THE ROLE OF TRANSLESION SYNTHESIS POLYMERASES REV1 AND POL η IN DNA DAMAGE RESPONSE

by

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ABSTRACT

MELISSA MARIE MCLEOD. The role of translesion synthesis polymerases REV1 and Pol η in DNA damage response (Under the direction of DR. SHAN YAN)

Our cells endure continuous challenges from byproducts of endogenous metabolic processes as well as outside sources such as UV light and environmental polltants. The DNA Damage Response (DDR) helps organisms cope with damage and coordinate an appropriate response pathway. It involves the initial detection of DNA damage and subsequent activation of the appropriate repair pathway, coordination of cell cycle progression, transcription activation, or activation of apoptosis or cellular senescence. ATR-Chk1 is one of the most studied DDR pathways and controls progression through the S-phase of the cell cycle. The translesion synthesis (TLS) pathway, once believed to act independently of the DDR pathways, ensures bypass of a DNA lesion in a rapid and often error-prone fashion. However, it remains largely unknown the interplay between the DDR and TLS pathways. In this research, we investigated the roles of two TLS polymerases, REV1 and Polη, to determine their function in the ATR-Chk1 pathway. Our data suggest that REV1 is important for the activation of ATR-Chk1 DDR pathway in response to interstand crosslinks and stalled replication forks, but is dispensable for the recruitment of checkpoint protein complex onto DNA damage sites or stalled forks. In addition, Polη is important for Chk1 phosphorylation in response to oxidative stress but not DNA replication stress. This research will help to understand how the TLS polymerases contribute to the DDR pathway to maintain genomic stability. Thus, our findings from this study will provide insight into how cells respond to chemotherapy

drugs and environmental agents, and ultimately lead to new potential drug targeting mechanisms.

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LIST OF ABBREVIATIONS

9-1-1 complex: Rad9-Rad1-Hus1 complex

AP site: Apurinic/apyrimidinic site

APH: Aphidicolin

ATM: ataxia-telangiectasia mutated

ATR: ATM and Rad3-related

ATRIP: ATR interacting protein

BER: Base excision repair

BRCT: BRCA1 C-terminal domain

Chk1: Checkpoint kinase 1

Chk2: Checkpoint kinase 2

CPDs: Cyclobutane pyrimidine dimers

DSBs: double-strand breaks

DDR: DNA damage response

DDT: DNA damage tolerance

dsDNA: double-stranded DNA

ELB: Egg lysis buffer

HR: Homologous recombination

ICLs: inter-strand crosslinks

MCM: mini-chromosome maintenance

MMC: mitomycin C

NER: Nucleotide excision repair

NHEJ: Non-homologous end joining

Ni-NTA: Nickel-nitrilotriacetic acid

PBS: Phosphate-buffered saline

PCNA: Proliferating cell nuclear antigen

PIKKs: Phosphatidylinositol 3-kinase-related kinases

PIP box: PCNA-interacting protein (PIP) box

PRR: Post-replication repair

ROS: Reactive oxygen species

RPA : Replication protein A

SSBs: single-stand breaks

ssDNA: single-stranded DNA

TopBP1: Topoisomerase IIβ binding protein 1

TLS: Translesion synthesis

TS: template switching

UBD: Ubiquitin-binding domain

UBM: ubiquitin-binding motifs

XP: Xeroderma pigmentosum

CHAPTER 1: INTRODUCTION

All cells are exposed to a variety of exogenous and endogenous insults, which lead to DNA damage or DNA replication stress on a daily basis. Various DNA repair pathways are activated to resolve DNA damage to maintain genomic stability (Lindahl et al., 1997). Our cells rely on DNA damage response (DDR) pathways to coordinate DNA repair, transcription, and cell cycle progression (Ciccia and Elledge, 2010). In addition, translesion synthesis (TLS) is a type of DNA damage tolerance (DDT) that involves the bypass of different DNA lesion types in order to continue replication if damage can't be repaired. This tolerance mechanism utilizes TLS polymerases to bypass and extend primers beyond the lesion until the normal replicative pols can take over. DDT is important for preventing a delay in replication that can lead to translocations, replication fork collapse, or cell death (Waters et al., 2009).

The conventional approach to understanding DDT mechanism is that it is a cellular response that acts rather independently of the DDR pathway. There is some evidence, however, to suggest that some interplay occurs between these two cellular responses to damage (Betous et al., 2013). Whereas DNA damage tolerance involves lesion bypass in an error-prone mutagenic fashion, DNA damage response will activate cellular kinases that initiate the appropriate cellular response to damage, such as cell apoptosis, cell-cycle arrest, or repair. Thus, we will determine the role of TLS polymerases in DDR pathways in this study.

