resection of DSBs mediated by CtIP nuclease also activates the ATR-Chk1 pathway (18, 19). ATR is recruited to RPA-coated ssDNA via direct interaction between RPA and ATRIP (20). ATR activation requires several mediator proteins including its interacting protein ATRIP, TopBP1 and the 9-1-1 (Rad9-Rad1-Hus1) complex (12, 20-22). Activated ATR phosphorylates multiple substrates including Chk1 (23). Chk1 is activated upon phosphorylation, serving as an indicator of ATR activation (24). The ATR-Chk1 pathway can also be activated in response to oxidative stress, inter-strand crosslinks (ICLs), and ultraviolet light (UV) (25-27). In addition, ATR is reported to be autophosphorylated after DNA damage at its Thr 1989 residue (28).

In response to DSBs, ATM can be activated by autophosphorylation and dimer dissociation (29, 30). This ATM kinase activation requires the Mre11-Rad50-Nbs1

(MRN) complex as well as other factors (31). Once activated, ATM kinase phosphorylates a number of substrates including Chk2 and p53 (32, 33). Defective ATM kinase is associated with neurodegenerative disease ataxia-telangiectasia (34). Interestingly, the MRN complex is phosphorylated by activated ATM, suggesting the MRN complex serves as both sensor and adaptor for the ATM DDR signaling pathway (31, 35). Accumulating evidence suggests that ATM is activated by conformational change during oxidative stress, which is independent of the MRN complex and DNA (36-38).

Dysfunctions in DDR signaling pathways are implicated in cancer development and characterized in primary patient tumors (39, 40). Importantly, multiple DDR proteins are potent therapeutic targets for anti-cancer therapy in preclinical and clinical studies (41-43). For example, ATR, Chk1, ATM, and Chk2 are targets for anti-cancer therapy via inhibiting their kinase activities (44-47). Studies of pharmacological inhibitors targeting DDR pathways provide evidence of improved efficacy in chemotherapeutic drugs (48). Inhibitors of DDR pathways have also been tested as single agents. Thus, basic research in ATR-Chk1 and ATM-Chk2-mediated DDR pathways will help to better understand tumorigenesis and may identify new anti-cancer targets.

1.2 The *Xenopus* egg extracts

Xenopus egg extracts derived from eggs of African clawed frogs have been utilized in studies of DNA replication, DNA repair, and DDR pathways (25, 49-55). There are several different types of *Xenopus* egg extracts: low-speed supernatant (i.e., LSS), high-speed supernatant (i.e., HSS), and nucleoplasmic extracts (i.e., NPE) (**Figure 2**). Briefly,

Xenopus eggs are crushed by centrifugation at low speed (20,000*g*) to prepare LSS. Then LSS can be further centrifuged at a high-speed (260,000*g*) to prepare HSS. In LSS system, sperm chromatin can be assembled into nuclei, which are further centrifuged into NPE at a high-speed (260,000*g*) (**Figure 2**). The approaches of how these different *Xenopus* egg extracts are made have been described previously (56).

After being added to the LSS, sperm chromatin DNA or bacteriophage lambda DNA can form nuclear envelope and be replicated in a semi-conservative manner, reconstituting an *in vitro* cell-free DNA replication system that mimics the *in vivo* DNA replication program in mammalian cells (57, 58). When DNA damaging agents are used to stress chromatin DNA in LSS system, immunoblotting analysis of proteins of interest (e.g., Chk1 phosphorylation at Ser 344 and ATM phosphorylation at Ser 1981) can dissect molecular mechanisms of DDR pathways (**Figure 3**). Chromatin bound fractions can be isolated through sucrose cushion and analyzed via immunoblotting analysis (**Figure 3**). Defined DNA structures, such as wild type plasmid DNA or plasmid DNA with an ICL at a defined location, can initiate pre-replication complex assembly in the HSS. However, the DNA replication of plasmid DNA can't be elongated without further addition of the NPE, which contain kinase activities of S-CDK (S-phase cyclin-dependent kinase) and DDK (Dbf4-dependent kinase Cdc7-Dbf4) (**Figure 3**). This unique characteristic of the *Xenopus* HSS/NPE system uncouples DNA replication initiation from replication elongation. Importantly, plasmid DNA with well-defined damage can be repaired in the HSS/NPE system, and cellular signaling mechanisms can be further dissected (**Figure 3**).

The main advantages of the LSS system and the HSS/NPE system are that target

proteins can be removed via immunodepletion with specific antibodies and that recombinant wild type or mutant proteins can be added back to depleted egg extracts. Another feature of *Xenopus* system is that small molecules (e.g., ATM specific inhibitor KU55933) can be added to LSS or HSS to certain concentrations and the roles and mechanisms of these small molecules with respect to DDR pathways can be analyzed ((**Figure 3**). In addition, *Xenopus* egg extracts can be aliquoted, frozen and stored in freezers at -80°C for multiple experiments.

1.3 Investigating DDR pathways using *Xenopus* egg extracts

DNA replication stress

DNA replication includes initiation, elongation, and termination, and is a fundamental cellular process that ensures accurate duplication of the genetic information stored in the double helix of DNA (59, 60). Generally defined as the stalling or impediment of DNA replication fork progression, DNA replication stress may result from limited nucleotides, ribonucleotide incorporation, impaired replicative DNA polymerases (δ/ε), DNA secondary structures, and fragile sites, as well as oncogene overexpression (15, 61, 62). Stalled replication forks can be stabilized and lead to cell cycle arrest and late-origin firing inhibition. Replication forks can be restarted downstream of the lesion, leaving an ssDNA gap (63, 64). The ssDNA gaps then are filled via DNA damage tolerance mechanisms such as lesion bypass or template switching (5). Unresolved stalled replication forks will collapse, resulting in replisome dissociation, nuclease digestion, and broken DNA. The physical structure and protein components of stalled and collapsed

replication forks are under intense investigation (62), and DNA replication stress is now accepted as a hallmark of cancer (65).

To study the DNA replication stress response, aphidicolin is widely used to stall DNA replication forks in *Xenopus* egg extracts. As an efficient inhibitor of DNA polymerase δ and ε, aphidicolin was utilized in the LSS system to trigger a robust Chk1 phosphorylation at a low concentration (100ng/μL) (51, 66). However, Chk1 phosphorylation is compromised when Pol α is inhibited by aphidicolin at a higher concentration (~300ng/μL) (17). More mechanistic studies have elucidated molecular details of the ATR-Chk1 pathway in DNA replication stress response from various research laboratories (51, 66-69). A primed M13-derived ssDNA mimics the ATR-activating structure and activates the ATR-dependent DDR pathway activation in the *Xenopus* HSS/NPE system (11). This primed ssDNA structure was further used to demonstrate that the MRN complex recruits TopBP1 for ATR activation (70).

Double-strand breaks (DSBs)

DSBs are one of the most deleterious types of DNA damage. Failures to detect DSBs and activate DDR signaling pathways for repair will compromise a cell's ability to maintain genomic stability, which is involved in the development of cancer and aging (3). ATM can be activated in response to DSBs and phosphorylates hundreds of substrates including Chk2 (29, 71). The ATM-Chk2-mediated DDR pathway was suggested as an anti-cancer barrier in early human tumorigenesis (72). The ATR-Chk1-mediated DDR pathway can also be activated after DSB end resection (73).

Restriction enzymes have been used to cleave circular DNA into linear version for studies of DSB-induced DDR pathways in *Xenopus* egg extracts. DSB-containing chromatin DNA can be generated by the addition of a restriction enzyme (e.g., EcoRI or PflMI) to the LSS system, triggering an ATR-mediated checkpoint response (74). EcoRI-treated chromatin DNA can also trigger ATM and Nbs1 phosphorylation in the LSS system (75). After HaeIII treatment, DSB-containing plasmid pBR322 triggers ATM-dependent checkpoint signaling that inhibits chromosomal DNA replication (76). After generation by digestion with restriction enzymes or by PCR using pBluescript as template, linear DNA fragments with different lengths were used in the *Xenopus* HSS system to demonstrate that ATM activation by DSBs requires at least ~200 bps of linear dsDNA (double-stranded DNA) and the binding of ATM to dsDNA region flanking DSB ends (77).

DSBs can also be generated after exposure to ionizing radiation or chemotherapeutic drugs. Chromatin DNA can be damaged by γ-radiation to generate DSBs, which can be added to *Xenopus* LSS, triggering the ATM-mediated DDR pathway (50). Exposure to etoposide, an inhibitor of topoisomerase II, can induce an ATR-mediated, but ATM-independent, DDR pathway activation that prevents DNA replication initiation in *Xenopus* LSS system (78, 79). Camptothecin, an inhibitor of topoisomerase I, was used to generate DSBs to study the ATM/ATR-dependent replication restart mechanism in *Xenopus* (80). In addition, a DNA DSB-mimic structure named AT70, an annealed complex of two oligonucleotides poly-(dA)70 and poly-(dT)70, was initially characterized in the Dunphy lab and utilized widely to investigate ATM- and ATR-mediated DDR pathways in *Xenopus* (52, 81, 82).

Oxidative stress

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the capacity of antioxidant defenses (83, 84). ROS include hydrogen peroxide and hydroxyl radicals and can be generated from cellular metabolism, such as oxidative phosphorylation in mitochondria, and exogenous sources, such as chemotherapeutic agents (85, 86). Oxidative stress can induce different forms of DNA damage including base damage, such as 8-oxo-7,8-dihydroguanine (8-oxo-G) and apurinic/apyrimidinic (AP) sites (87, 88). Oxidative DNA damage is repaired primarily by base excision repair (BER) while other repair pathways, such as nucleotide excision repair (NER), nucleotide incision repair (NIR), and mismatch repair (MMR), are backup mechanisms (1, 89). Oxidative stress has been implicated in the pathogeneses of cancer, aging, and neurodegenerative diseases (90).

Chromatin DNA can be damaged by hydrogen peroxide to generate oxidative DNA damage, which triggers the activation of ATR- and ATM-mediated DDR pathways in *Xenopus* LSS system (25). Notably, a base excision repair protein APE2 was demonstrated to play an essential but previously uncharacterized role in the hydrogen peroxide-induced ATR-Chk1 pathway activation (25). This study led to a more general conception that various DNA repair proteins interplay functionally with DDR pathways in oxidative stress (1).

Inter-strand crosslinks (ICLs)

ICLs are extremely cytotoxic lesions because irreparable ICLs prevent DNA replication and transcription programs, thereby threatening genome stability (91, 92). Although DNA crosslinking agents such as mitomycin C (MMC) are widely used in chemotherapy, tumor cells also acquire resistance to such agents (93). The chemotherapeutic drug MMC was used to generate crosslinks in chromatin DNA, which can activate the ATR-Chk1 DDR pathway in a *Xenopus* LSS system (94). This MMC-induced system has been utilized to elucidate the requirements of nuclear import of TopBP1 and FANC complex for DDR pathway activation (91, 95).

Our understanding of ICL repair and signaling pathways has been advanced using a defined plasmid-based ICL in the *Xenopus* HSS/NPE system, in which DNA replication of plasmid DNA is initiated in the HSS first, and subsequently elongated once NPE is added (**Figure 3**) (26, 53). ICLs activate the DDR pathway, which requires the Fanconi anemia (FANC) complex (26, 96).

Role of DNA polymerase especially TLS in DDR pathway

There are several steps for the general mechanism of how ATR-Chk1 DDR pathway is activated (**Figure 4**). ATR is recruited to RPA-coated ssDNA via direct interaction of ATRIP with RPA, though it is currently unknown whether TopBP1's recruitment to stalled replication forks requires direct TopBP1-RPA association (Step 1, **Figure 4**). A model is proposed for how checkpoint activation on the leading strand is coupled to replication restart in response to stalled replication forks, in which TopBP1 recruits Pol α, and then TopBP1 and Pol α work together to recruit the 9-1-1 complex to

stalled replication forks in *Xenopus* egg extracts (Step 2, **Figure 4**) (12, 64). Moreover, primer synthesis is initiated by Pol α and continued by Pol δ and Pol ε on stalled replication forks, which contributes to checkpoint activation in *Xenopus* egg extracts (Step 3, **Figure 4**) (66). TopBP1 bridges ATR-ATRIP with the 9-1-1 complex via direction protein-protein interactions, while the 9-1-1 complex is preferentially recruited to the ssDNA/dsDNA junction (Step 4, **Figure 4**). Lastly, ATR is directly activated by TopBP1, and Chk1 is then phosphorylated by activated ATR (Step 5, **Figure 4**).

It's significant to determine how TLS polymerases and DDR pathways regulate each other. Several recent studies have shed lights on the role of TLS polymerases for DDR pathway activation. Notably, TLS polymerase Pol κ is required for the primer synthesis, the recruitment of the 9-1-1 complex onto stalled replication forks, and subsequent activation of the ATR-Chk1 DDR pathway in both *Xenopus* egg extracts and human cells lines (Step 4, **Figure 4**) (97). Consistent with this observation, Pol κ depletion facilitates temozolomide (TMZ)-induced ubiquitination and proteasome-mediated degradation of Rad17 and severely compromises ATR-Chk1 DDR pathway activation in human glioblastoma cell lines (Step 4, **Figure 4**) (98). These findings suggest that TLS polymerases play a previously uncharacterized role in ATR-Chk1 DDR pathway via their catalytic and non-catalytic functions.

Importantly, another TLS polymerase REV1 is required for the activation of the ATR-Chk1 DDR pathway but is dispensable for the recruitment of ATR, ATRIP, TopBP1, the 9-1-1 complex, and RPA onto stalled replication forks and ICLs, suggesting a role of REV1 in the downstream of ATR activation but before Chk1 phosphorylation (Step 5, **Figure 4**) (99). Thus, TLS polymerases Pol κ and REV1 are involved in a

positive regulation for the DDR pathway. It remains to be determined whether other TLS polymerases or replicative DNA polymerases also regulate the DDR pathways directly. Defects in TLS polymerases have been implicated in human tumorigenesis and inhibitors to TLS polymerases such as Pol κ are being developed (40, 100).

1.4 Base Excision Repair

The Base Excision Repair Pathway (BER) is the most active DNA damage repair pathway. It corrects small base lesions that do not have a significant structural effect on the DNA helix. This kind of damage is usually the result of either; single strand breaks (SSBs), alkylation, depurinations, deamination, methylation, or oxidation. Most endogenous DNA damage is processed through the BER pathway and, indeed, much of the damage processed is the result of spontaneous DNA decay (101). As a result, the proper function of this pathway is integral to genomic integrity. Which makes it understandable why it is a highly conserved pathway in which the sensing proteins have structural or functional homologs from mammalian to bacterial cells(102). It is now believed that BER is important with respect to cancer, neurodegeneration, and aging (103, 104). Thus, a full understanding of this pathway and its players is paramount.

First, the DNA glycosylases search the DNA for lesions. In humans eleven of these enzymes exist, five are monofunctional (in which they remove the damaged base only) and six are bifunctional (in which they remove the damaged base and cleave the DNA backbone). It has been estimated that each glycosylase enzyme is responsible for analyze roughly 70,000 base pairs (105). The glycosylases accomplish this task by sliding up and down the DNA, interacting with the bases weakly as it passes by (106). Once the

enzyme encounters a damaged base it undergoes a conformational change ,which causes the base to flip out of the helix and into the binding pocket of the glycosylase. Once there the enzyme can distinguish if the base is damaged or not via chemical structure and excise it from the DNA (105).

In the case of a monofunctional glycosylase recognizing the damage and removing the damaged base, the resulting abasic site is cleaved by apurinic (AP) endonuclease (APE1) leaving the sugar attached to the 5' side. APE1 has been shown to be the primary molecule in initiating AP site repair with 95% of AP cleavage in HeLa cell extracts utilizing APE1 (107). AP sites are detrimental as they block transcription and replication fork progress during DNA replication. Furthermore, due to translesion synthesis, they can also lead to single base mutations (substitutions, insertions, deletions) (108, 109). After APE1 cleavage the opposing 3' hydroxyl group is the substrate for the repair polymerase in BER polymerase β (Pol β), which not only adds the corrected base to the DNA but also removes the sugar attached to the 5' phosphate via it's lyase activity. The gap is then filled in and sealed by a DNA ligase.

The bifunctional glycosylases are responsible for sensing oxidative damage and have a slightly different path. After the enzyme removes the base and cleaves the backbone it leaves an unsaturated aldehyde, which gets removed by APE1's phosphodiesterase activity, or the glycosylase leaves a phosphate group, which is removed by polynucleotide kinase (PNK). The remainder of the steps follows the path of the monofunctional glycosylase steps to completion. This process is called short patch BER and proceeds to fix the damage unless the repair encounters problems. The most common of the hiccups is that the sugar moiety is inefficiently removed from the 5' DNA

end. In the event of a problem the pathway is redirected to a long patch process in which, the damaged strand is misplaced by polymerase ε or polymerase δ, which normally function as replicative polymerases. After the polymerase has detached a short stretch of bases on the DNA from their Watson-Crick partners, by replacing them and leaving an overhang, flap endonuclease 1 (FEN1) removes the stranded stretch of DNA by cutting the backbone and leaving a 5' phosphate. The nick left over from this process is sealed by a DNA ligase.

In order to coordinate this complex and multistep process Poly(ADP)ribose polymerase 1 (PARP1) binds the AP site (110) and recruits the scaffolding protein XRCC1 (111). XRCC1 has not been shown to have any enzymatic activity of its own but it is nonetheless integral for Single Strand Break Repair (SSBR) and BER as a scaffolding protein to which protein binding partners have been characterized in the greatest detail. Of note for this work is the knowledge that XRCC1 and Pol β have been shown to be binding partners (112, 113) in addition to being considered essential for Base Excision Repair as a scaffold for various other factors and the primary polymerase in the pathway, respectively.

1.5 XRCC1

X-ray cross complementing group 1 protein (XRCC1) is a scaffolding protein that forms complexes with other enzymes to coordinate BER and SSBR. A plethora of work has been done to establish which proteins XRCC1 bind to in order to coordinate these complex multi-step repair processes. Despite this the abundant protein-protein interaction work, structural mechanisms underlying XRCC1's interactions with binding to DNA and

recruitment for BER are poorly understood. Nevertheless, the extensive interaction work done by the field reveals that XRCC1 has a myriad of binding partners at almost all point in the BER pathway and utilizes its distinct domains and linker regions to accomplish these interactions.

XRCC1 was the first mammalian gene identified that confers protection to the cell against the effects of ionizing radiation, alkylating agents, and UV irradiation (114). XRCC1-deficient cancer cell lines are viable but this genotype confers embryonic lethality in mice around day 7 of gestation (115). Restoring less than 10% of the normal level of XRCC1 is however sufficient to rescue development and results in healthy adults (116). Heterozygous $XRCC1^{+/-}$ mice show increased sensitivity upon ingestion of alkylating agents, which manifests in precancerous colon lesions and liver toxicity (117). These findings suggest that the cellular concentration of XRCC1 and the protein complexes it generates are important for genome integrity. Human XRCC1 is 33 kilobases long and located on the 19^{th} chromosome (Ensembl ID: ENSG00000073050). This DNA sequence gets transcribed into a 633 amino acid (aa) protein that weighs ~70 kDa (Uniprot ID: p18887).