1.1. DNA damage response pathways

In response to various DNA damage or replication stress, DNA damage response pathways are activated to maintain genomic stability. The proteins involved in DDR pathways include sensors, which sense and process the damage, and transducers, which will initiate the cascade of signal transduction (Fig. 1) (Andersen et al., 2008; Ghosal and Chen, 2013; Poland et al., 2014; Recolin et al., 2014). Effector proteins will then relay the signal from the transducers, leading to cell cycle arrest, DNA repair, transcription activation, apoptosis, or senescence (Fig. 1)(Cha and Yim, 2013). There are three main checkpoints that function within the cell cycle: G1/S, intra-S, and G2/M. The kinases involved in activating these checkpoints carry information about the condition of DNA, which is transduced by proteins such as Checkpoint kinase 1 (Chk1) and Checkpoint kinase 1 (Chk2).The most integral kinases to genomic maintenance are the ataxia-telangiectasia mutated (ATM) and ATM and Rad3 (Radiation sensitive protein 3)-related (ATR) kinases. ATM and ATR belong to the family of phosphatidylinositol 3-kinase-related kinases (PIKKs), a family of protein kinases that function though phosphorylation of Serine or Threonine residues with a neighboring Glutamine residue (SQ or TQ) on downstream targets (Cimprich and Cortez, 2008; Marechal and Zou, 2013).

Unlike ATM-Chk2 pathway, which responds primarily to double-strand breaks (DSBs), ATR-Chk1 activation occurs in response to exposed single-strand DNA (ssDNA) and culminates in the downstream activation of Chk1. This Chk1 protein, when activated, will ultimately prevent Cdc2-cyclin B complex activation, thereby preventing mitotic entry via regulating CDC25A (Kumagai et al., 2004; Patil et al., 2013). Many

effector proteins involved in the ATR pathway have been elucidated via a large-scale proteomic analysis (Matsuoka et al., 2007).

As of late, a hot topic for DNA damage researchers involves how ATR-Chk1 DDR pathway is activated in response to various DNA damage and replication stress. Stalled replication forks or other damage types that create ssDNA, such as UV light-induced dimers, are optimal for ATR activation. Abasic sites that occur as a result of removal of a damaged or mismatched nucleotide are also efficient activators of ATR. The major sensor for single-stranded DNA in eukaryotes is replication protein A (RPA), a heterotrimeric protein with a DNA binding domain as well as several protein-protein interaction domains (Vaithiyalingam et al., 2010). Stalled replication forks occur when the normal replicative polymerases uncouple from the mini-chromosome maintenance (MCM) helicase (Byun et al., 2005), and continued DNA unwinding leaves long stretches of ssDNA which will quickly bind to the ubiquitous RPA molecules (Yan and Michael, 2009b). RPA-bound ssDNA (RPA-ssDNA) has a direct interaction with ATRIP (ATR interacting protein), which in turn recruits ATR to the chromatin (Zou and Elledge, 2003). At the 5'-primed ssDNA/dsDNA junction, the 9-1-1 (Rad9-Rad1-Hus1) complex is recruited and the protein TopBP1 (topoisomerase IIβ binding protein 1) bridges the gap between ATR and the 9-1-1 complex. The interaction between TopBP1 and the Rad 9 subunit of the 9-1-1 complex is important for the ATR activation (Furuya et al., 2004; Lee et al., 2007). It has is recently been demonstrated that TopBP1 is recruited to RPA-coupled ssDNA via direct interaction between TopBP1 and RPA (Acevedo et al., 2016). An adaptor protein, Claspin, then recruits Chk1 protein to ATR to catalyze its phosphorylation (Kumagai et al., 2004). In addition, several other proteins and protein

kinases can contribute to Chk1 activation independently of or in cooperation with ATR (Zhang and Hunter, 2014). For example, a base excision repair (BER) protein, APE2, associates with Chk1 and is required for its activation in a similar manner as Claspin (Willis et al., 2013a).

1.2. DNA damage repair pathways

A host of DNA repair pathways and associated proteins function in correcting damage to the DNA so the cell can bypass the checkpoint and continue onto the next phase of the cell cycle. One type of damage, inter- and intra-strand crosslinks (ICLs), can occur in response to genotoxic agents such as DNA crosslinking drugs mitomycin C (MMC) or cisplatin, or UV and IR radiation. Intra-strand crosslinks involve unnatural bonds between bases on the same DNA strand and can be repaired via nucleotide excision repair (NER). Inter-strand crosslinks, however, occur between nucleotides on complimentary strands and pose a threat to cells, as they can prevent DNA strand separation during replication and transcription (Vaithiyalingam et al., 2010). For this review, we will use the term ICL to refer to inter-strand crosslinks specifically. When the replicative machinery encounters an ICL in S-phase, it will stall due to an inability to replicate past the lesion. If these ICLs are not repaired promptly, the replication machinery including MCM helicase and replicative pols will uncouple, compromising cellular viability (Byun et al., 2005).

Oxidative damage to DNA is predominately repaired via NER and Base Excision Repair (BER), which repairs base lesion and oxidatively damaged DNA (Yan et al., 2014). The first step in BER is the formation of an apurinic/apyrimidinic (AP) site via DNA glycosylases which cleave the N-glycosidic bond between the damaged nitrogenous

base and the sugar-phosphate backbone (Lindahl, 1986; Meira et al., 2005). AP sites need to be rapidly repaired, as their instability can lead to double-strand breaks (DSBs) and the replicative machinery cannot bypass ssDNA (Meira et al., 2005). Afterwards, endonuclease enzymes (e.g. APE1 and APE2) will cleave the polynucleotide chain and exonuclease activity will create a stretch of ssDNA (Boiteux and Guillet, 2004; Hegde et al., 2008). Repair is then coordinated by DNA polymerase β in conjunction with the sliding clamp proliferating cell nuclear antigen (PCNA) via gap-filling nucleotide extension, and the phosphodiester bond is created by DNA ligase I. BER is classified into either short-patch or long-patch BER depending upon the size of the ssDNA gap, and the enzymes FEN1, XRCC1, and Replication Factor C are also involved in repair (Liu et al., 2007).

DSBs are repaired by Homologous Recombination (HR) or Non-homologous End Joining (NHEJ) pathways. The main difference between these two types of repair is that HR requires a homologous chromosome as a template for repair and thus must occur within or after S-phase, whereas NHEJ can occur in any phase of the cell cycle, yet is more error-prone than HR (Cha and Yim, 2013).

1.3. DNA damage tolerance and translesion synthesis polymerases

DNA damage tolerance (DDT), also known as post replication repair (PRR) is a rapid form of repair that allows for continued DNA synthesis downstream of a lesion. The lesion will then be efficiently repaired after DNA replication is completed (Ghosal and Chen, 2013). There are two main types of tolerance: translesion synthesis (TLS) and template switching (TS), however we will be focusing on the former rather than the later form. Although mutagenic, the DDT pathway is important for cell viability, as it prevents

replication fork collapse in the case of a normal replicative polymerase encountering a DNA lesion. As ssDNA is much more likely to generate DSBs (which can lead to chromosomal rearrangements), the ability to rapidly synthesize past DNA lesions and extend ssDNA is essential to efficient replication (Branzei and Szakal, 2016).

When a DNA lesion is encountered during replication, replicative polymerases are often unable to bypass the lesion. For certain lesion types such as those creating a distorted helix structure, the 3'-5' exonuclease activity of replicative polymerases may not be sufficient to facilitate DNA repair, and proliferating cell nuclear antigen (PCNA), a sliding clamp that provides a scaffold for DNA polymerases, is covalently modified (Masuda et al., 2015). Accordingly, error-prone TLS polymerases are recruited to the replication machinery at the site of damage to bypass the lesion and continue transcription. The type of post-translational PCNA modification will determine which tolerance pathway is utilized. For instance, the TLS pols will be recruited to mono-ubiquitinated PCNA (error-prone bypass), whereas poly-ubiquitinated PCNA will result in error-free lesion bypass via template-switching (TS) (Waters et al., 2009). An ubiquitin ligase complex consisting of Rad6 and Rad18 monoubiquitinates PCNA on a lysine (K164) residue at stalled replication forks to signal for recruitment of damage-tolerant polymerases. Next, a Ubc13/MMS2/Rad5 complex can form K63-linked polyubiquitin chains on modified PCNA to initiate error-free DNA repair (Kermi et al., 2015). When a lesion such as a modified base is encountered, the replicative polymerases uncouple from PCNA, and, once ubiquitinated, PCNA recruits the TLS polymerases. TLS Pols likely bind PCNA at two sites, the interdomain connector loop via their PCNA-interacting

protein (PIP) domain and the K164-linked Ub moiety via their K164 ubiquitin-binding domain (UBD) (Poland et al., 2014).