XRCC1 is comprised of three globular domains joined by two linker regions, the first of which is ~150 aa and the second of which is ~120 aa (**Figure 5A**). The *N*-terminal domain (NTD) of XRCC1 spans aa 1-183 and binds specifically to pol β. This interaction with XRCC1 is considered to have high affinity and a stabilizing effect on the protein (112, 118). The aa region 166 to 403 binds to DNA glycosylases NEIL2, NTH1, OGG1, and UNG2 (119-121). APE1 also binds to XRCC1 in this amino acid region (120). Half of this region (between aa 155-310) is comprised of the Linker 1 region (XL1) in addition

to being part of the region the glycosylases bind to this is also the region of XRCC1 that houses its nuclear localization signal (NLS) (122). Housed in this region also is a Rev1-interacting region RIR sequence that has been shown to interact with Rev1 for the purpose of recruiting translesion synthesis polymerases (123). XRCC1 has also been shown to interact with proliferating cell nuclear antigen (PCNA) between aa 166-310 even though it lacks a traditional PCNA interaction motif (PIP box) (124, 125). The central domain of XRCC1 is the first of two BRCA 1 C terminus (BRCT) domains (**Figure 5A**). BRCT domains are found traditionally among proteins associated with the DNA Damage Response (DDR), and appear to act as phosphorylation dependent protein interaction domains (126). Between the two BRCT domains in XRCC1, BRCT1 is the most evolutionarily conserved and is necessary for efficient DNA SSBR (127). The BRCT1 domain is located at aa 310-405 and contains a poly(ADP-ribose) (PAR)-binding motif (128). This motif allows for XRCC1 to be recruited to DNA following the formation of polymeric ADP-ribose chains at the site of single strand breaks by PARP-1 (122). The BRCT1 domain also shows direct binding with the PARP1 and PARP2. The Linker 2 region of XRCC1 (XL2) aa 405-529 interacts with the forkhead-associated (FHA) domains of at least three binding partners: polynucleotide kinase phosphatase (PNPK), aprataxin (APTX), and aprataxin- and PNPK-like factor (APLF) (129, 130). FHA domains are involved in protein-protein interactions and are found in more than 700 eukaryotic proteins including; kinases, phosphatases, kinesins, transcription factors, RNA binding proteins and metabolic enzymes (131). The final domain of XRCC1 is the BRCT2 domain, which spans from aa 529 to 633 (**Figure 5A**). This domain of XRCC1 binds to the Ligase 3 (LIG3) through LIG3's own BRCT domain (132, 133). Much like

the interaction in the NTD to pol β the BRCT2 interaction with LIG3 has high affinity and stabilizes expression levels of LIG3 (134).

As described above XRCC1 has been known to associate with enzymes involved in every step of the BER pathway from DNA damage sensing glycosylases to DNA sealing ligases. In addition to this polymorphisms and mutations/deletions of the protein have been implicated in innumerable cancer data sets and populations over the years. Thus, it is of critical importance that as much is discovered as possible regarding XRCC1's role in genomic integrity.

1.6 Polymerase beta

DNA Polymerase β (Pol β) was discovered and purified in 1971 and was characterized as a low molecular weight DNA polymerase (**Figure 19A**) (135). Years later it was uncovered that Pol β is a 39 kDa protein with DNA polymerase and deoxyribose phosphatase activity (136-138). This makes it the smallest DNA polymerase and despite this also happens to be one of the most well studied due to its key function in DNA repair and the fact that it is expressed in all stages of the cell cycle (139). Pol β is a key enzyme in BER which is one of the major genome maintenance repair pathways in mammalian cells. Pol β's role in this pathway has already been described above but, in brief, Pol β is responsible for catalyzing removal of the 5' deoxyribose phosphate (dRP) left by APE1 and filling in the gap in the DNA so that DNA Ligase can come and seal the backbone. During BER Pol β fills DNA gaps of 1 to 6 nucleotides though evidence of Pol β having the ability to fill gaps over 300 nucleotides has been reported (140).

Pol β is a 335 amino acid polypeptide with two distinct domains responsible for separate functions of the enzyme's activity. The amino terminus domain is 8 kDa in size and is responsible for the lyase activity of Pol β that removes the 5' dRP after the APE1 incision step in BER (137, 141). This 8 kDa domain is attached to the 31 kDa polymerase domain via a protease sensitive hinge region (142). This polymerase domain catalyzes the nucleotidyl transferase reaction. The 8 kDa domain binds to ssDNA and the 31 kDa polymerase domain binds to dsDNA (143, 144). Upon binding to gapped DNA, the C-terminus has been shown to close around the correct dNTP and its complementary base in addition to significant movement of the amino terminus domain (145, 146). Pol β despite being a crucial polymerase is highly error prone when tested in vitro. On a 5-nucleotide gap it produces a base substitution error at a rate of 10^{-3}, in contrast if the gap is a single nucleotide the fidelity increases about 5-fold (140). As reference the replication complex has a mistake rate of ~10^{-9} if not lower. To make up for this obvious lack of fidelity it has been shown that DNA ligases involved in BER inefficiently ligate DNA mismatches (147). This has led to the theory that the delay provided by the ligase on a mismatched base may give a 3'-exonuclease the opportunity to remove to improper base. In addition to its well characterized role in BER, recent work has been done to show that Pol β assists in DSB repair during Prophase I of meiosis (148, 149). Indeed, meiotic synapsis in mice is defective in spermatocytes and oocytes that were modified to have the Pol β gene deleted. Intentional DSB formation in these spermatocytes have reduced removal of the Spo-11 complex from the 5' end of the break which makes it impossible for repair enzymes to be recruited due to stochiometric restrictions at the site. It is thus believed that Pol β has an important role associated with the removal of this complex (150).

Pol β has been shown to be mutated in 30 to 40 percent of human tumors, including colon, gastric, and prostate carcinomas. Of note is that the amino acid alterations in these mutations are not localized to a specific domain but are found all throughout the protein (151, 152). These finding show that further work on Pol β to establish a wider knowledge base on how this critical protein impacts genomic integrity.

CHPATER 2: DISTINCT ROLES OF XRCC1 IN GENOME INTEGRITY IN XENOPUS EGG EXTRACTS

2.1 Introduction

Cells of all organisms are constantly exposed to threats, such as oxidative stress, from endogenous sources or environmental agents (3, 153, 154). Oxidative stress-induced DNA damage includes oxidized base damage or sugar moiety damage, apurinic/apyrimidinic (AP) sites, single-strand breaks (SSBs), double-strand breaks (DSBs), interstrand crosslinks (ICLs), and oxidatively-generated clustered DNA lesions (1, 88, 90). Oxidative DNA damage is repaired primarily by base excision repair (BER) while other DNA repair pathways such as nucleotide excision repair (NER), mismatch repair (MMR), and nucleotide incision repair (NIR) are backup options (1, 89, 155-157). The molecular mechanism of BER pathway includes damaged base removal by DNA glycosylase to generate AP site, SSB generation by APE1 or bi-functional glycosylase, and subsequent gap filling and ligation reactions (101). BER pathway is composed of short-patch and long-patch sub-pathways, which has been reconstituted with purified human proteins in vitro (112, 155).

Representing about 10 percent of all DNA lesions, SSBs are generated from oxidative stress, intermediate products of DNA repair, or aborted activity of cellular enzymes such as DNA topoisomerase 1 (101, 158, 159). SSBs are repaired by a rapid global SSB repair mechanism (160). A four-step mechanism of SSB repair including SSB detection, DNA end processing, DNA gap filling, and DNA ligation has been proposed previously (161). In addition, recent studies suggest that SSBs can also be

resolved by homologue recombination or alternative homologue-mediated SSB repair (160, 162, 163). Unrepaired SSBs hinder proper DNA transcription and accurate DNA replication of the genome, leading to cancer and neurodegenerative disorders (90, 161, 164). For example, defective SSB repair is responsible for senescence and neoplastic escape of epithelial cells (165). Others and we have demonstrated that oxidative stress triggers both ATM-Chk2 and ATR-Chk1 DNA damage response (DDR) pathways. ATM can be activated through a disulfide bond formation and conformation change in a DNA-independent manner following oxidative stress (36, 38, 166, 167). ATR DDR pathway can be activated by oxidative stress-damaged chromatin DNA and defined SSB structure (25, 168). Although ATM is proposed to be activated by SSBs, it is not known how exactly SSBs activate ATM (167).

X-ray Repair Cross Complementing Protein 1 (XRCC1) has been implicated in different types of DNA repair pathways, including BER, NER, SSB repair, non-homologous end joining (NHEJ), and microhomology-mediated end joining (MMEJ) pathways (114, 169-172). XRCC1 acts as a scaffolding protein to recruit a multitude of factors to the site of DNA damage (169, 173, 174). Furthermore, XRCC1 interacts with PCNA and DNA polymerase α, participating in DNA replication (124, 175, 176). XRCC1-deficient mice are embryonically lethal, suggesting its physiological significance for development (115). While over-expression or under-expression of XRCC1 has been linked to cancer, XRCC1 variants with Arg194Trp, Arg280His, or Arg399Gln mutant have been studied via epidemiological analysis and meta-analysis (169, 177-179). DNA Polymerase β (Pol β) complexes with XRCC1 and acts as the main repair DNA polymerase in the BER pathway (180, 181). Pol β has the unique ability to repair DNA

gaps smaller than 6 nucleotides but is incredibly error prone (140). More recently, numerous XRCC1 case studies have come out uncovering specific mutations in these proteins that are believed to have a correlation to cancer progression (182-184). These mutations are believed to play a role in genome instability by compromising some aspect of XRCC1 functions. However, exact roles and mechanisms of XRCC1 in DNA repair and DDR pathways in response to oxidative DNA damage and SSBs remain unclear.

Xenopus egg extracts have been widely used as biochemical system in studies of DNA metabolism, and findings from *Xenopus* system can be verified in mammalian system (49, 56, 185). Low-speed supernatant (LSS), high-speed supernatant (HSS), and nucleoplasmic extracts (NPE) are three different types of Xenopus egg extracts (54, 186, 187). We have demonstrated that the ATR-Chk1 DDR pathway is activated by hydrogen peroxide-induced oxidative stress in *Xenopus* LSS system, and that a BER protein APE2 (AP endonuclease 2) plays an essential role for ssDNA generation and assembly of checkpoint protein complex including ATR, ATRP, and TopBP1 to activate the ATR-Chk1 DDR pathway in oxidative stress (1, 25). Furthermore, APE2's conserved Zf-GRF motif in its extreme C-terminus is required for binding to ssDNA and its 3'-5' exonuclease activity in the activation of ATR-Chk1 DDR following oxidative stress (188). In addition, ATR-Chk1 DDR is activated by a defined SSB plasmid in *Xenopus* HSS system (168). However, it remains unknown whether APE2 plays a direct role in the repair of oxidative DNA damage and SSBs.

In this work, I demonstrate evidence that XRCC1 is dispensable for ATR-Chk1 DDR pathway following oxidative stress, and that XRCC1 depletion enhances DDR pathway activation. Surprisingly, XRCC1 is dispensable for the repair of defined SSB

and gapped plasmids with a 5'-OH or 5'-P terminus. In vitro protein-protein interaction suggests that XRCC1 interacts with APE2, but XRCC1 only mildly enhanced PCNA-mediated APE2 exonuclease activity. Lastly, I have shown that XRCC1 is important of the repair of DNA damage following oxidative stress in *Xenopus* egg extracts.

2.2 Materials and methods

Experimental procedures for Xenopus laevis egg extracts and sperm chromatin

Xenopus laevis care and use was approved by UNC Charlotte's Institutional Animal Care and Use Committee (IACUC). The preparation of *Xenopus* LSS, HSS, and sperm chromatin was described previously (56, 68, 186). Immunodepletion of APE2 from HSS was performed as previously described (25, 168, 188). Immunodepletion of XRCC1 from LSS and HSS was performed using a similar approach as APE2 depletion in LSS/HSS. To deplete XRCC1 from LSS, 200 μL of LSS was incubated with ~40 μL of ProteinA Sepharose beads (GE Healthcare) pre-coupled with anti-XRCC1 antibodies, for ~40 min at 4°C with constant mixing. Typically, 3-round depletion is needed to get ~150 μL of XRCC1-depleted LSS from 200 μL of LSS. Antibodies against XRCC1 were raised in rabbits against recombinant GST-XRCC1 (Cocalico Biologicals, see below section for the preparation).

For experiments in *Xenopus* egg extracts, hydrogen peroxide (100mM) was added to mock- or XRCC1 depleted LSS or HSS, which was supplemented with sperm chromatin (4,000/μL). After incubation of different time as indicated at room temperature, 5 μL of reaction mixture was added with 45 μL of sample buffer for immunoblotting analysis.

For DNA-bound protein isolation from LSS system, after room temperature incubation, 50 μL of reaction mixture was diluted with 200 μL of egg lysis buffer (ELB, 250 mM sucrose, 2.5 mM MgCl2, 50 mM KCl, 10 mM HEPES, pH 7.7) and spin through a 1 ml of sucrose cushion (0.9 M sucrose, 2.5 mM MgCl2, 50 mM KCl, 10 mM HEPES, pH 7.7) at 11,000 rpm for 2 min at 4°C with a swinging bucket. After centrifugation, the supernatants were removed down to 100 μL, and another round of centrifugation was performed. For the second round the remaining reaction mixture was diluted with 200 μL of ELB supplemented with 0.06% Triton X-100 detergent. The remaining DNA-bound protein factions collected after removing the supernatants were resuspended with sample buffer and examined via immunoblotting analysis.

For DNA-bound protein isolation from HSS, after room temperature incubation, 50 μL of reaction mixture was diluted with 150 μL of Buffer XB (50 mM sucrose, 100 mM KCl, 100 μM CaCl2, 2 mM MgCl2, 10 mM Hepes, pH 7.7) and layered on a 400 μL of sucrose cushion (1.1 M sucrose in Buffer XB), and spun (11,000 rpm, 30 min, 4°C) with a swinging bucket. After centrifugation, the supernatants were removed, and the chromatin-bound protein factions were resuspended with sample buffer and examined via immunoblotting analysis.

Preparation of SSB and gapped plasmids

There are four recognition sites on pUC19 for Nt.BstNBI, designated as site1 (nt 427–431 on (+) strand), site2 (nt 1177–1181 on (+) strand), site3 (nt 706–710 on (–) strand), and site4 (nt 1694–1698 on (–) strand). The plasmid pS was generated by mutant pUC19 on three sites (i.e. site2–site4) sequentially with three pairs of primers using

QuikChange II XL site-directed mutagenesis kit. The mutations were verified and confirmed by DNA sequencing. Qiagen plasmid midi kit was utilized to obtain large amounts of the pS plasmid.

To generate a defined SSB between C435 and T436, the pS was treated with Nt. BstNBI (10 U/μg) for 2 h at 55°C and CIP (calf intestine phosphatase, 10 U/μg) for 1 h at 37°C to remove the 5′-P of T436. Furthermore, SSB plasmid with 5'-P was prepared by treating pS with Nt.BstNBI but without subsequent CIP. The SSB plasmid with a 5'-OH or 5'-P was further purified from agarose gel with QIAquick gel extraction kit and then optionally purified by phenol–chloroform extraction. To generate a DSB plasmid, the pS was treated with SbfI-HF at 37°C and subsequently with CIP at 37°C. The DSB plasmid was purified from agarose via QIAquick gel extraction kit and then optionally purified by phenol–chloroform extraction. This procedure has been described previously (168).

To prepare gapped plasmids with 5'-OH or 5'-P, the SSB plasmid with a 5'-OH or 5'-P was treated with recombinant GST-APE1, respectively, in an exonuclease buffer (20 mM KCl, 10 mM MCl2, 2 mM DTT, 50 mM HEPES, pH 7.5) at 55°C for 20 min to generate 1-3nt gap in the 3'-5' direction, followed by phenol-chloroform extraction and purification. As shown in a recent study published by my lab, this APE1-pretreatment can generate ~1-3 nt gap at the plasmid's nick in the 3' to 5' direction (168). The gapped plasmids with 5'-OH or 5'-P was also further purified from agarose gel with QIAquick gel extraction kit.

Analysis of DNA repair products of SSB or gapped plasmids in Xenopus HSS system

The SSB or gapped plasmid with 5'-OH or 5'-P was added to mock-, XRCC1-, or APE2-depleted HSS (final concentration 75 ng/μL). After incubation at room temperature for different times, nuclease-free water was added to each reaction, followed by DNA repair product isolation procedure including phenol-chloroform extractions, as described previously in my lab's recent studies (168). Then the purified DNA repair products were examined via agarose electrophoresis.

Recombinant DNA and proteins

Recombinant pGEX-4T1-XRCC1 was generated by cloning the coding region (nt 164-2119) of *Xenopus laevis* XRCC1 (GenBank: BC045032, Xenopus Gene Collection IMAGE ID: 5543195) into EcoRI- and XhoI-digested pGEX-4T1, as previously described [26]. pGEX4T-XRCC1-BRCT1 (307-414aa) and pGEX4T-XRCC1-BRCT2 were kind gifts from Dr. Domenico Maiorano (Institute of Human Genetics, France) (175). pCS2+MT-APE2 FL and ΔZF were constructed in our lab previously (25, 188). GST-tagged recombinant proteins were expressed and purified in E. coli DE3/BL21 according to standard protocol. Purified recombinant proteins were confirmed on SDS-PAGE gels with coomassie staining. FL and ΔZF Myc-APE2 were expressed in TnT SP6 Quick coupled transcription/translation system in vitro according to the manufacturer's protocol (Promega).

Immunoblotting analysis and antibodies

For immunoblotting analysis, samples were denatured in the presence of reducing Laemmli buffer for 10 min at 95 °C and run on 5-15% polyacrylamide gels at a constant

25 mA per gel. Separated proteins were transferred to PVDF membranes (Immobilon-P 0.45 μm, Millipore) using wet transfer at 110 V for 80 min. PVDF membranes will be washed, blocked, and incubated with appropriate primary and secondary antibodies. Membranes were washed again 3 times with TBST and incubated with WesternBright ECL or Sirius substrate for 10 minutes (Advansta, USA). Fluorescence was observed using X-ray film or a ChemiDoc MP Imaging System (BIO-RAD, USA).

Anti-XRCC1 antibodies were raised in rabbits against GST-XRCC1 (Cocalico Biologicals). Anti-*Xenopus* APE2 antibodies was described previously (25). Antibodies against ATR were provided by Dr. Karlene Cimprich (49, 189). Antibodies against ATRIP were provided by Dr. Howard Lindsay (190). Antibodies against RPA70 were provided by Dr. Matthew Michael (12). Antibodies against Chk1 phosphorylation at Ser345 were purchased from Cell Signaling Technology. Antibodies against Histone 3 were purchased from Abcam. Antibodies against Chk1, GST, and Myc were purchased from Santa Cruz Biotechnology.

GST pulldown assays

For the GST-pull-down experiments, 3 μg of GST or GST-tagged recombinant proteins were added to 10uL TNT SP6 reaction with 1uL of Myc tagged protein and the mixtures were volume balanced to 100uL with Interaction Buffer. After an hour of incubation, an aliquot of the mixture was collected as Input and the remaining mixture was diluted with 200 μL of Interaction Buffer (200 mM NaCl, 5 mM MgCl2, 10% (vol/vol) glycerol, 0.1% Nonidet P-40, 20 mM Tris–HCl at pH 8.0). Then, 30 μL of glutathione beads that were resuspended in 200 μL interaction buffer were added to the

diluted egg extracts. After 1h-incubation at room temperature, beads were washed with Interaction Buffer two times. Then, the bead-bound fractions and Input were analyzed via immunoblotting.

In vitro exonuclease assays

For the APE2 exonuclease analysis in vitro, I prepared APE1-pretreated FAM-labeled gapped DNA substrate as my lab recently reported (168). The purified gapped dsDNA structure (50 nM) was incubated in 1× reaction buffer (50 mM NaCl, 1 mM TCEP, 1 mM MnCl2, 10 mM Tris–HCl, pH 8.0) with the presence of GST or GST-XRCC1 (final concentration 200ng/μL). After a 30-min incubation at 37°C, samples were loaded and examined on a 20% TBE–Urea gel. Gels were imaged via a Typhoon imager (GE Healthcare).

COMET assays

COMET assays were preformed using the OxiSelect Comet Assay Kit from Cell BioLabs, Inc. The procedure was modified and tailored to *Xenopus laevis* cell lysates as opposed to mammalian cell samples. LSS reactions were performed as described above until the sample buffer would be added. From that point the entire ~50μL reaction was diluted with 1mL of cold PBS and spin in a swinging bucket tabletop centrifuge at 2,000rpm for 5 min at 4°C. After centrifugation, the supernatant was aspirated and the pellet was resuspended with 0.2mL of cold PBS. From this point the procedure follows the OxiSelect standard procedure for both the alkaline (pH>13) and neutral conditions (pH=~7.0) with the only exception being SYBR Gold was used, and diluted 1:10,000 in

TE buffer pH 7.5. After the slides were allowed to dry the nuclei were observed with fluorescent microscope using a FITC filter. Images were taken using DP Controller software (Olympus Corporation, JPN) and analyzed using Comet Assay IV Lite software (Instem, UK).