The TLS polymerases are divided into two families according to their structural homologies: Y family and B family (Fig. 2). The Y-family consists of Polymerases (Pols) η, ι, κ and REV1, whereas Pol ζ is a member of the B-family of polymerases, which also includes some normal replicative polymerases (Ohmori et al., 2009). The eukaryotic replicative polymerases include pols α, δ, and ε. Pol α is responsible for inserting short DNA primers at the start of origin site replication, whereas Pols δ and ε function in strand elongation of template DNA (Albertson et al., 2009). One feature of the TLS pols that separates them from the classical replicative polymerases is their inability to perform 3'-5' exonuclease activity, which occurs rapidly during replication via the B-family of polymerases after incorrect base insertion (Waters et al., 2009).

The TLS polymerase REV1 is implicated in the bypass of certain types of irreparable DNA damage, including ICLs and TT dimers. REV1 contains five domains including a BRCA1 C-terminal (BRCT) domain, two ubiquitin-binding motifs (UBMs), and a C-terminal protein-protein interaction domain (CTD) (Fig. 2). The UBMs are likely necessary for REV1's interaction with PCNA, as well as the BRCT domain. The CTD is involved in interactions with the other TLS polymerases η, κ, and the Rev7 subunit of Pol ζ. Because of its protein-protein interactions, REV1 is often referred to as a "scaffolding protein," facilitating TLS pols recruitment to chromatin (Waters et al., 2009). Interestingly, however, REV1 is the only TLS polymerase that lacks a "PIP-box" (PCNA interacting protein region) for PCNA binding. The protein in also the only Y-family polymerase to have a single BRCT domain in its N-terminal region (Otsuka et al., 2005).

REV1 therefore plays a dual role in the TLS mechanism involving both its nucleotidyl transferase activity (incorporating dCMPs onto template DNA), as well as its partnership with PCNA in the DNA polymerase-switching aspect of TLS.

The error-prone Pol η functions by insertion of adenine residues across from cyclobutane pyrimidine dimers (CPDs) and potentially other lesion types in an error-free manner (Bomgarden et al., 2006). Not only is Pol η involved in bypass of UV-dimers, it may contribute to the recruitment of other TLS polymerases to DNA damage sites (Ito et al., 2012). Evidence for the requirement of Pol η in effective lesion bypass exists in Xeroderma pigmentosum (XP) patients. In these individuals, mutations in Pol η prevent bypass of UV-light-induced lesion bypass, accounting for the high risk of skin cancer amongst XP patients. Pol κ (kappa) is shown, along with Pol η, to play a role in nucleotide excision repair (NER), a type of DDR that corrects UV damaged DNA (Maiorano and Hoffmann, 2013; Ogi and Lehmann, 2006). Pol κ-deficient mouse embryonic stem cells, for instance, are highly sensitive to UV-radiation (Takenaka et al., 2006).

1.4. Oxidative stress

Genomic instability resulting from oxidative stress comprises a major source of mutational load on somatic cells. A major source of DNA damage, oxidative stress occurs in approximately 1 in 10^5 DNA bases or more (Yan et al., 2014). In humans, this amounts to approximately 10^4 DNA lesions per cell each day (Ciccia and Elledge, 2010). This damage results from endogenous metabolic processes (e.g. oxidative phosphorylation within mitochondria) or exogenous sources such as ionizing and UV-radiation, chemotherapeutic agents, and other environmental toxins. Oxidative damage

can occur within cells when reactive oxygen species, or ROS, create deleterious modifications to DNA, RNA, lipids, or proteins. ROS include free radicals and peroxides such as the hydroxyl radical (-OH), hydrogen peroxide (H_2O_2), and the superoxide anion (O^{2-}). Along with endogenous processes, UV light exposure can produce ROS, causing specific types of base modifications (e.g. 5-methylcytosine) or strand breaks (Cadet and Wagner, 2013; Halliwell, 2007; Hanson et al., 2006). Our cells have evolved means of neutralizing ROS using antioxidant compounds- certain enzymes (superoxide dismutase) and glutathione or ingestible antioxidants like vitamins A, C, and E, zinc, and selenium (Halliwell, 2007).