Quantification and statistical analysis

ImageJ was utilized to quantify gels from exonuclease assays. Statistical analysis was performed using GraphPad Prism8. T-test was performed for statistical analysis on DNA repair capacity experiments. Tail moment representing DNA damage from COMET Assays was quantified using Comet Assay IV software (Perceptive Instruments/Instem, UK). Ordinary one-way ANOVA (Tukey's multiple comparisons test) was chosen for statistical analysis on COMET Assays.

2.3 Results

XRCC1 is not required for ATR-Chk1 DDR pathway activation following oxidative stress in Xenopus LSS system

Xenopus XRCC1 contains a N-terminal domain (NTD), a nuclear localization signaling (NLS) in the middle, and two BRCT domains (i.e., BRCT1 and BRCT2) in the C-terminus (**Figure 5A**), which is very similar to its homologue in humans (191). Clustral Omega analysis of XRCC1 shows high identity or similarity in the amino acid sequence in Xenopus, human, and mouse (**Figure 5B**), suggesting that XRCC1 may have similar or conserved functions during evolution. In particular, BLASTP analysis shows

that there are 54% (366/677) identities and 67% (458/677) positives between *Xenopus* XRCC1 (GenBank#: AAH45032) and human XRCC1 (GenBank#: NP_006288) (**Figure 5**).

We recently reported that XRCC1 is not required for the activation of ATR-Chk1 DDR pathway in response to a defined SSB plasmid in *Xenopus* HSS system (168). As briefly mentioned previously (168), I constructed recombinant GST-XRCC1, which was expressed in *E. coli* DE3 cells with IPTG induction and purified following vendor's protocol (**Figure 6A**). SDS-PAGE analysis verified the purified recombinant GST-XRCC1, which was shown at ~100kD position on gel, as expected (**Figure 6A**). The purified recombinant GST-XRCC1 was utilized for custom antibodies production in rabbits from Cocalico Biologicals Inc. Anti-XRCC1 antibodies were used to immunodeplete endogenous XRCC1 successfully from LSS and HSS, respectively (**Figure 6B-6C**). To test whether XRCC1 is important for ATR-Chk1 DDR pathway following oxidative stress, I added hydrogen peroxide and sperm chromatin into mock- or XRCC1-depleted LSS. After a 45-min incubation, total egg extracts examination via immunoblotting analysis demonstrated that hydrogen peroxide triggered Chk1 phosphorylation in mock-depleted LSS (**Figure 7A**), consistent with previous studies (25, 174). However, hydrogen peroxide-induced Chk1 phosphorylation was enhanced when XRCC1 was depleted in LSS (Lane 4 vs. Lane 2, **Figure 7A**). Notably, XRCC1-depletion also triggered Chk1 phosphorylation without the treatment of hydrogen peroxide (Lane 3 vs. Lane 1, **Figure 7A**). Chromatin fraction analysis shows that the recruitment of ATR and RPA70 to chromatin with the presence and absence of hydrogen

peroxide was increased when XRCC1 was depleted from LSS (**Figure 7B**), which is consistent with the ATR activation.

Consistent with our recent report (25), hydrogen peroxide-induced oxidative stress triggered both Chk1 phosphorylation and ATM phosphorylation when sperm chromatin DNA was added to LSS system (Lanes 1&2, **Figure 8A**). As expected, hydrogen peroxide addition did not trigger Chk1 phosphorylation when no DNA was added to LSS system (Lanes 3&4, **Figure 8A**). It has been shown that hydrogen peroxide can trigger ATM activation in an DNA-independent manner (38). However, we did not observe any ATM phosphorylation after the addition of hydrogen peroxide into LSS with the absence of chromatin DNA (Lanes 3&4, **Figure 8A**).

A recent study demonstrates that, to prevent DSB formation, ATM is activated by DNA damage induced by XRCC1-deficiency in mammalian cells (167). To determine whether the enhanced Chk1 phosphorylation in XRCC1-depelted LSS is due to ATM activation, I added ATM specific inhibitor KU55933 in XRCC1-depleted LSS. The incubation of KU55933 reversed the increased Chk1 phosphorylation with the presence and absence of hydrogen peroxide in XRCC1-depleted LSS (Lanes 1&2, Lanes 3&4, Lanes 7&8, **Figure 8B**), suggesting the role of ATM in response to XRCC1-deficiency-induced DNA damage. Furthermore, ATR specific inhibitor VE-822 impaired Chk1 phosphorylation in XRCC1-depeleted LSS, regardless of hydrogen peroxide (Lanes 5&6, **Figure 8B**). These observations suggest that although XRCC1 is not required for hydrogen peroxide-induced ATR-Chk1 DDR activation, XRCC1 depletion may trigger both ATR and ATM activation under non-perturbed conditions. Consistent with this interpretation, XRCC1 depletion triggered mild γ-H2AX under normal conditions in LSS,

which is similar to hydrogen peroxide-induced γ-H2AX in mock-depleted LSS (Lane 3 vs Lane 2, **Figure 8B**).

There are several different types of *Xenopus* egg extracts: LSS, HSS, and NPE (56, 186). Generally speaking, chromatin DNA can't form nuclear membrane for DNA synthesis in HSS, due to the lack of membrane fractions and CDKs and DDKs (56). Taking advantage of DNA replication-deficiency in HSS, I sought to determine whether XRCC1 deficiency induces Chk1 phosphorylation in HSS. As shown in **Figure 9A**, XRCC1 depletion in HSS did not result in noticeable Chk1 phosphorylation under normal conditions, although XRCC1 depletion increased hydrogen peroxide-induced Chk1 phosphorylation. Chromatin fraction analysis also shows that neither ATR nor RPA70 was noticeably increased on chromatin under normal conditions in XRCC1-depleted HSS (**Figure 9B**). These observations suggest that DNA replication may be needed to generate necessary DNA damage for DDR pathway activation when XRCC1 is absent. Overall, XRCC1 depletion leads to ATM- and ATR-dependent Chk1 phosphorylation in *Xenopus* LSS system, while XRCC1 is not required for ATR-Chk1 DDR pathway activation.

XRCC1 is dispensable for the repair of plasmid with defined SSB or gapped structures in Xenopus egg extracts

Our lab recently demonstrated that a defined SSB plasmid with a 5'-OH can be repaired in the *Xenopus* HSS system and that SSB-induced ATR activation is required for SSB repair (168). To test whether XRCC1 is required for repairing the defined SSB plasmid, I added SSB plasmid with a 5'-OH in mock- or XRCC1-depleted HSS. After different timepoints, DNA repair products were isolated and examined via agarose gel

electrophoresis. The SSB repair in the absence of XRCC1 is similar to that in the presence of XRCC1 (**Figure 10A**). Quantification of DNA repair capacity at 5 min and 30 min from four independent experiments show no significance between the mock- and XRCC1-depletion HSS (**Figure 10B**). This observation suggests that XRCC1 is dispensable for repairing the defined SSB plasmid with a 5'-OH in the *Xenopus* HSS system. Next, I tested whether the presence of a 5'-P at the SSB site may affect potential role of XRCC1 in DNA repair. The DNA repair capacity of the SSB plasmid with a 5'-P in XRCC1-depleted HSS was similar to that of mock-depleted HSS (**Figure 11A**). Quantification of DNA repair capacity at 5 min and 30 min from three independent experiments shows no significance between the mock- and XRCC1-depletion HSS, suggesting that XRCC1 is not required for the repair of SSB plasmid with 5'-P (**Figure 11B**). Then, I sought to determine whether a gapped plasmid with 5'-OH or 5'-P can be repaired when XRCC1 is depleted in HSS. Notably, the repair of the gapped plasmid with 5'-OH in XRCC1-depleted HSS is not noticeably affected in comparison to that in mock-depleted HSS (**Figure 12A**). Quantification of DNA repair capacity at 5 min and 30 min from three independent experiments show no significance between the mock- and XRCC1-depletion HSS, suggesting that XRCC1 is dispensable for repairing the gapped structure with a 5'-OH (**Figure 12B**). I found similar results using a gapped plasmid with a 5'-P (**Figure 13**). Together, these observations suggest that XRCC1 is dispensable for the repair of SSB or gapped structures with either 5'-OH or 5'-P terminus in *Xenopus* egg extracts.

This finding implied that BER was not the primary repair pathway for dealing with our defined SSB structure in *Xenopus* system. To further test this hypothesis, I used

inhibitors of Poly(ADP-ribose) polymerase 1 (PARP-1) to see if traditional BER or SSBR were part of the process of repairing the SSB plasmid with 5'-OH. PARP-1 has been implicated in almost every major DNA repair pathway but the most work has been done on its involvement in SSBR and BER (192). Where it is responsible for the detection of single-stand break damage and the poly(ADP-ribose)-mediated recruitment of the scaffolding factor XRCC1 to DNA strand breaks (122, 193-196). Olaparib is a PARP-1 inhibitor approved for treatment of 'platinum sensitive' ovarian cancers (197). To test whether PARP-1 is required for repairing the defined SSB plasmid, I added SSB plasmid with a 5'-OH to HSS adding either DMSO or Olaparib to the extract (DMSO acts as a control because it is the solution Olaparib is dissolved into). I again found that the repair capacity was not noticeably different in the presence or absence of Olaparib (**Figure 14**). Similar result was found using another PARP-1 inhibitor Iniparib. These observations with PARP1 inhibitors suggest that the traditional SSB and BER pathways are not involved in the repair of the defined SSB plasmid in our *Xenopus* HSS extract.

We recently reported that APE2 is required for SSB end resection in the 3'-5' direction and SSB-induced ATR-Chk1 DDR pathway in the HSS system (168). To test whether APE2 is important for SSB repair, we removed APE2 from HSS, and found that the repair of SSB plasmid with a 5'-OH was significantly compromised in APE2-depleted HSS (**Figure 15**). To determine whether SSB repair deficiency in APE2-depleted HSS is due to APE2 absence, recombinant Myc-APE2 was added back to APE2-depleted HSS (**Figure 15**). Notably, adding back Myc-APE2 rescued the SSB repair deficiency (**Figure 15**). These observations suggest that APE2 is required for the repair of SSB plasmid with a 5'-OH in the HSS system.

XRCC1 interacts with APE2 but plays a minimal role of APE2's PCNA-mediated exonuclease activity in vitro

Our data so far suggest that XRCC1 is dispensable for SSB repair, which is different from APE2's essential role for SSB repair. To test whether XRCC1 regulates APE2 functions, I first sought to determine whether XRCC1 interacts with APE2. Importantly, GST-XRCC1 but not GST interacted with recombinant Myc-APE2 protein in an interaction buffer, suggesting that XRCC1 associates with APE2 directly (**Figure 16A**). My lab recently demonstrated that the Zf-GRF domain in APE2 C-terminus is important for DNA binding and PCNA interaction to promote its exonuclease activity (168, 188). Notably, Myc-APE2 lacking Zf-GRF domain (designated as Myc-APE2-ΔZF) still interacts with GST-XRCC1 but not GST, suggesting that the Zf-GRF domain within APE2 is dispensable for XRCC1 association (**Figure 16A**). In addition, the BRCT1 and BRCT2 domains of XRCC1 do not have a very strong ability to bind full length Myc-APE2 (Figure 16B). This suggests that XRCC1's BRCT domains are no sufficient for the interaction between XRCC1 and APE2. Previous studies have shown that PCNA promotes APE2's exonuclease activity (168, 198, 199). The addition of GST-XRCC1, but not GST, mildly enhanced the PCNA-mediated APE2 exonuclease activity (**Figure 16C**). I interpret that this effect of XRCC1 for APE2's exonuclease is very minimal.

XRCC1 is important for repairing DNA damage following oxidative stress in Xenopus egg extracts

Our observations suggest that XRCC1 is dispensable for the repair of the defined SSB plasmid, although XRCC1 interacts with APE2. Next, I sought to determine whether XRCC1 is important for DNA damage repair following oxidative stress using COMET assays under alkaline and neutral conditions (200). Using COMET assays under alkaline condition, I found that the Tail Moment was enhanced after hydrogen peroxide treatment in the LSS system, suggesting that more SSBs and AP sites are generated following oxidative stress (**Figure 17A**). Notably, XRCC1 depletion significantly increased the Tail Moment with the absence and presence of hydrogen peroxide in the LSS system, suggesting that XRCC1 is important for repairing AP sites and SSBs following oxidative stress (**Figure 17B**). Using COMET assays under neutral condition, I found that the Tail Moment was increased after hydrogen peroxide treatment and that XRCC1 depletion increased the Tail Moment in the presence of hydrogen peroxide in the LSS system (**Figure 18**), suggesting that XRCC1 is important for repairing DSBs induced by oxidative stress. Overall, our evidence suggests that XRCC1 is important for the repair of oxidative stress-derived DNA damage in *Xenopus* egg extracts.

2.4 Conclusion

Our results from this Chapter demonstrate that XRCC1 is dispensable for ATR-Chk1 DDR pathway following oxidative stress, and that XRCC1 depletion enhances DDR pathway activation. The significant increase in DDR activation clearly demonstrates that XRCC1 plays an important role in preserving genomic integrity.

Furthermore, I show that XRCC1 is dispensable for the repair of defined SSB or Gapped plasmids, while important for total nuclear integrity. Indeed, my data provides evidence that the BER pathway in totality is not required to repair these defined DNA structures in *Xenopus* egg extracts. I also report here that while XRCC1 can bind to APE2 in vitro its effect on APE2's endonuclease activity is minimal. Lastly, I have shown that XRCC1 plays an important role in the repair of DNA damage in *Xenopus* egg extracts following oxidative stress. These results will be further discussed in Chapter 4.

CHAPTER 3: INVOLVEMENT OF POL BETA IN GENOME INTEGRITY IN XENOPUS EGG EXTRACTS

3.1 Introduction

There are about fifteen different DNA polymerases encoded in mammalian genomes. These polymerases are fine tuned for replication and repair/tolerance of DNA damage. As a result, deficiencies in this class of proteins or improper regulation of these proteins can lead to genomic instability and cancer. Therefore, there is great value to further elucidate the mechanisms, functions, and substrates for DNA polymerases.

Of interest to this work is DNA polymerase β (Pol β), which is essential to BER and meiotic recombination (148). The BER pathway's major role in cells is to maintain genomic integrity by removing oxidatively damaged DNA. Reactive oxygen species (ROS) are generated as a by-product of normal mitochondria activity. When more ROS are generated that the cell can reasonably deal with the condition is called oxidative stress. Under this cellular condition extensive damage can be generated on DNA as every Watson-Crick base is subject to oxidation. Guanine is the most frequently oxidized nucleotide due to its comparatively low redox potential (201). The major, and most widely recognizable, oxidation product of Guanine is 8-oxoG (a guanine with an oxygen double bonded to the 8^{th} carbon on its ring structure). This aberrant DNA structure alone accounts for ~400-1500 DNA lesions per cell per day (202). Pol β is responsible for two steps in BER pathway: excision of 5'-terminal deoxyribose phosphate (dRP) residue from incised AP site and gap filling (137, 203). Both Pol α and Pol β are required for repairing UV-induced DNA damage including cyclobutane pyrimidine dimers and (6-4)

photoproducts in nuclear extracts from *Xenopus* oocytes (204). As described, *Xenopus* egg extracts provide an ideal platform for understanding how repair and replication processes work in an in vitro system that can be verified in mammalian systems (49, 56, 185). Evidence has shown that Pol β functions in DNA repair during Prophase 1 of meiosis (148, 149). Pol β maintains genomic stability by participating in DNA BER. Thus, somatic and germline deficiencies in Pol β can lead to faulty DNA repair which will result in the accumulation of BER intermediates or inefficient DSB repair, respectively (150). This accumulation could be a cancer driver and impact cancer therapy. In addition, Pol β variants have been described in colon tumors, gastric carcinoma, and prostate cancer.

This goal of this research was to better understand if and how Pol β is utilized in the repair of oxidative stress, as well as in defined SSB repair in the *Xenopus* cell-free egg extract system. I hypothesized that since Pol β was known to be a key participant in the BER pathway, in addition to the fact that it complexes very strongly with XRCC1, that it would in fact have a prominent role in repairing oxidative stress and defined SSB repair. To this end, a vast quantity of *Xenopus* polymerase β was grown in E. Coli and was then used to create anti-Pol β antibodies in rabbits. This antibody was then used for detection and immunodepletion in *Xenopus* LSS and HSS. LSS was treated with hydrogen peroxide to induce oxidative stress and Western blot analysis was performed to determine what effect Pol β had on DDR pathway activation. A defined DNA plasmid structure was added to HSS and the repair of the DNA was evaluated to determine what impact Pol β had on this defined SSB repair. To ensure that removal of Pol β was in fact leading to more DNA damage in a more direct way than measuring protein levels, Comet

Assays were performed in LSS. These COMET assays were imaged using Fluorescence Microscopy and quantified using the COMET IV software.

3.2 Materials and methods

Experimental procedures for Xenopus laevis egg extracts and sperm chromatin

Xenopus laevis care and use was approved by UNC Charlotte's Institutional Animal Care and Use Committee (IACUC). The preparation of *Xenopus* LSS, HSS, and sperm chromatin was described previously (56, 68, 186). Immunodepletion of proteins of interest (i.e., Pol β and Pol α) from LSS and HSS was performed using similar approach as Chapter 2 and previously described (25, 168, 188). Antibodies against Pol β were raised in rabbits against recombinant GST-XRCC1 (Cocalico Biologicals, see below section for the preparation).

For experiments in *Xenopus* egg extracts, hydrogen peroxide (100mM) was added to mock- or Pol β depleted LSS or HSS, which was supplemented with sperm chromatin (4,000/μL). After incubation of different time as indicated at room temperature, 5 μL of reaction mixture was added with 45 μL of sample buffer for immunoblotting analysis. Approaches for DNA-bound protein isolation from LSS system and HSS system have been described in Chapter 2.

Preparation of SSB plasmid

The SSB plasmid with 5'-OH was prepared as described earlier and previously (168). Briefly, the plasmid pS was treated with Nt.BstNBI to generate a site-specific nick

between C435 and T436, followed by CIP treatment to generate 5'-OH. The SSB plasmid with 5'-OH was further purified from agarose gel with QIAquick gel extraction kit.

Analysis of DNA repair products of SSB plasmid in Xenopus HSS system

The SSB plasmid with 5'-OH was added to mock-, Pol β- and Pol α-depleted HSS (final concentration 75 ng/μL). After incubation at room temperature for different times, nuclease-free water was added to each reaction, followed by DNA repair product isolation procedure including phenol-chloroform extractions, as described previously in our recent studies (168). Then the purified DNA repair products were examined via agarose electrophoresis.

Recombinant DNA and proteins

Recombinant pGEX-4T1-Pol β was made by cloning the coding region (nt 245-1249) of *Xenopus laevis* Pol β (GenBank: BC106329, Xenopus Gene Collection IMAGE ID: 7203966) into pGEX-4T1 with EcoRI- and XhoI sites. GST-tagged recombinant proteins were expressed and purified in E. coli DE3/BL21 according to standard protocol. Purified recombinant proteins were confirmed on SDS-PAGE gels with coomassie staining.

Immunoblotting analysis and antibodies

For immunoblotting analysis, detailed procedure has been described in Chapter 2. Anti- Pol β antibodies were raised in rabbits against GST- Pol β (Cocalico Biologicals). Antibodies against ATR were provided by Dr. Karlene Cimprich (49, 189). Antibodies

against ATRIP were provided by Dr. Howard Lindsay (190). Antibodies against RPA32 and Pol α were provided by Dr. Matthew Michael (12). Antibodies against Chk1 phosphorylation at Ser345 were purchased from Cell Signaling Technology. Antibodies against Histone 3 were purchased from Abcam. Antibodies against Chk1 and GST were purchased from Santa Cruz Biotechnology.

COMET Assays

COMET assays under alkaline and neutral conditions have been described in Chapter 2. Images were taken using DP Controller software (Olympus Corporation, JPN) and analyzed using Comet Assay IV Lite software (Instem, UK).

Quantification and statistical analysis

ImageJ was utilized to quantify gels from exonuclease assays. Statistical analysis was performed using GraphPad Prism8. t-test was performed for statistical analysis in DNA repair capacity experiment. Ordinary one-way ANOVA (Tukey's multiple comparisons test) was chosen for statistical analysis on Comet Assays.

3.3 Results

Role of Pol β in ATR-Chk1 DDR pathway in Xenopus egg extracts

Xenopus Pol β contains a DNA binding domain and a Catalytic domain containing finger, palm, and thumb domains found in all DNA polymerases (**Figure 19A**). Clustral Omega analysis of Pol β shows high identity or similarity in the amino

acid sequence in *Xenopus*, human, and mouse (**Figure 19B**). In particular, BLASTP analysis shows that there is 82% identity and 276 out of 335 identical sequences (i.e., 82%) between *Xenopus* (GenBank#: AAI06330), Mouse (GenBank#: NP_035260), and Human (GenBank#: NP_002681) Pol β. Of the 56 positions in the aa sequence that are not identical, 49 of them are similar. These amino acid sequence alignment result suggests that Pol β may have similar or conserved functions during evolution.

In conjunction with our work on XRCC1 I wanted to determine if Pol β, as the primary polymerase for the BER pathway to which XRCC1 complexes with strongly, could also influence ATR-Chk1 signaling when in the presence of oxidative stress. As BER is the first responder and primary repair pathway for oxidative stress-induced DNA damage it stands to reason that the loss of the pathway's primary polymerase would cause problems. The least of which would be an accumulation of unrepaired DNA caused by inefficient BER. I constructed recombinant GST-Pol β, which was expressed in *E. coli* DE3 cells with IPTG induction and purified following vendor's protocol (**Figure 20A**). SDS-PAGE analysis verified the purified recombinant GST-Pol β, which was shown at ~70kDa position on gel, as expected (**Figure 20A**). The purified recombinant GST-Pol β was utilized for custom antibodies production in rabbits from Cocalico Biologicals Inc. Anti-Pol β antibodies were used to immunodeplete endogenous Pol β in LSS and HSS successfully (**Figure 20B-20C**). To test whether Pol β is important for ATR-Chk1 DDR pathway following oxidative stress, I added hydrogen peroxide and sperm chromatin into mock- or Pol β-depleted LSS. After a 45-min incubation, total egg extracts examination via immunoblotting analysis demonstrated that hydrogen peroxide triggered Chk1 phosphorylation in mock-depleted LSS (**Figure 21A**), consistent with previous studies

(25, 188). However, hydrogen peroxide-induced Chk1 phosphorylation was diminished when Pol β was depleted from LSS (**Figure 21A**). Notably, there was a slight increase in the amount of Chk1 phosphorylation without the treatment of hydrogen peroxide but the phosphorylation did not increase at all when hydrogen peroxide was added. Chromatin fraction analysis shows that RPA and TopBP1 recruitment to DNA remains largely unchanged from Mock to depleted extracts (**Figure 21B**). PCNA recruitment itself stays the same (Figure 23A) but shows a reduction in SUMOylation and Ubiquitination modification in the Pol β depleted lanes potentially implying increased DSB formation (205). ATR and ATRIP however, are completely absent from the chromatin in the Pol β depleted extracts (**Figure 21B**).

It has been previously observed that Pol α is necessary for the establishment of ATR-Chk1 activation in response to DNA replication stress in *Xenopus* (206). I hypothesized that perhaps Pol β had a similar function. To address this, I repeated the same experiment in HSS in an effort to determine if the same Chk1 phosphorylation phenotype would be observed in a replication free system. Chk1 phosphorylation was observable under oxidative stress in the mock depleted extracts when hydrogen peroxide was added but there was a complete lack of phosphorylation in the Pol β depleted extract (**Figure 22**). Even the slight increase in the Pol β depleted non-peroxide treated extract was absent. Based on these results I tested to see if ATR and ATRIP were being co-depleted with anti-Pol β antibodies during the depletion process. The same 45-min time point experiment with hydrogen peroxide was used, as described above, in LSS to assess whether co-depletion was in fact occurring. When Pol β is depleted in LSS, ATR is mildly reduced and ATRIP is almost completely co-depleted (**Figure 21B**). These

observations imply that while depleting Pol β in *Xenopus* LSS can mildly increase Chk1 phosphorylation in unstressed conditions it co-depletes proteins vital for the increased phosphorylation observed when introduced to oxidative stress. Overall, Pol β depletion with our antibody leads to depletion of proteins necessary for the very signaling pathway being studied. This unfortunately also rules out the possibility of accomplishing a reasonable rescue experiment to restore the Mock depleted phenotype. Overall, it is difficult to distinguish the reduction of oxidative stress-induced Chk1 phosphorylation when Pol β was removed between the absence of Pol β or co-depletion of its interaction protein ATRIP.

Role of Pol β in repairing the defined SSB plasmid in Xenopus egg extracts

We recently demonstrated that a defined SSB plasmid with a 5'-OH can be repaired in the *Xenopus* HSS system and that SSB-induced ATR activation is required for SSB repair (168). To test whether Pol β is required for repairing the defined SSB plasmid, I added SSB plasmid with a 5'-OH in mock- or Pol β-depleted HSS. After different timepoints, DNA repair products were isolated and examined via agarose gel electrophoresis. As shown in **Figure 23A**, repair capacity of the SSB plasmid with a 5'-OH in Pol β-depleted HSS was not visibly different than the repair capacity in mock-depleted HSS. Quantification of DNA repair capacity at 5 min and 30 min from three independent experiments show no significance between the mock- and Pol β-depletion HSS (**Figure 23B**). This observation suggests that Pol β is dispensable for repairing the defined SSB plasmid with a 5'-OH in the *Xenopus* HSS system.

This finding led us to ask; which DNA polymerase was repairing the SSB plasmid if it depleting Pol β had no effect on the repair capacity? To this end I again added SSB plasmid with a 5'-OH to *Xenopus* HSS with the addition of either DMSO or Aphidicolin (Apx). Apx is a chemical that inhibits DNA replication via inhibiting DNA Polymerase α, δ, and ε. As shown in **Figure 24**, repair capacity of the SSB plasmid was negatively affected when Apx was added compared to the DMSO control. This observation suggests that one of the three polymerases Apx targets or some combination of them in tandem are required for efficient repair of the defined SSB in HSS.

Next, I wanted to see if I could narrow down which of the polymerases Apx inhibits is responsible for the decreased repair capacity. Our lab has an antibody for *Xenopus* Pol α and it has been shown to inhibit Chk1 phosphorylation in LSS when depleted from the extract (**Figure 25C**). Mock and Pol α depleted extracts were prepared with the addition of SSB plasmid with 5'-OH. At various timepoints the DNA repair products were isolated, purified, and examined via electrophoresis. Depleting Pol α from HSS was sufficient to visibly inhibit the repair capacity of the SSB plasmid (**Figure 25A-25B**). This data suggests that Pol α may be involved in the repair of the SSB plasmid with 5'-OH in *Xenopus* HSS.

Role of Pol β in the repair of oxidative DNA damage in Xenopus egg extracts

Our observations suggest that Pol β is dispensable for the repair of the defined SSB plasmid in HSS, instead utilizing Pol α for this repair activity. Next, I sought to determine whether Pol β is important for DNA damage repair following oxidative stress using COMET assays under alkaline and neutral conditions (200). This would be a direct

way to measure DNA damage than simply looking at Chk1 phosphorylation. Comet assays were preformed using LSS either mock or Pol β depleted at a 30-minute time point. Using COMET assays under alkaline condition, I found that the Tail Moment was not enhanced when Pol β was removed regardless of the addition of hydrogen peroxide in the LSS system, suggesting that a similar SSBs and AP sites are generated following oxidative stress with the presence and absence of Pol β (**Figures 26A-26B**). This result suggests that Pol β can be replaced when necessary for the repair of AP sites and SSBs following oxidative stress.

Using COMET assays under neutral condition, I found that the Pol β depletion again did not modify the Tail Moment in the presence of hydrogen peroxide in the LSS system (**Figures 27A-27B**), suggesting that the damage induced by the oxidative stress is not progressing to DSB more than would be expected when Pol β has been depleted. Overall, our evidence suggests that Pol β depletion may be compensated by another DNA polymerase in the repair of oxidative stress-derived DNA damage in *Xenopus* egg extracts.

3.4 Conclusion

The aim of this Chapter was to discern what role, if any, Pol β plays in the maintenance of genomic integrity. Pol β was shown to have a dampening effect on DDR signaling in *Xenopu*s egg extracts following oxidative stress. This however may be due to a co-depletion effect and not an effect of Pol β itself. In addition, I discovered that Pol β is dispensable for the repair of the defined SSB plasmid in HSS. Instead I found evidence that Pol α may be involved in the repair of this defined DNA structure. An analysis of the

total DNA damage in nuclei following oxidative damage showed that when Pol β is depleted there is no significant increase in the damage load on the DNA. These findings imply that when Pol β's function is compromised the *Xenopus* egg extracts can compensate for it and carry on seemingly unhindered. The results will be further discussed in Chapter 4.

CHAPTER 4: SUMMARY AND DISCUSSION FOR FUTURE STUDIES

4.1 XRCC1 and DDR pathway activation

I have shown evidence that XRCC1 is dispensable for the ATR-Chk1 DDR pathway activation in response to hydrogen peroxide in *Xenopus* LSS system (**Figure 9**). Consistent with this, my lab recently reported that XRCC1 is not required for defined SSB-induced ATR-Chk1 DDR pathway in *Xenopus* HSS system (168). These finding of the independence of XRCC1 for ATR-Chk1 DDR activation in *Xenopus* system are consistent with several prior studies using mammalian cells or cell lines. The lack of the BER protein expression including XRCC1, PARP1, and Ligase III α in human monocytes results in more SSBs and DSBs accumulation following oxidative stress (207). Notably, the ATR-Chk1 and ATM-Chk2 DDR pathways still can be activated upon treatment with tert-butyl hydroperoxide in XRCC1-deficient monocytes [58]. Furthermore, XRCC1 is dispensable for the MMS-induced ATR-Chk1 DDR pathway activation in human breast cancer cell line MDA-MB-549 (208). In addition, ATR inhibition is synthetically lethal in XRCC1 deficient cells with increased cytotoxicity and accumulation of DSBs (209). All these studies support the notion that XRCC1 is dispensable for ATR DDR pathway activation.

What are the potential roles of DDR pathway activation for XRCC1 functions? ATM-Chk2 DDR pathway may promote BER pathway via Chk2-dependent XRCC1 phosphorylation on Thr284 residue, suggesting that DDR pathway activation is earlier event than BER pathway in response to oxidative stress (210). Furthermore, activated ATM can phosphorylate transcription factor Sp1 to downregulate XRCC1 expression for

cell elimination (211). It remains unknown whether ATR DDR pathway directly regulates XRCC1 functions.

4.2 Distinct functions of XRCC1 and APE2

It is widely accepted that XRCC1 function as a scaffolding protein to interact with many repair proteins, such as APE1, NEIL1, NEIL2, OGG1, UNG1, PCNA, NTH1, Pol β, PARP1, PNKP, and Ligase 3α (212). For example, XRCC1 physically interacts with APE1 and stimulates its enzymatic activity and such XRCC1-APE1 interaction is essential for repairing DNA AP site in Chinese ovary cell lines (213). My results suggest that XRCC1 interacts with APE2 directly (**Figure 16A**). However, the role of XRCC1 interaction for APE2 exonuclease activity is very minimal (**Figure 16C**). I also sought to characterize the regions of XRCC1 and APE2 that were responsible for their binding. Our findings show that BRCT1 and to a slightly larger extent the BRCT2 domain both bind to APE2 in vitro (**Figure 16B**). Binding of these regions is much weaker than full length XRCC1 implying that those domains on their own are not sufficient to reconstitute the binding between XRCC1 and APE2. Further research must also be done to determine what region of APE2 and XRCC1 are responsible for their interaction. XRCC1 has been shown to interact with APE1 in the region 141-572aa (213). This encompasses a relatively large region of XRCC1 encoding BRCT1 part of BRCT2 and both Linker regions. An educated next step toward determining which region of XRCC1 is responsible for APE2 binding would be to probe a similar region as the one involved in APE1 binding.

APE2, but not XRCC1, is required for ATR-Chk1 DDR pathway following oxidative stress in *Xenopus* LSS system (**Figure 7**) (25, 188). Furthermore, APE2, but not XRCC1, is required for defined SSB-induced ATR-Chk1 DDR pathway in *Xenopus* HSS system (**Figure 9**) (168). In addition, APE2, but not XRCC1, is required for the repair of defined SSB plasmid in *Xenopus* HSS system (**Figures 10-15**). Whereas XRCC1 interacts with APE2 directly, these observations clearly indicate different requirements of APE2 and XRCC1 for SSB repair and ATR-Chk1 DDR pathways in maintaining genome integrity.

4.3 Role of XRCC1 in the repair of different types of DNA damage

XRCC1 has been implicated in several different types of DNA repair pathways including BER, NER, SSB repair, NHEJ, and MMEJ (169, 212). To the best of our knowledge, it is the first time to show that XRCC1 is not required for the repair of defined SSB plasmids with simple termini such as 5'-OH or 5'-P (Figure 9-12). Many prior studies on the role of XRCC1 in SSB repair primarily measure DNA repair of SSBs indirectly generated from stressful conditions, such as gamma-irradiation and alkylation agent methyl methanesulfonate (MMS) (114, 208). It has been shown that XRCC1 stimulates PNKP activity to promote SSB repair using in vitro reconstitution system with recombinant proteins and defined SSB structures (173). The different experimental systems (i.e., reconstitution system with purified proteins vs. Xenopus *HSS* system) and two different SSB structures may explain this discrepancy to our result. In addition, XRCC1 interacts with Pol β and Ligase III to serve as a scaffolding protein in a reconstituted BER system (112). Notably, in vitro biochemical analysis indicates that

XRCC1 is dispensable for BER activity of 8-OH-dG, 5-hydroxycytosine, ethanoadenine, and uracil lesions, and that XRCC1 is important for the ligation step of BER and SSB repair (214).

Oxidative stress can induce several different types of DNA damage, including but not limited to, base damage, SSBs, DSBs, and AP sites (1). My result does not exclude the potential role of XRCC1 for the repair of DSBs and SSBs with complex termini that may be generated in oxidative stress. Consistent with this, evidence from the COMET assays demonstrates that XRCC1 is important for the repair of oxidative stress-derived DNA damage, such as DSBs, AP sites, and SSBs with complexed termini (**Figures 17-18**). Future studies are needed to directly determine the exact roles of XRCC1 for repairing these different types of oxidative stress-derived DNA damage.

Overall, the evidence presented demonstrates that, in contrast to APE2, XRCC1 is dispensable for oxidative stress-induced ATR-Chk1 DDR pathway in *Xenopus* system. XRCC1 may play important roles for repairing oxidative stress-derived DSBs and AP sites, but not SSBs. Targeting XRCC1 deficiency in breast cancer has been proposed for personalized therapy (215). Therefore, a better understanding of the roles and mechanisms of XRCC1 in genome integrity will provide insight into how design novel avenues to cancer therapies.

4.4 DNA polymerase for SSB repair in *Xenopus* egg extracts

I have shown that, when using our Pol β antibody to remove Pol β from LSS and HSS it would appear at first glance that Pol β is indispensable for ATR-Chk1 activation in response to oxidative stress (**Figure 21**). Unexpectedly, the Pol β antibody also

depletes ATRIP and to a lesser extent ATR which themselves are vital for chk1 phosphorylation (**Figure 21B**). It is due to this co-depletion of ATR and ATRIP, not Pol β itself, that appears to be causing the decrease in checkpoint phosphorylation. In trying to determine if there was any known reason why a polymerase antibody might also be targeting ATR or ATRIP I theorized that Pol β might have a similar action as pol α, which is an important component of the ATR-Chk1 signaling cascade (206, 216). Unlike Pol α, Pol β does not have primase activity, which is theorized to be the mechanism by which ATR is recruited to damaged DNA by the polymerase (64). Attempts at pulling down or immunoprecipitating ATR or ATRIP using recombinant Pol β or our Pol β antibody proved fruitless. In addition, analysis of Pol β protein sequence did not show any known binding motif that would imply that Pol β can bind to ATR. Taken together these results do not elucidate a direct role for Pol β in DDR pathway activation. However, due to the complications I have listed it would be premature to imply that no such role exists.

The interaction between Pol β and XRCC1 has been widely studied and one would not be faulted for thinking that it is through this interaction that Pol β is transported into the nucleus, as Pol β lacks a traditional Nuclear Localization Signal (NLS) in its sequence. Since Pol β is a very small protein it has long been assumed that this size conferred to the protein an ability to circumvent active nuclear import. While Pol β lacks a traditional NLS, recent sequence and structural analysis suggests that a monopartite nuclear localization signal may reside in the N-terminal Lyase domain. To this end binding of this domain to Importin α1 was shown and uptake of Pol β to mouse embryonic fibroblasts nuclei in absence if binding partners was observed (217). Since Pol

β shows the ability to be transported the nucleus independent of XRCC1 its role in checkpoint signaling beyond its association with XRCC1 cannot be ruled out. Pol β has also been shown to promote the recruitment of the XRCC1-LigaseIII heterodimer to sites of BER (218). This finding illustrates that while XRCC1 might be a "strong" binding partner for Pol β and their association together may be crucial for Pol β's function as the primary BER polymerase, these observations do not rule out the possibility that Pol β's presence alone or interactions therein could be sufficient for now uncharacterized functions in related DNA damage pathways. Thus, the earlier finding that XRCC1 is dispensable for ATR-Chk1 signaling does not necessitate that Pol β is similarly dispensable.

My lab recently reported that XRCC1 is not required for defined SSB-induced ATR-Chk1 DDR pathway in *Xenopus* HSS system (168). I then wanted to test if Pol β was involved in repair of the defined SSB plasmid in HSS. HSS is a replication free nuclear system so it is ideal for testing replication independent repair of DNA. As with XRCC1, Pol β was found to not be required to repair the defined SSB plasmid in HSS (**Figure 23**). Pol β has been implicated in BER and meiotic DSB repair (150), so this finding in combination with the result that XRCC1 is similarly not required leads me to the conclusion that the conventional BER Pathway is not active in removing this lesion from the DNA.

Instead I have shown evidence that Pol α is required for the defined SSB repair in HSS. Apx was sufficient to inhibit the repair capacity of the HSS when the defined SSB was added, implying that a replicative DNA polymerase is involved in the reduction. This was further narrowed by the result that when Pol α is depleted from *Xenopus* HSS extract

defined SSB repair was visibly reduced (**Figure 25**). In isolation it is very strange to think that a replicative polymerase known for synthesizing a 10 nucleotide RNA primer followed by a 30 to 40 nucleotide stretch of DNA would be involved in the repair of an SSB nick in the DNA backbone. My lab recently reported, however, that APE2 is essential for the repair if the defined SSB plasmid in HSS. APE2 is also necessary for checkpoint activation in HSS and is required for ATR-Chk1 checkpoint activation in response to oxidative stress (25, 168). Based on these findings it is reasonable to conclude that the DNA damage response pathway is being activated in response to the defined SSB plasmid. Under this line of logic, it begins to make sense why Pol α is involved in this repair. After APE2 resects the SSB, RPA molecules coat the exposed Single Stranded DNA. Then ATR-ATRIP are recruited to the RPA coated DNA along with TopBP1 and the 9-1-1 complex. These factors together are sufficient to propagate the DDR. It has been shown that Pol α is recruited to the RPA coated ssDNA by TopBP1 and that this interaction allows an RNA primer (synthesized by Pol α's primase) to be created which serves as a platform for 5' 9-1-1 loading onto the DNA (12). It was similarly discovered that Pol α's primase activity is required to activate the ATR-Chk1 pathway (51).

Based on this information and the results of my own findings, the evidence suggests that in the repair of a defined SSB plasmid in a replication independent system the DDR pathway is being activated to repair the nick. In order to activate that DDR pathway and efficiently repair Pol α is recruited and necessary to initiate the activation of the pathway and repair of the damage.

In a further effort to take advantage of my Pol β antibody I needed to be sure that there was still damage occurring when Pol β was being depleted. I found that there was no significant increase in DNA damage in either the Alkaline or Neutral Comet Assays (**Figures 26-27**). This is in opposition to my experiments involving XRCC1 where, when XRCC1 is depleted there is an increase in the amount of DNA damage.

In *Xenopus* LSS a loss of Pol β does not appear to be the arbiter of increased DNA damage. When the 5' end of damaged DNA is resistant to lyase activity of Pol β there is a switch from short patch BER to long Patch BER (219). This causes polymerase involved in the repair process to switch from Pol β to the replicative DNA polymerases δ and ε (220). When Pol β is absent it is possible that the repair machinery treats the lesion as if it cannot be processed by Pol β and moves to fix the damage via the Long Patch repair pathway. As the XRCC1 scaffold is still in place when Pol β is depleted in the LSS the pathway itself may not be disrupted to the point of being nonfunctional. Instead the damage may be processed in much the same way making a concession for the lack of the normal polymerase in the pathway. Another possibility is that, with ATR and ATRIP gone, instead of DDR pathway activation in response to oxidative stress translesion synthesis takes over. Translesion synthesis prevents genomic instability caused by replication stress at nucleotide lesions (221). This allows the DNA to be repaired quickly at the sacrifice of fidelity so that the cell does not have to face catastrophic mass replication fork collapse. Interestingly activation of TLS has been shown to inhibit ATR-dependent DDR and in mammalian cells deficient in TLS unchecked DDR signaling causes irreversible cell cycle arrest in G_2 (221, 222), implying that while one pathway is activated the other is suppressed. With TLS activated and repairing oxidatively stressed

replicating nuclei it is possible that DNA damage itself would not see an increase but rather the fidelity of the repair would be decreased.

As discussed earlier disruption of the Pol β gene is embryonically lethal in mice (223). It is not beyond the pale to assume that, based on this knowledge, if my Pol β antibody did not co-deplete ATR and ATRIP an increase in DNA damage might be observed. However, the *Xenopus* LSS system that I used for these experiments only undergoes one round of replication. It cannot be overlooked that part of why Pol β's loss is so detrimental may in fact be due to a build-up of damage that gets worse as time goes on, from processive rounds of replication. More research on Pol β must be undertaken to provide a clearer picture of what previously uncharacterized roles this protein may play in genome stability and how exactly its loss/mutation affect the pathways in which it participates.

Taken together, in my PhD thesis I have investigated the role of XRCC1 and Pol β in genome integrity in *Xenopus* egg extracts (**Figure 28**). In particular, my findings provide a better understanding of how XRCC1 and Pol β play distinct roles in the DNA repair and DDR pathways in eukaryotic systems.

FIGURES AND LEGENDS

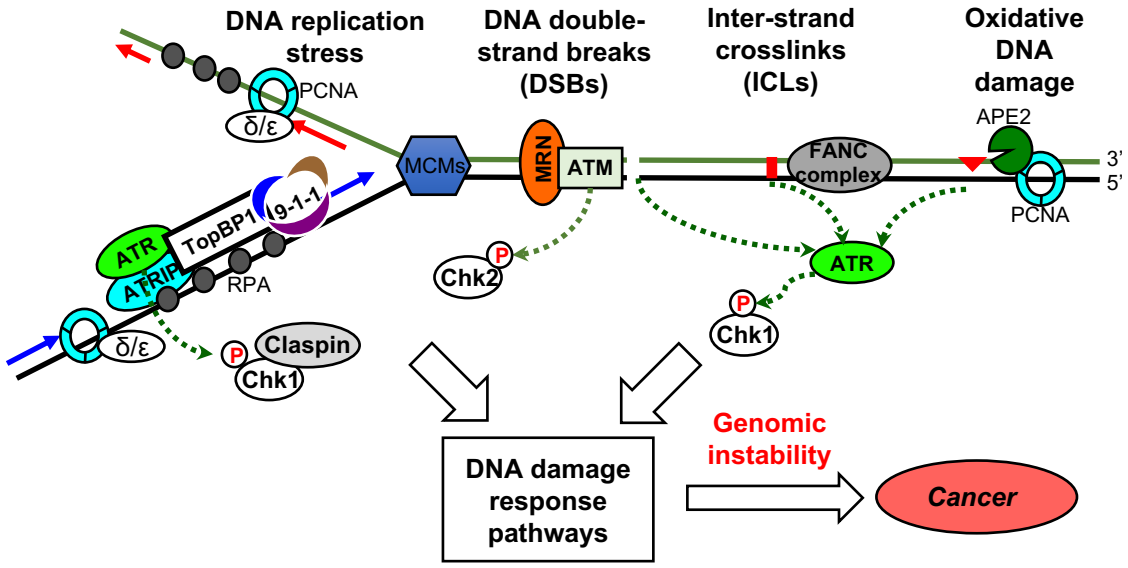


Figure 1. Schematic diagram of DNA damage response (DDR) pathways in response to DNA replication stress, DNA double-strand breaks, inter-strand crosslinks, and oxidative DNA damage. The black and orange lines represent two strands of DNA that is unwounded by DNA helicase during DNA replication. The blue and red lines with arrows represent newly synthesized DNA. Individual proteins are designated as ATR, ATRIP (ATR-interaction protein), TopBP1, 9-1-1 (Rad9-Rad1-Hus1 complex), δ/ε (DNA Polymerase δ or Polymerase ε), MCMs (minichromosome maintenance complexes), MRN (Mre11-Rad50/Nbs1 complex), FANC complex, APE2, PCNA, Chk1 (Checkpoint kinase 1), Chk2 (Checkpoint kinase 2), and Claspin. The circled "P" in red indicates phosphorylation event. See text for details.

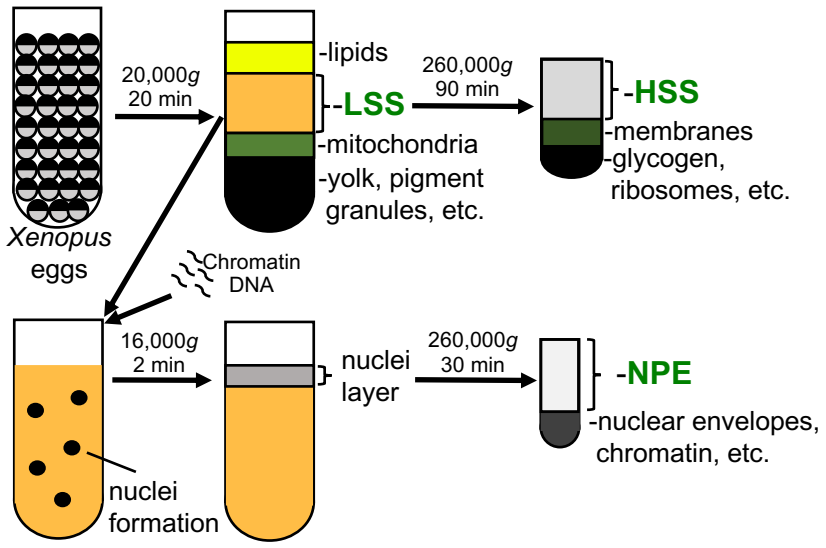


Figure 2. Diagram of how LSS, HSS, and NPE are prepared from *Xenopus* eggs. LSS, Low-speed supernatant; HSS, high-speed supernatant; NPE, nucleoplasmic extracts. After PMSG & HCG stimulation, *Xenopus* eggs are collected, processed, and centrifuged at 20,000 *g* to prepare the LSS fraction, while the top lipids layer and bottom mitochondria and yolk as well as pigment granules are discarded. The LSS can be further centrifuged with a speed of 260,000 *g* to separate the HSS from membrane fractions and glycogen as well as ribosomes. Sperm chromatin DNA can be added to the LSS, which form nuclear envelop. The nuclei formed from LSS are centrifuged and collected from the top layer, as indicated. The nuclei fraction will be spun again with a speed of 260,000 *g* to separate to distinguish the NPE fraction from nuclear envelopes and chromatin. Details of how LSS, HSS, and NPE are prepared can be found from previously studies (56, 68).

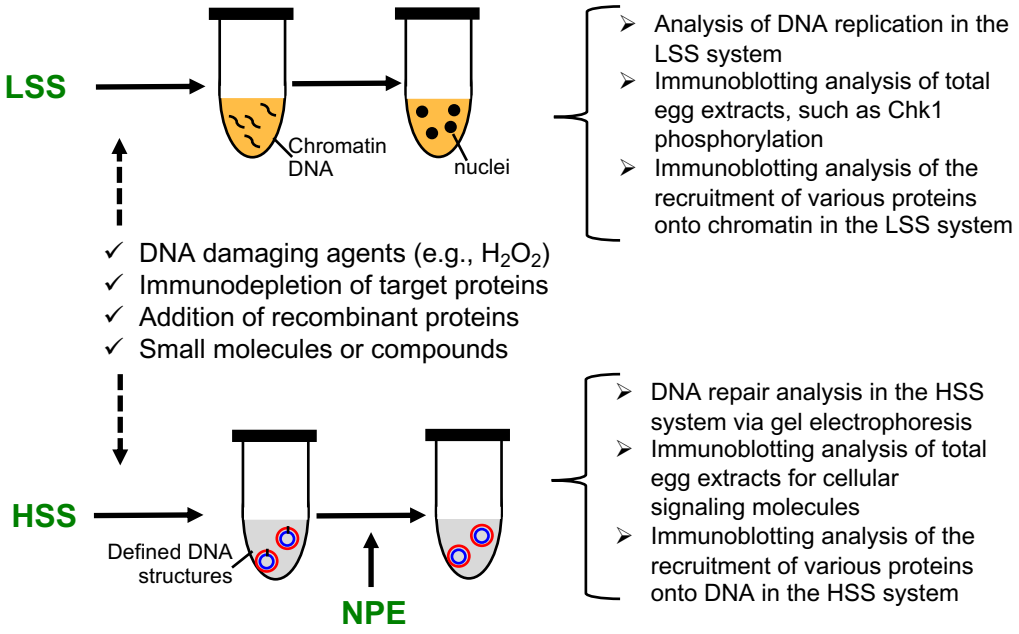


Figure 3. *Xenopus* LSS or HSS/NPE system is utilized to study DDR pathways. LSS, Low-speed supernatant; HSS, high-speed supernatant; NPE, nucleoplasmic extracts. Two approaches are utilized in Xenopus system: (I) Chromatin DNA can be added to the LSS, in which chromatin is surrounded with nuclear envelope into nuclei and chromatin DNA can be replicated. DNA damaging agents such as hydrogen peroxide can be added to damage chromatin DNA. (II) Plasmid DNA with defined DNA damage such as a single ICL at a defined location can be added to the HSS, which is subsequently supplemented with the NPE. In this HSS/NPE system, defined DNA structures can be replicated and repaired. In both approaches, customized antibody-based immunodepletion can remove target proteins from the LSS or HSS, which can be added with wild type or mutant recombinant proteins. In addition, small molecules or compounds can be easily added to the LSS or HSS/NPE systems to perform dose-dependent assays. See text for details.

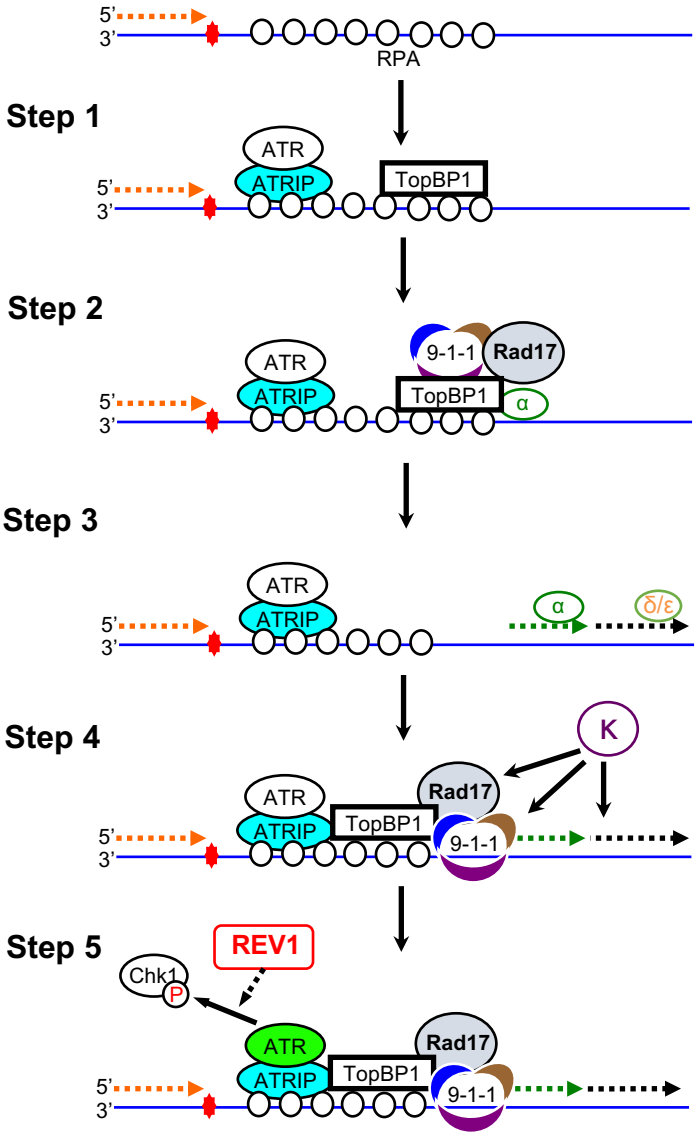
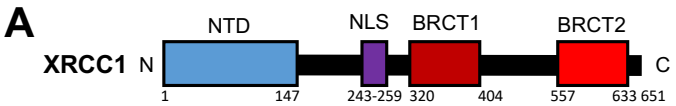


Figure 4. A model for the role of TLS polymerases Pol κ and REV1 in the ATR-Chk1 DDR pathway. Pol κ has three mechanisms: primer synthesis, 9-1-1 recruitment, and Rad17 stabilization. REV1 is dispensable for the recruitment of ATR, ATRIP, TopBP1, 9-1-1, and RPA onto stalled forks, but is important for Chk1 phosphorylation by activated ATR. Please see the text for details.



B

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HS XRCC1 MPEIRLRHVVSCSSQDSTHCAENLLKADTYRKWRAAKAGEKTISVVLQLEKEEQIHSVDI 60
MM XRCC1 MPEISLRHVVSCSSQDSTHCAENLLKADTYRKWRAAKAGEKTISVVLQLEKEEQIHSVDI 60
         ** * *:******* *:** *************:**: *** **::**:********:**

XL XRCC1 GNEGSAFVEVLVGRSTS---VSEQEYEVLLGMSSFMSPSESKNESSLNRTRMFGPDKLVK 117
HS XRCC1 GNDGSAFVEVLVGSSAGG--AGEQDYEVLLVTSSFMSPSESRSGSNPNRVRMFGPDKLVR 118
MM XRCC1 GNDGSAFVEVLVGSSAGGATAGEQDYEVLLVTSSFMSPSESRSGSNPNRVRIFGPDKLVR 120
         **:********** *:.   ..**:*****  *********:. *. **.*:*******:

XL XRCC1 GAAEKNWDRVKIVCTQPYTKNLAYGLSFIRLNSPPEDGSPTDPSP--KLTKLGQFMVKEE 175
HS XRCC1 AAAEKRWDRVKIVCSQPYSKDSPFGLSFVRFHSPPDKDEAEAPSQKVTVTKLGQFRVKEE 178
MM XRCC1 AAAEKRWDRVKIVCSQPYSKDSPYGLSFVKFHSPPDKDEAEATSQKVTVTKLGQFRVKEE 180
         .****.********:***:*:  :****::::***:...    *   .:****** ****

XL XRCC1 ENSSPSMLPGSLFFNRTSKPQVTPPKTPPATQSYAAAALQGTAESSSSTEKQTVKTPPSN 235
HS XRCC1 DESANSLRPGALFFSRINKTSPVT-ASDPAGPSYAAATLQASSAASSASPVS--RAIGST 235
MM XRCC1 DDSANSLKPGALFFSRINKTSSAS-TSDPAGPSYAAATLQASSAASSASPVP--KVVGSS 237
         ::*: *: **:***.* .* . .   : **  *****:**.:: :**::     :.  *.

XL XRCC1 NMTKEPSSGKRKFEFNKEPSSHSTVKKPEVKESPSSTKESLSQPVPKKPKVETHTVAPTK 295
HS XRCC1 SKPQESPKGKRKLDLNQEEKK-----------TPSKPPAQLSPSVPKRPKLPAPTRTPAT 284
MM XRCC1 SKPQEPPKGKRKLDLSLEDRK-----------PPSKPSA--GPSTLKRPKLSVPSRTPAA 284
         .  :*  .****:::. *  .            **.     .  . *:**: . : :*:

XL XRCC1 KPSPSEK-------PT-Q--KKSPSAPQSMELGRILQGTVFVLSGFQNPFRADLRDKALE 345
HS XRCC1 APVPARAQGAVTGKPRGEGTEPRRPRAGPEELGKILQGVVVVLSGFQNPFRSELRDKALE 344
MM XRCC1 APASTPAQRAVPGKPRGEGTEPRGARTGPQELGKILQGVVVVLSGFQNPFRSELRDKALE 344
          *  :         *  :  :         ***:****.*.**********::*******

XL XRCC1 MGAKYRPDWTPDSTHLICAFANTPKFSQVKAAGGIIVRKEWVLDCYKKRQRLPYKQYLLG 405
HS XRCC1 LGAKYRPDWTRDSTHLICAFANTPKYSQVLGLGGRIVRKEWVLDCHRMRRRLPSQRYLMA 404
MM XRCC1 LGAKYRPDWTPDSTHLICAFANTPKYSQVLGLGGRIVRKEWVLDCHHMRRRLPSRRYLMA 404
         :********* ***************:*** . ** **********:: *:*** ::**:.

XL XRCC1 AAESSSEEEDDSD----EDEPPKAPLHKPHPDPSRTNHKKPLP--SPAKAKEKRAP--IK 457
HS XRCC1 GPGSSSEEDEASHSGGSGDEAPKLPQKQPQTKTKPTQAAGPSSPQKPPTPEETKAASPVL 464
MM XRCC1 GLGSSSEDEGDSHSESGEDEAPKLPQKRPQPKAK-TQAAGPSSPPRPPTPKETKAPSPGP 463
         .  ****:: *.      ** ** * ::*: . . *:   *     * . :*.:*

XL XRCC1 SEDEDTEDENPRLVPSKANHAIKQEDEYDASTDEETTGDRQRHQDDSGEDTEDELRRFQE 517
HS XRCC1 QEDIDIE-------------------------GV-QSEGQDNGAEDSGDTEDELRRVAE 497
MM XRCC1 QDNSDTE-------------------------GE-ESEGRDNGAEDSGDTEDELRRVAK 496
         .:: * *                          .: : :  :.. ********. :

XL XRCC1 EKQ-RKKAAVKVEPEDPYAGSTDENTDVEEQ-KELDLPIPELPDLFLGKKFFLYGEFPAA 575
HS XRCC1 QKEHRLPPGQEENGEDPYAGSTDENTDSEEHQEPPDLPVPELPDFFQGKHFFLYGEFPGD 557
MM XRCC1 QREQRQPPAPEENGEDPYAGSTDENTDSETP-SEADLPIPELPDFFEGKHFFLYGEFPGD 555
         ::: *   . : : ************* *   .  ***:*****:* **:********.

XL XRCC1 ERRMLLRYIIAFNGELEEYMNEKVQFVITAQEWDDSFEDALNENENVSFVRPRWIYNCND 635
HS XRCC1 ERRKLIRYVTAFNGELEDYMSDRVQFVITAQEWDPSFEEALMDNPSLAFVRPRWIYSCNE 617
MM XRCC1 ERRRLIRYVTAFNGELEDYMNERVQFVITAQEWDPNFEEALMENPSLAFVRPRWIYSCNE 615
         *** *:**: *******:**.::*********** .**:** :* .::********.**:

XL XRCC1 RQKCIPHQPYVVVPKA 651
HS XRCC1 KQKLLPHQLYGVVPQA 633
MM XRCC1 KQKLLPHQLYGVVPQA 631
         :** :*** * ***:*
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Figure 5. Schematic diagram and amino acid sequence analysis of XRCC1. (A) Schematic diagram of *Xenopus laevis* XRCC1. (B) Amino acid sequence alignment of XRCC1 using the Clustal Omega software. Abbreviations: XL XRCC1 (*Xenopus laevis* XRCC1), AAH45032.1; HS XRCC1 (*Homo sapiens* XRCC1), NP_006288.2; MM XRCC1 (*Mus musculus* XRCC1), NP_033558.3. "*" indicates identical residues; "-" represents gaps in the alignment; ":" indicates highly conserved residues; "." represents moderately conserved residues.

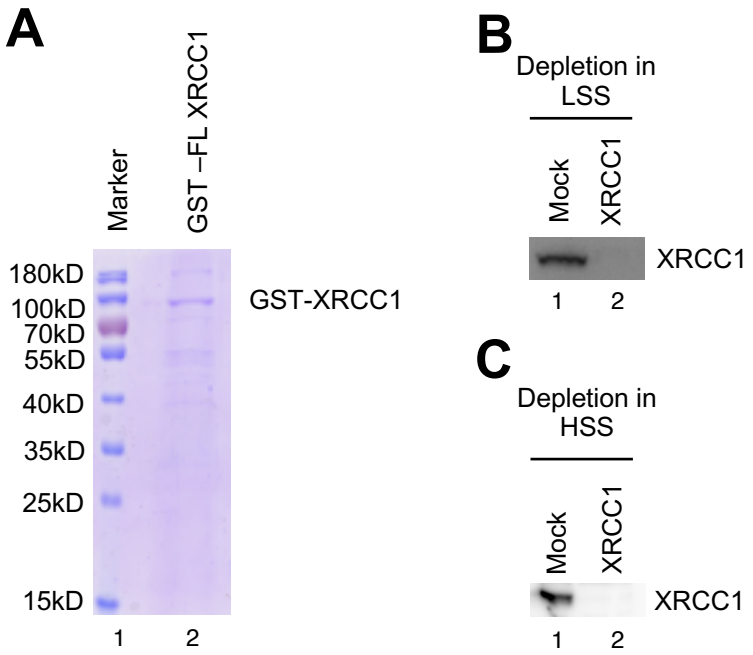


Figure 6. Validation of purified recombinant GST-XRCC1 proteins and immunodepletion efficiency of XRCC1 in *Xenopus* LSS and HSS system. (A) Verification of purified GST-XRCC1 (1 μg) on SDS-PAGE gel. (B) Mock- or XRCC1-depleted LSS was examined via immunoblotting analysis. (C) Mock- or XRCC1-depleted HSS was examined via immunoblotting analysis.

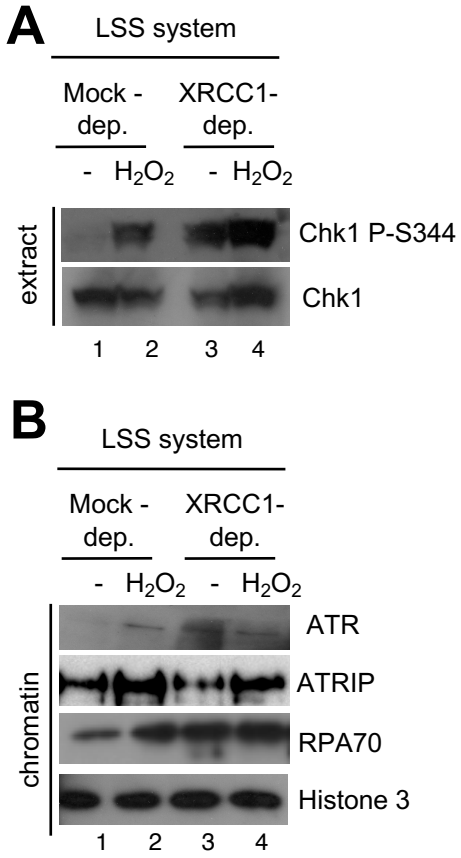


Figure 7. XRCC1 is not required for ATR-Chk1 DNA damage response pathway in Xenopus LSS system. (A) Hydrogen peroxide was added to mock- or XRCC-depleted LSS, which was supplemented with sperm chromatin and incubated for 45 minutes. Extracts were examined via immunoblotting analysis for Chk1 phosphorylation (i.e., Chk1 P-Ser344) and total Chk1. (B) Chromatin fractions from Experiments in Panel (A) were isolated and examined via immunoblotting as indicated. Histone 3 serves as loading control.

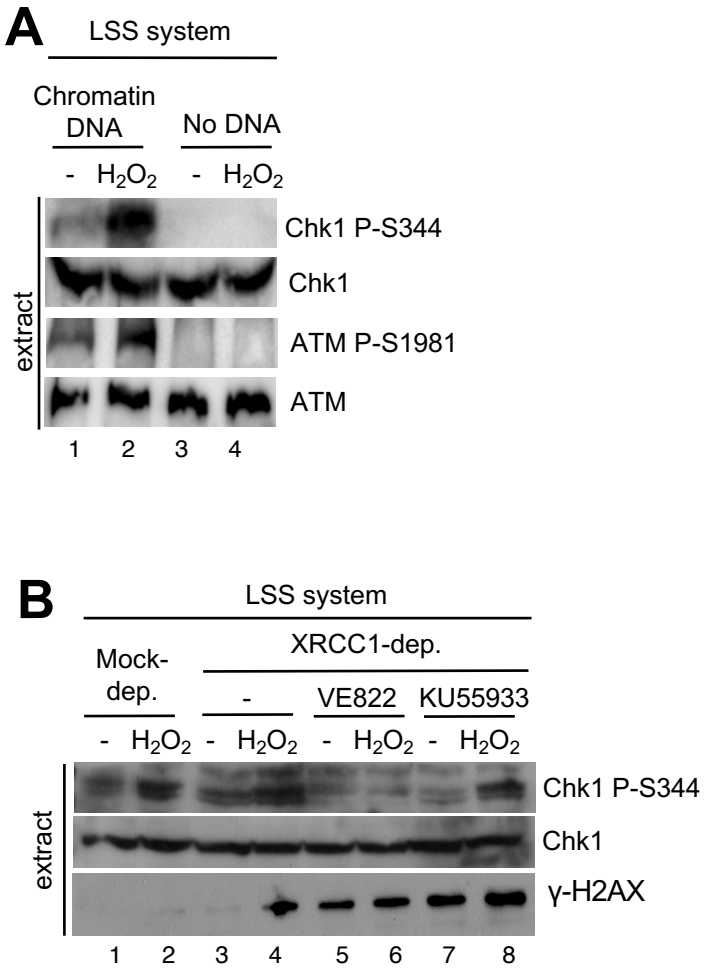


Figure 8. Role of ATM and ATR in Chk1 phosphorylation in oxidative stress in *Xenopus* LSS system. (A) Chk1 phosphorylation and ATM phosphorylation in oxidative stress with the presence or absence of DNA in Xenopus LSS system. (B) Mock- or XRCC1-depleted LSS was examined via immunoblotting analysis. (B) ATR inhibitor VE-822 (final concentration 10 μM) or ATM inhibitor KU55933 (final concentration 100 μM) was added to XRCC1-depleted LSS, then supplemented with hydrogen peroxide and sperm chromatin. After a 45-minute incubation, total egg extracts were examined via immunoblotting as indicated.

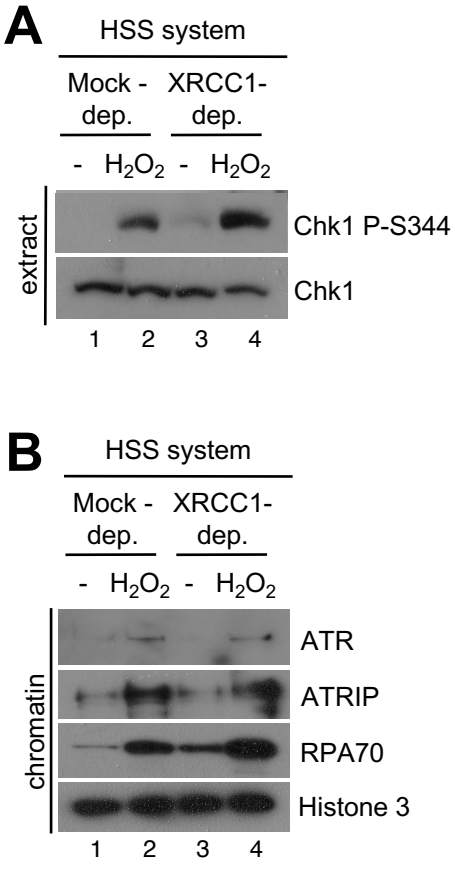


Figure 9. XRCC1 is not required for ATR-Chk1 DNA damage response pathway in Xenopus HSS system. (A) Hydrogen peroxide was added to mock- or XRCC-depleted HSS, which was supplemented with sperm chromatin and incubated for 45 minutes. Extracts were examined via immunoblotting analysis for Chk1 phosphorylation (i.e., Chk1 P-Ser344) and total Chk1. (B) Chromatin fractions from Experiments in Panel (A) were isolated and examined via immunoblotting as indicated. Histone 3 serves as loading control.

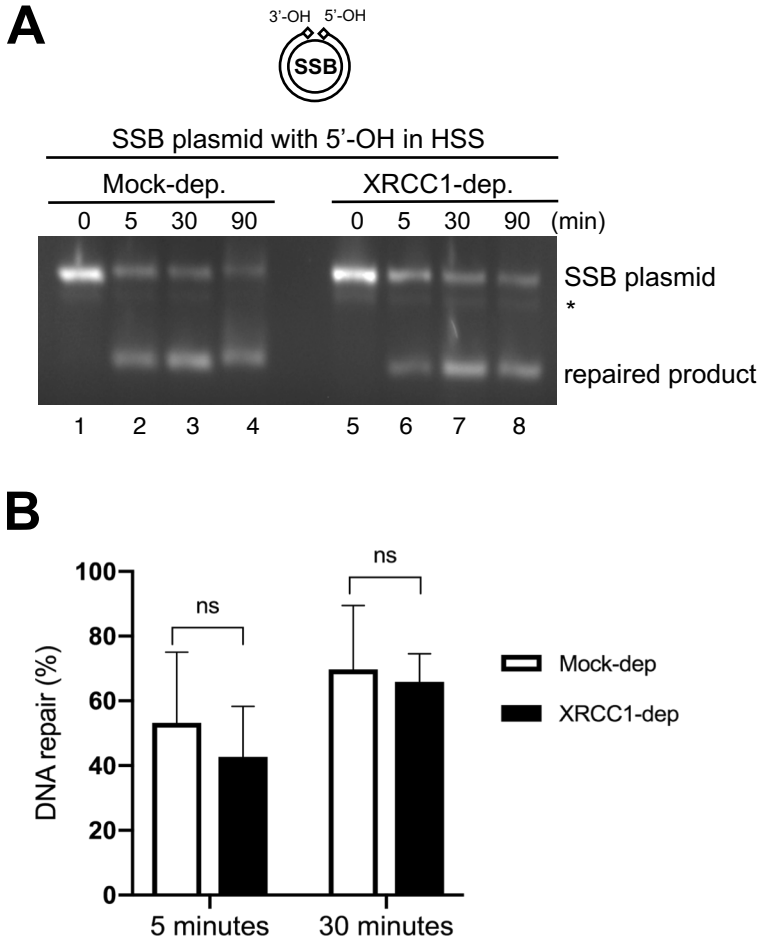


Figure 10. XRCC1 is dispensable for the repair of site-specific SSB plasmid with 5'-OH in *Xenopus* HSS system. (A) SSB plasmid with 5'-OH was incubated in mock- or XRCC1-depleted HSS. After different timepoints (0, 5, 30, 90 min), DNA repair products were isolated and examined on agarose gel. (B) DNA repair capacity (%, i.e., intensity of DNA repair products / intensity of DNA repair products and SSB plasmid) from Panel (A) was analyzed using Image J. "n.s." represents no significance (p>0.05, n=4).

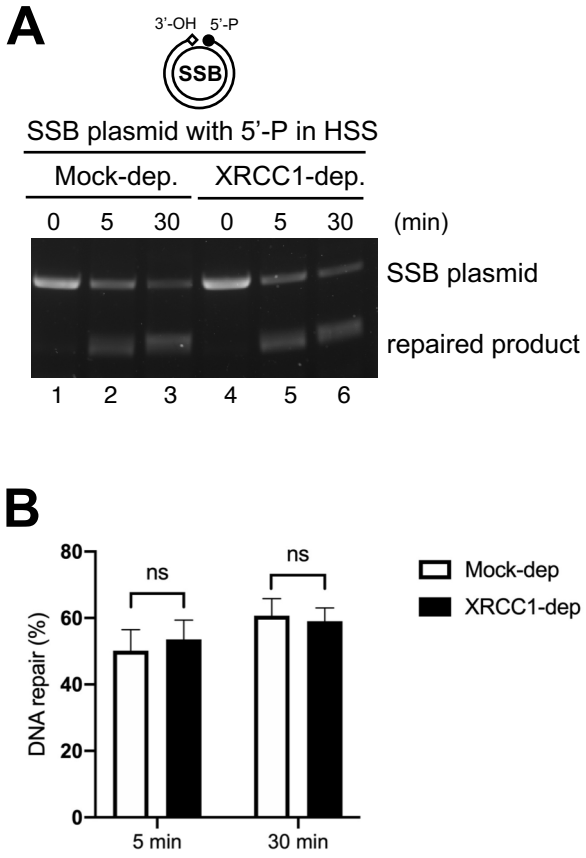


Figure 11. XRCC1 is dispensable for the repair of site-specific SSB plasmid with 5'-P in *Xenopus* HSS system. (A) SSB plasmid with 5'-P was incubated in mock- or XRCC1-depleted HSS. After different timepoints (0, 5, 30 min), DNA repair products were isolated and examined on agarose gel. (B) DNA repair capacity (%, i.e., intensity of DNA repair products / intensity of DNA repair products and SSB plasmid) from Panel (A) was analyzed using Image J. "n.s." represents no significance (p>0.05, n=3).

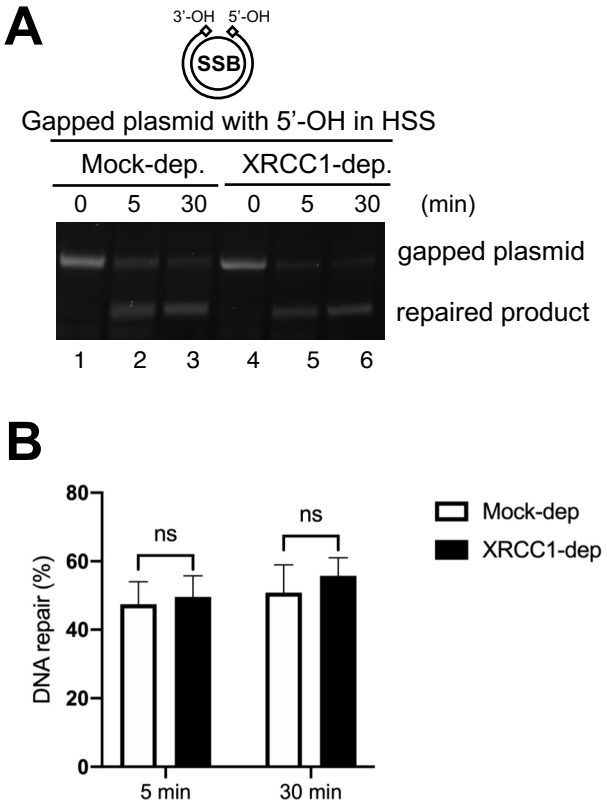


Figure 12. XRCC1 is dispensable for the repair of gapped plasmid with 5'-OH in *Xenopus* HSS system. (A) Gapped plasmid with 5'-OH was incubated in mock- or XRCC1-depleted HSS. After different timepoints (0, 5, 30 min), DNA repair products were isolated and examined on agarose gel. (B) DNA repair capacity (%, i.e., intensity of DNA repair products / intensity of DNA repair products and SSB plasmid) from Panel (A) was analyzed using Image J. "n.s." represents no significance (p>0.05, n=3).

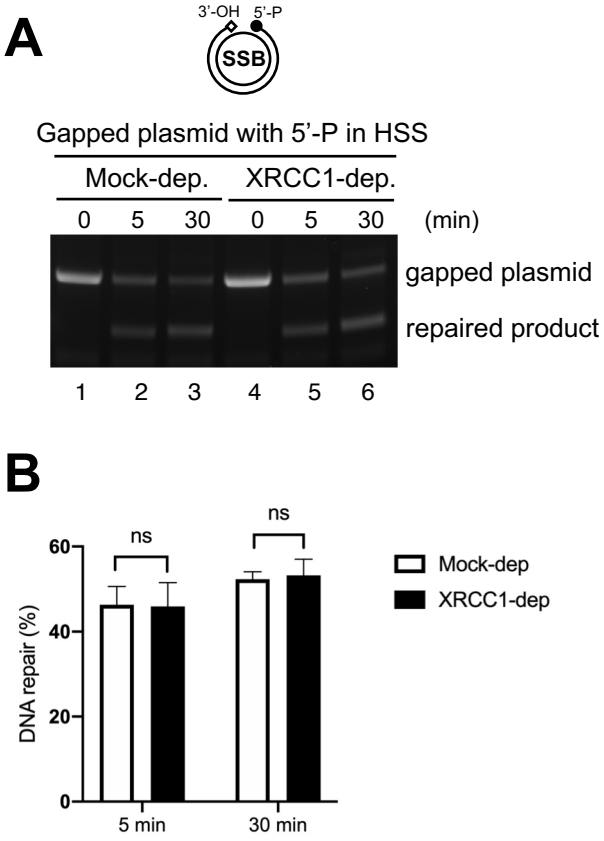


Figure 13. XRCC1 is dispensable for the repair of gapped plasmid with 5'-P in *Xenopus* HSS system. (A) Gapped plasmid with 5'-P was incubated in mock- or XRCC1-depleted HSS. After different timepoints (0, 5, 30 min), DNA repair products were isolated and examined on agarose gel. (B) DNA repair capacity (%, i.e., intensity of DNA repair products / intensity of DNA repair products and SSB plasmid) from Panel (A) was analyzed using Image J. "n.s." represents no significance (p>0.05, n=3).

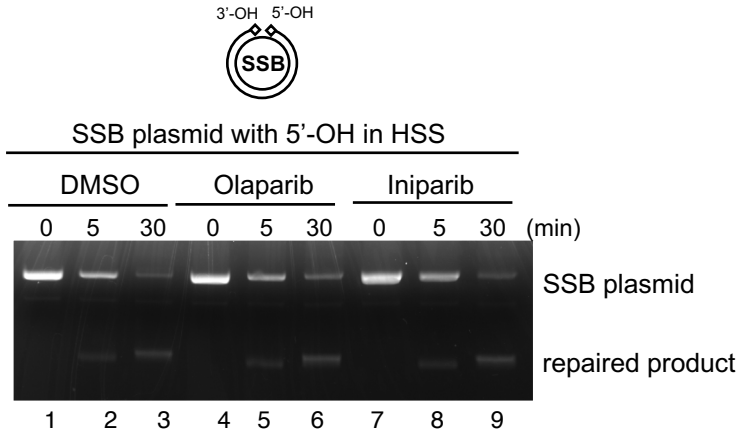


Figure 14. PARP1 inhibitors have no noticeable effect on the repair of site-specific SSB plasmid with 5'-OH in *Xenopus* HSS system. SSB plasmid with 5'-OH was incubated in HSS supplemented with DMSO or PARP1 inhibitors Oliparib (200 μM) or Iniparib (1 mM) . After different timepoints (0, 5, 30 min), DNA repair products were isolated and examined on agarose gel.

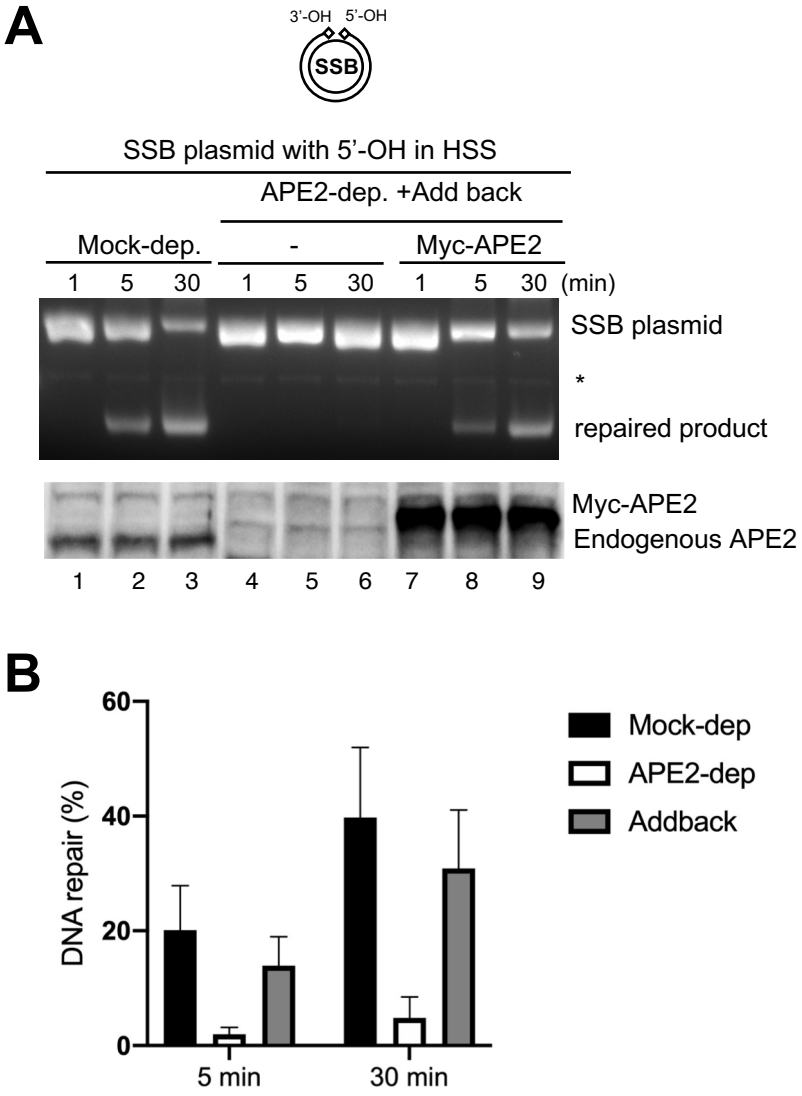


Figure 15. APE2 is required for the repair of site-specific SSB plasmid with 5'-OH in *Xenopus* HSS system. (A) Recombinant Myc-APE2 was added back to APE2-depleted HSS. Then, SSB plasmid was added to mock- or APE2-depleted HSS. After different timepoints (1, 5, 30 min), DNA repair products were isolated and examined on agarose gel. (B) DNA repair capacity (%, i.e., intensity of DNA repair products / intensity of DNA repair products and SSB plasmid) from Panel (A) was analyzed using Image J.

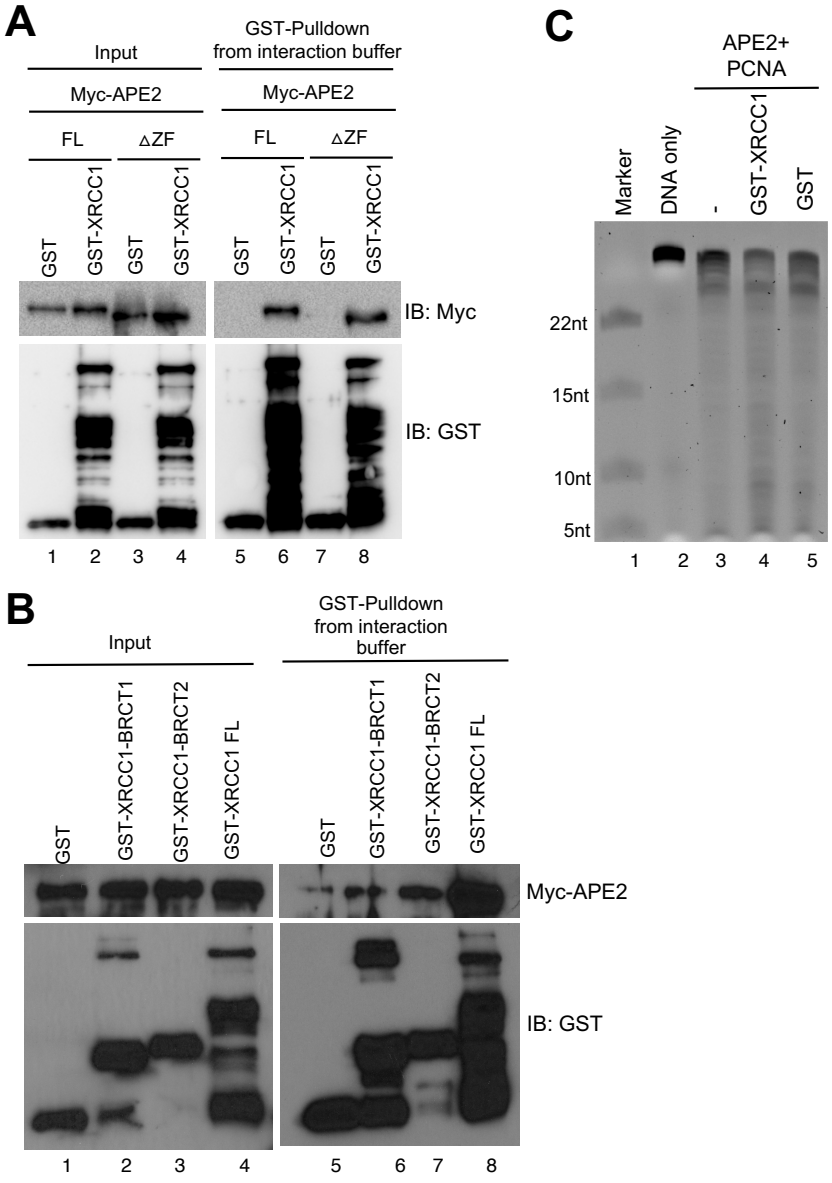


Figure 16. XRCC1 interacts with APE2 but plays a minimal role in PCNA-mediated APE2 exonuclease activity in *vitro*. (A) GST-pulldown assays using GST or GST-XRCC1 with recombinant Myc-APE2 or Myc-APE2-ΔZF in interaction buffer. Input and beads-bound fractions were examined via immunoblotting analysis as indicated. (B) GST-pulldown assays using GST, GST-XRCC1 FL, BRCT1, or BRCT2 fragment with recombinant Myc-APE2 in interaction buffer. Input and beads-bound fractions were examined via immunoblotting analysis as indicated. (C) *In vitro* endonuclease activity of APE2 with the addition of PCNA and GST-XRCC1 or GST. Samples were examined via TBE-Urea gel and Viewed via Typhoon imager.

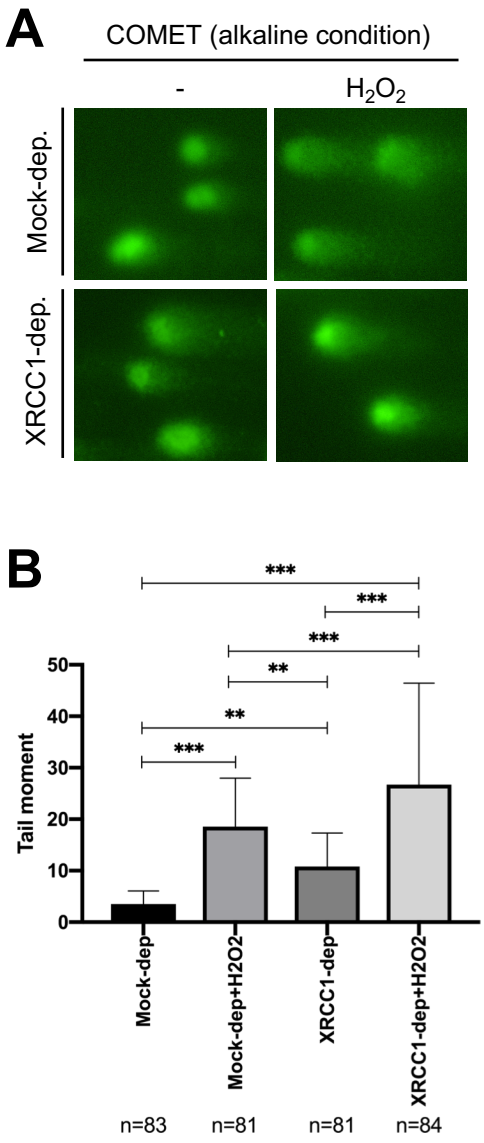


Figure 17. XRCC1 is important to repair DNA damage following oxidative stress in *Xenopus* egg extracts using alkaline COMET assays. (**A**) Hydrogen peroxide and sperm chromatin were added to mock- or XRCC1-depleted LSS. After a 30-minute incubation, reaction mixture was further examined with COMET assays under alkaline condition. Representative images are shown. (**B**) Quantification of DNA damage from four reactions shown in panel (A). *** indicates $p<0.0001$; ** indicates $p<0.001$.

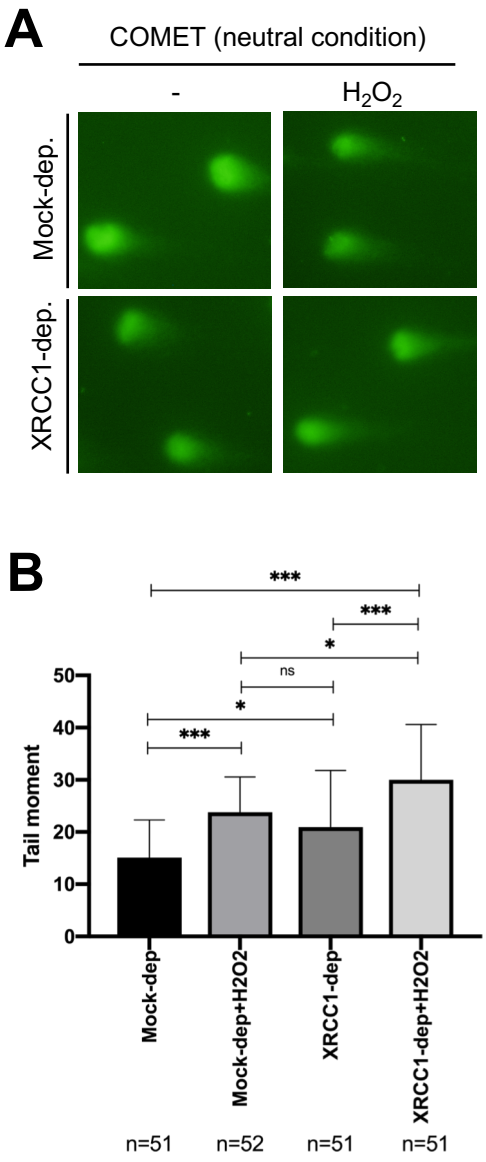
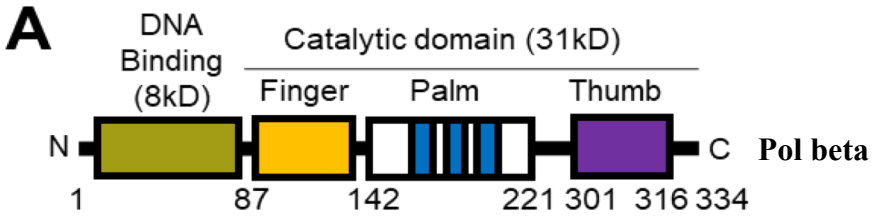


Figure 18. XRCC1 is important for the repair of DNA damage following oxidative stress in *Xenopus* egg extracts using neutral COMET assays. (A) Hydrogen peroxide and sperm chromatin were added to mock- or XRCC1-depleted LSS. After a 30-minute incubation, reaction mixture was further analyzed using COMET assays under neutral condition. Representative images are shown. (B) Quantification of DNA damage from four reactions shown in panel (A). *** indicates $p<0.0001$; * indicates $p<0.01$. "n.s." shows no significance.



B

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XL Pol beta MSKRKAPQESPNEGITDFLVELANYERNVNRAIHKYNAYRKAASVIAKYPTKIKSGTEAK 60
Mm Pol beta MSKRKAPQETLNGGITDMLVELANFEKNVSQAIHKYNAYRKAASVIAKYPHKIKSGAEAK 60
Hs Pol beta MSKRKAPQETLNGGITDMLTELANFEKNVSQAIHKYNAYRKAASVIAKYPHKIKSGAEAK 60
           *********: * ****:*.****:*:**.:****************** *****:***

XL Pol beta KLDGVGAKIAEKIDEFLATGKLRKLEKIRQDDTSSSINFLTRVTGIGPAAARKFFDEGIK 120
Mm Pol beta KLPGVGTKIAEKIDEFLATGKLRKLEKIRQDDTSSSINFLTRVTGIGPSAARKFVDEGIK 120
Hs Pol beta KLPGVGTKIAEKIDEFLATGKLRKLEKIRQDDTSSSINFLTRVSGIGPSAARKFVDEGIK 120
           ** ***:************************************:****:*****.*****

XL Pol beta TLDDLRNNEHKLNHHQKIGLKHFDDFEKRIPRKEMLQMQEIILDKVNNLDPEYIATVCGS 180
Mm Pol beta TLEDLRKNEDKLNHHQRIGLKYFEDFEKRIPREEMLQMQDIVLNEIKKVDSEYIATVCGS 180
Hs Pol beta TLEDLRKNEDKLNHHQRIGLKYFGDFEKRIPREEMLQMQDIVLNEVKKVDSEYIATVCGS 180
           **:***:**.******:****:* ********:*******:*:*:*******  *********

XL Pol beta FRRGAESSGDMDILLTHPDFTSESAKQPRLLHQVVQCLEDCNFITDTLVKGDTKFMGVCQ 240
Mm Pol beta FRRGAESSGDMDVLLTHPNFTSESSKQPKLLHRVVEQLQKVHFITDTLSKGETKFMGVCQ 240
Hs Pol beta FRRGAESSGDMDVLLTHPSFTSESTKQPKLLHQVVEQLQKVHFITDTLSKGETKFMGVCQ 240
           ************:*****.*****:***:***:**: *:. :****** **:********

XL Pol beta LPCESD-QDYPYRRIDIRLIPKDQYYCGVLYFTGSDIFNKNMRTHALEKGFTLNEYTLRP 299
Mm Pol beta LPSEKDGKEYPHRRIDIRLIPKDQYYCGVLYFTGSDIFNKNMRAHALEKGFTINEYTIRP 300
Hs Pol beta LPSKNDEKEYPHRRIDIRLIPKDQYYCGVLYFTGSDIFNKNMRAHALEKGFTINEYTIRP 300
           **.:.* ::**:*********************************:********:****:**

XL Pol beta LGVTGIAGEPLPIDSEKDIFDYIQWKYREPKDRSE 334
Mm Pol beta LGVTGVAGEPLPVDSEQDIFDYIQWRYREPKDRSE 335
Hs Pol beta LGVTGVAGEPLPVDSEKDIFDYIQWKYREPKDRSE 335
           *****:******:***:********:*********
```

Figure 19. Schematic diagram and amino acid sequence analysis of Pol beta. (A) Schematic diagram of *Xenopus laevis* Pol beta. DNA binding domain and catalytic domain including Finger, Palm, and Thumb motifs are shown. (B) Amino acid sequence alignment of Pol beta in Xenopus (AAI06330), mouse (NP_035260), and humans (NP_002681) using the Clustal Omega software. "*" indicates identical residues; "-" represents gaps in the alignment; ":" indicates highly conserved residues; "." represents moderately conserved residues.

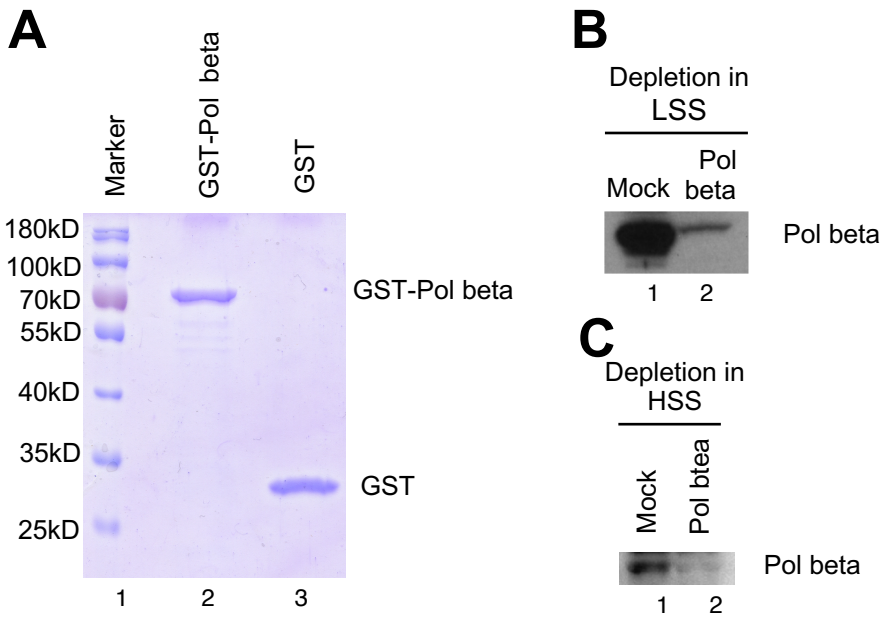

Figure 20. Validation of purified recombinant GST-Pol beta protein and immunodepletion efficiency of Pol beta in *Xenopus* LSS and HSS system. (A) Verification of purified GST-Pol beta (1 μg) on SDS-PAGE gel. (B) Mock- or Pol beta -depleted LSS was examined via immunoblotting analysis. (C) Mock- or Pol beta-depleted HSS was examined via immunoblotting analysis.

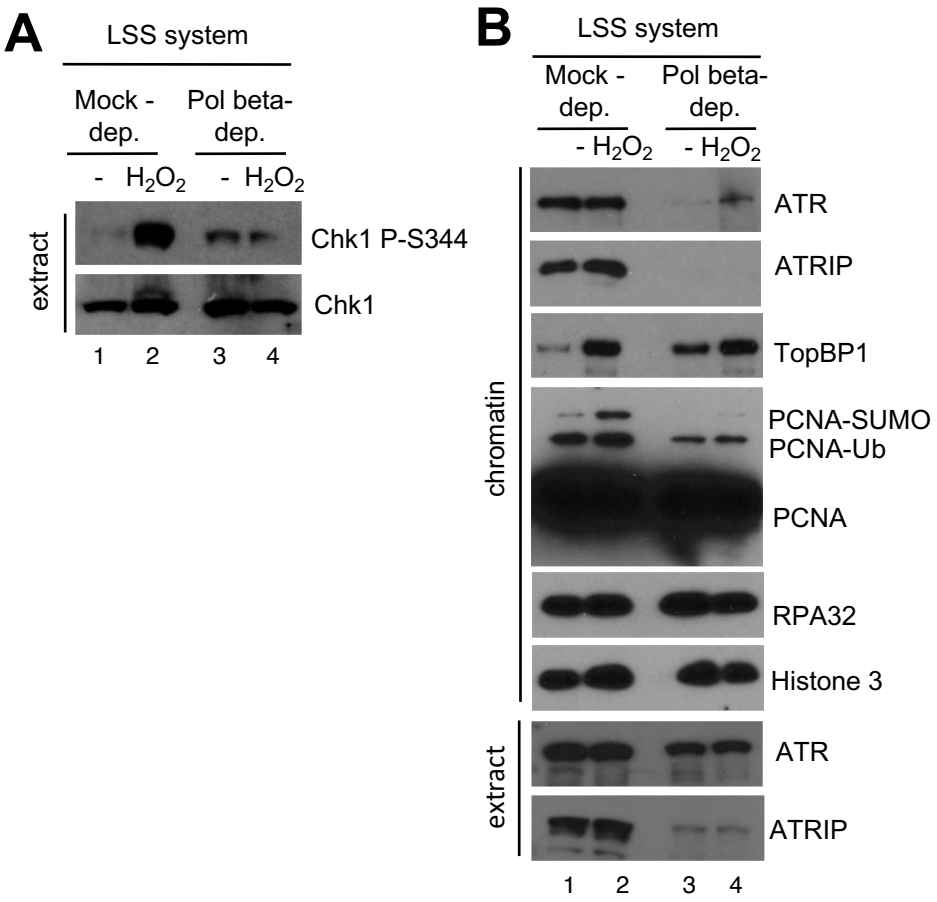

Figure 21. Role of Pol beta for the ATR-Chk1 DDR following oxidative stress in *Xenopus* LSS system. (A) Hydrogen peroxide was added to mock- or Pol beta-depleted LSS, which was supplemented with sperm chromatin and incubated for 45 minutes. Extracts were examined via immunoblotting analysis for Chk1 phosphorylation (i.e., Chk1 P-Ser344) and total Chk1. (B) Chromatin fractions and total extracts from Experiments in Panel (A) were examined via immunoblotting as indicated. Histone 3 serves as loading control.

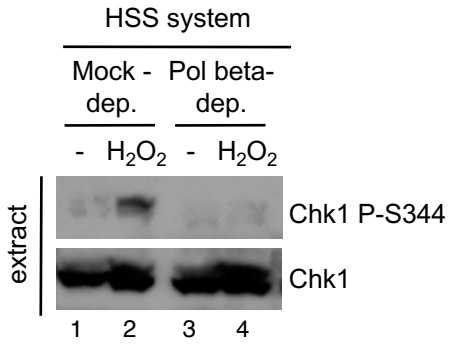

Figure 22. Role of Pol beta for the ATR-Chk1 DDR following oxidative stress in *Xenopus* HSS system. Hydrogen peroxide was added to mock- or Pol beta-depleted HSS, which was supplemented with sperm chromatin and incubated for 45 minutes. Extracts were examined via immunoblotting analysis for Chk1 phosphorylation (i.e., Chk1 P-Ser344) and total Chk1.

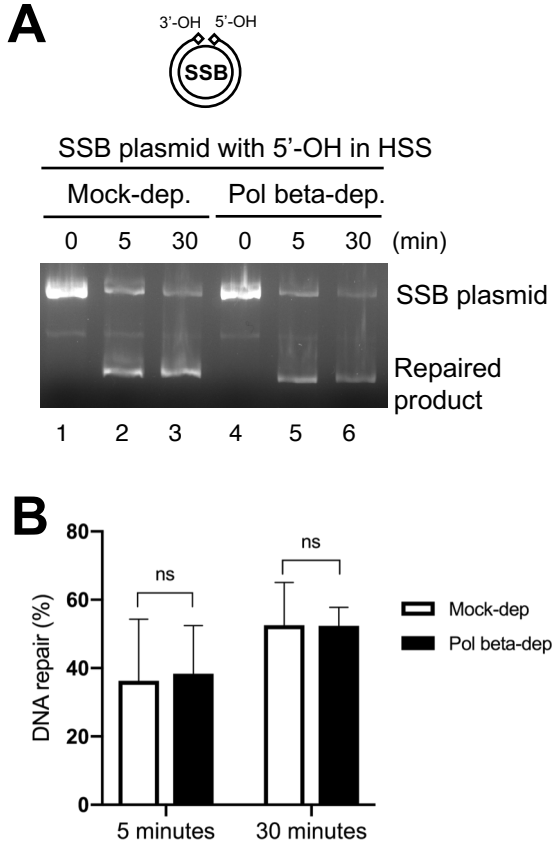

Figure 23. Pol beta is dispensable for the repair of site-specific SSB plasmid with 5'-OH in *Xenopus* HSS system. (A) SSB plasmid with 5'-OH was incubated in mock- or Pol beta-depleted HSS. After different timepoints (0, 5, 30 min), DNA repair products were isolated and examined on agarose gel. (B) DNA repair capacity (%, i.e., intensity of DNA repair products / intensity of DNA repair products and SSB plasmid) from Panel (A) was analyzed using Image J. "n.s." represents no significance (p>0.05, n=3).

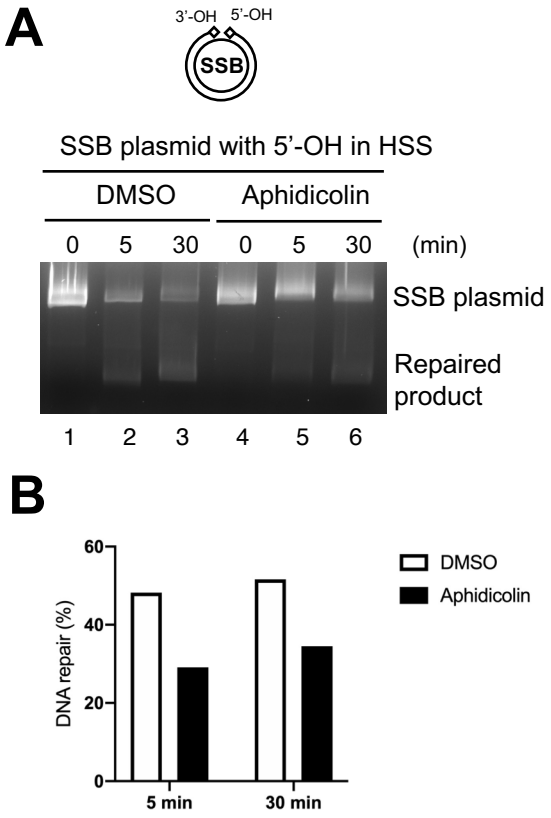

Figure 24. The repair of site-specific SSB plasmid with 5'-OH was compromised with the presence of Aphidicolin in *Xenopus* HSS system. (A) SSB plasmid with 5'-OH was incubated in HSS supplemented with DMSO or Aphidicolin (295 μM) . After different timepoints (0, 5, 30 min), DNA repair products were isolated and examined on agarose gel. (B) DNA repair capacity (%, i.e., intensity of DNA repair products / intensity of DNA repair products and SSB plasmid) from Panel (A) was analyzed using Image J.

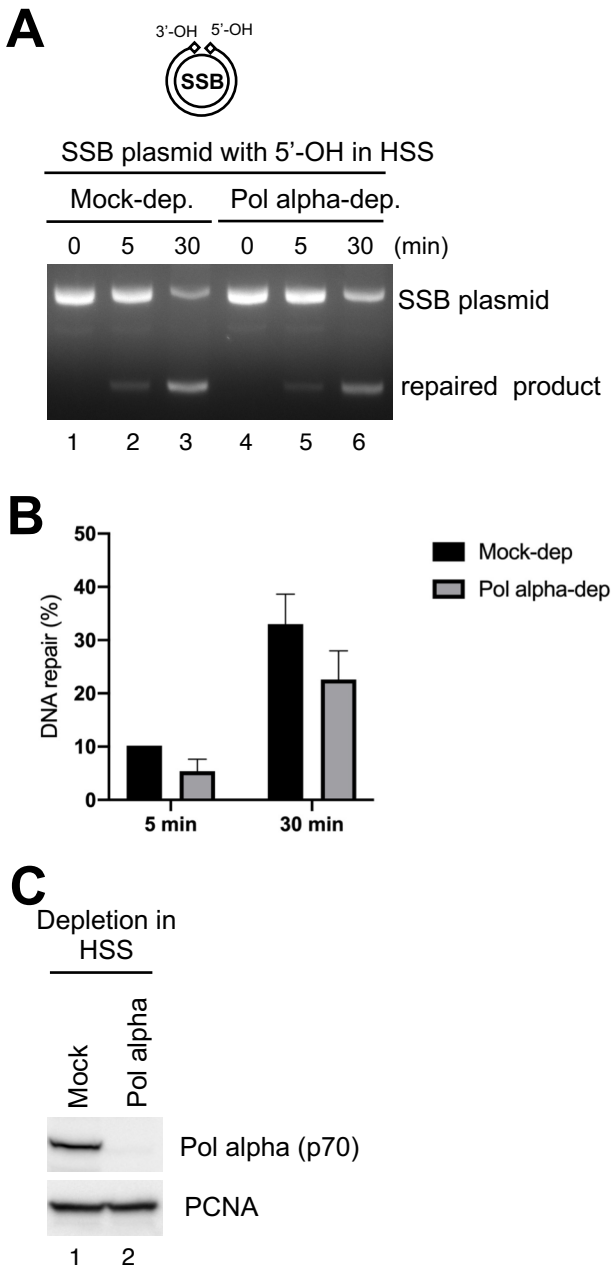

Figure 25. Pol alpha is important for the repair of site-specific SSB plasmid with 5'-OH in *Xenopus* HSS system. (A) SSB plasmid with 5'-OH was incubated in mock- or Pol alpha-depleted HSS. After different timepoints (0, 5, 30 min), DNA repair products were isolated and examined on agarose gel. (B) DNA repair capacity (%, i.e., intensity of DNA repair products / intensity of DNA repair products and SSB plasmid) from Panel (A) was analyzed using Image J. (C) Mock- or Pol alpha-depleted HSS was examined via immunoblotting assays as indicated.

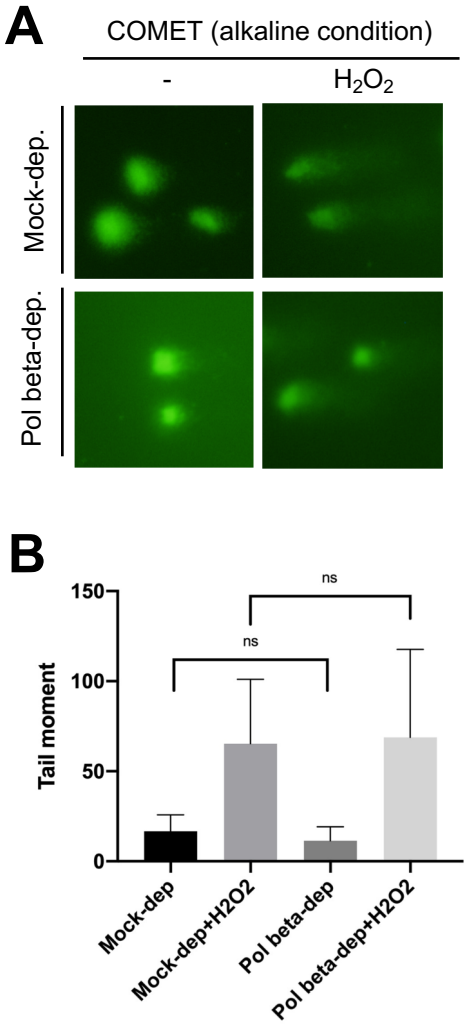

Figure 26. Pol beta is dispensable for the repair of DNA damage following oxidative stress in *Xenopus* HSS using alkaline COMET assays. (**A**) Hydrogen peroxide and sperm chromatin were added to mock- or Pol beta-depleted HSS. After a 30-minute incubation, reaction mixture was further examined with COMET assays under alkaline condition. Representative images are shown. (**B**) Quantification of DNA damage from panel (A). "ns" indicates no significance.

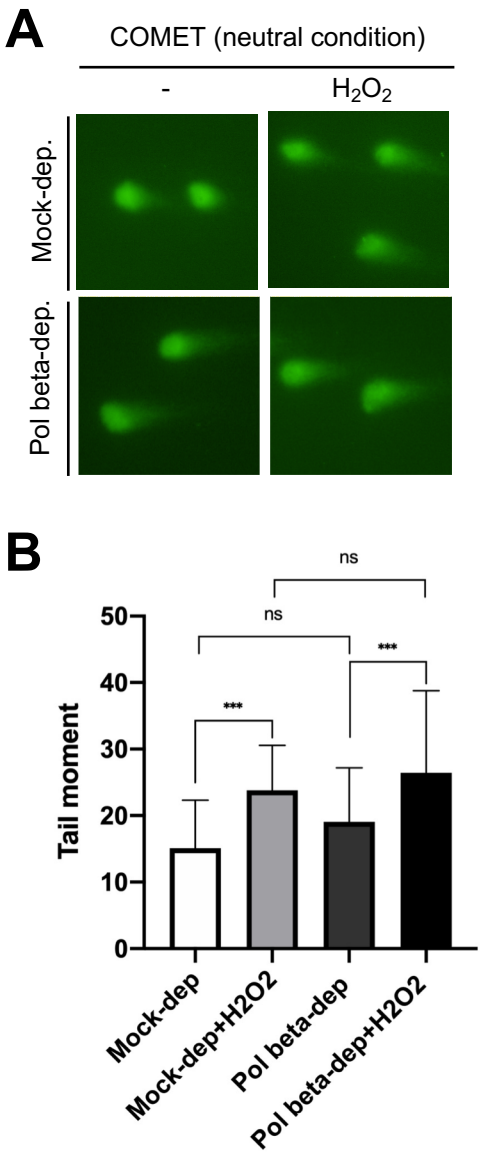

Figure 27. Pol beta is dispensable for the repair of DNA damage following oxidative stress in *Xenopus* HSS using neutral COMET assays. (**A**) Hydrogen peroxide and sperm chromatin were added to mock- or Pol beta-depleted HSS. After a 30-minute incubation, reaction mixture was further examined with COMET assays under neutral condition. Representative images are shown. (**B**) Quantification of DNA damage from panel (A). "ns" indicates no significance. *** indicates $p<0.0001$

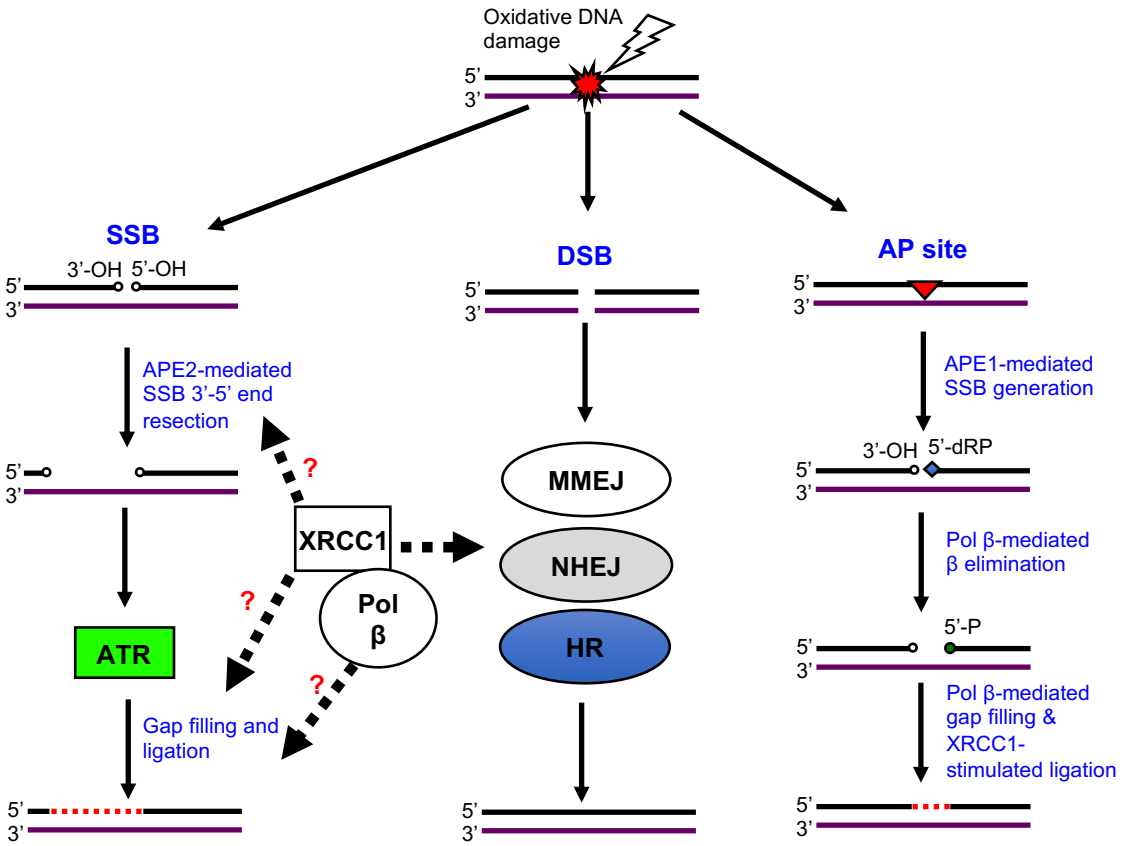

Figure 28. A working model for distinct roles of XRCC1 in genome integrity. Following oxidative stress, different types of DNA damage, including but not limited to SSBs, DSBs, and AP sites, are generated. (**Left**) **SSB:** XRCC1 interacts with APE2 but plays very minimal role for APE2's 3'-5' exonuclease activity. XRCC1 is dispensable for ATR-Chk1 DDR following oxidative stress. XRCC1 is not required for SSB repair. (**Middle**) **DSB:** Oxidative stress-induced DSBs may be repair by HR, NHEJ, and MMEJ. XRCC1 is important for repairing oxidative stress-derived DSBs. (**Right**) **AP site:** XRCC1 is important for repairing oxidative stress-induced AP sites, likely in the final step of ligation.

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APPENDIX

Awards, Papers, and Presentations

Awards:

2018 UNCC Graduate School Summer Fellowship: $6,000

Papers:

(1) Cupello S, Richardson C, and Yan S. **2016**. Cell-free *Xenopus* egg extracts for studying DNA damage response pathways. *International Journal of Developmental Biology*. 60 (7-8-9): 229-236. (PMCID: PMC5071109; PMID: 27160070) DOI: http://dx.doi.org/10.1387/ijdb.160113sy

(2) 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

(3) Cupello S, Lin Y, Yan S. **2019**. Distinct roles of XRCC1 in genome integrity in *Xenopus* egg extracts. *Biochemical Journal*. (In revision)

Presentations:

(1) Cupello S, and Yan S. (Oral presentation) Functional interplay between DNA repair and DNA damage response in oxidative stress. 49th Annual Meeting of Environmental Mutagenesis & Genomics Society. San Antonio, Texas. September 2018.

(2) Cupello S. (Oral presentation) Functional interplay between DNA repair and DNA damage response in oxidative stress. Department Friday Seminar Series. Department of Biological Sciences, UNC Charlotte. September 2018.

(3) Cupello S, Yan S. (Poster presentation) Functional interplay between DNA repair and DNA damage response in oxidative stress. 5th Department of Biological Science Symposium. April 2018.

(4) Cupello S, Yan S. (Poster presentation) Role of XRCC1 and Polymerase beta in DNA damage response. Poster Competition for Graduate Students at the Center for Biomedical Engineering and Science (CBES), UNC Charlotte. May 2017.

(5) Cupello S, and Yan S. (Poster presentation) The role of XRCC1 and Polymerase beta in DNA damage response. Annual Graduate Research Symposium at UNC Charlotte. March 2016.