ROS poses a threat to genomic integrity when there is an imbalance in the redox reactions taking place within cells. Oxidative DNA damage can lead to damaged nitrogenous bases, AP (apurinic/apyrimidinic) sites, single- and double-strand breaks (SSB and DSBs), and DNA inter- and intra-strand crosslinks (ICLs) among other lesion types (Poland et al., 2014; Yan et al., 2014). One of the more prevalent types of oxidative damage, 8-oxo-7,8-dihydroguanine (8-oxoG) occurs when an oxidized Guanine forms a Hoogsteen base pair with Adenosine rather than Cytosine, creating a G:C to A:T transversion (Cooke et al., 2003). Several different repair mechanisms are employed to correct these lesions, namely NER (nucleotide excision repair), BER (base excision repair), and single-strand and double-strand break repair (SSBR and DSBR). The progressive accumulation of oxidative damage contributes to the pathogenesis of a host of diseases and ailments such as chronic inflammation, neurodegenerative disorders, cardiovascular disease, aging, and cancer (Poland et al., 2014; Richardson et al., 2015; Yan et al., 2014).

1.5. Major hypothesis

Although investigations between DNA repair and DDR pathways are numerous and ongoing, the interplay between DDR pathway and TLS pathway is less understood. A recent report demonstrates that short DNA intermediates are synthesized in part by Pol κ at stalled replication forks, facilitating the recruitment of the 9-1-1 checkpoint clamp for ATR-Chk1 activation (Fig. 3) (Betous et al., 2013). Furthermore, Pol κ depletion in unstressed cells perturbs replication and affects genome stability (Betous et al., 2013; Maiorano and Hoffmann, 2013). Although Pol η was found to preferentially associate with UV-damaged chromatin at low nuclear/cytoplasm ratio, it remains unknown whether Pol η plays a role in the activity of ATR-Chk1 pathway in cellular response to DNA damage (Kermi et al., 2015). In addition, it is unknown whether REV1 plays an important role in the ATR-Chk1 DDR pathway.

The objective of this research is to determine the interplay between the TLS polymerases and DDR pathway. Our major hypothesis is that TLS polymerases REV1 and Pol η play important roles in the ATR-Chk1 DDR pathway in DNA damage response. We have two specific aims to test our major hypothesis:

Specific Aim 1: Determine the role of REV1 in the ATR-Chk1 DDR pathway

Specific Aim 2: Determine the role of Pol η in the DNA damage response pathway in oxidative stress

1.6. Significance

For our experiments, *Xenopus laevis* (African clawed frog) egg extract serves as a cell-free biochemical experimental system for elucidating the role of TLS polymerase in the DDR pathways (Cupello et al., 2016; Willis et al., 2012).

Importantly, a variety of DNA repair and DDR proteins have become therapeutic targets and are currently being tested in the laboratory and clinical studies (Curtin, 2012; Kastan and Bartek, 2004; Wallace et al., 2012). Pharmacological inhibitors targeting DDR proteins such as ATR and Chk1 provide improved efficacy of chemotherapy drugs for cancer patients (Bouwman and Jonkers, 2012; Fokas et al., 2012; Helleday et al., 2008; Toledo et al., 2011). Although DNA crosslinking agents, such as mitomycin C (MMC), are widely used in chemotherapy, tumor cells can develop resistance to such agents, possibly through bypass of the ICLs. Understanding of the role of TLS polymerases in DDR pathway has great potential for cancer research, as prevention of TLS would potentially augment the effectiveness of chemotherapy in cancer patients.

CHAPTER 2: DETERMINE THE ROLE OF REV1 IN THE ATR-CHK1 DDR PATHWAYS

2.1 Rationale and Hypothesis

When DNA lesions cannot be replicated by high-fidelity replicative DNA polymerases (Pol δ/ε), they can be bypassed by low-fidelity translesion DNA synthesis (TLS) polymerases, increasing the risk of mutagenesis as a tradeoff for survival (Chang and Cimprich, 2009; Goodman and Woodgate, 2013). TLS polymerases include the Y-family DNA polymerases (REV1, Pol η, Pol κ and Pol ι) and a B-family DNA polymerase Pol ζ (Ho and Scharer, 2010; You et al., 2013). Although the REV1 protein has deoxycytidyl transferase activity that transfers a dCMP to a damaged nucleotide in an error-free fashion, its non-catalytic function may play an essential role in mutagenesis and cell survival, possibly through its interaction with other TLS polymerases via a C-terminal fragment (Nelson et al., 1996; Ohashi et al., 2004). As REV1 lacks an obvious PCNA-interaction protein box (PIP box), this TLS protein may be recruited to damage sites through its unique N-terminal BRCT domain and ubiquitin-binding motifs (UBMs) (Bomar et al., 2010; Guo et al., 2006). Together with Pol ζ, REV1 facilitates various DNA repair programs including ICL repair and homologous recombination repair of DSBs, promoting or preventing genome instability (Ho et al., 2011; Sharma et al., 2012). However, it has not been determined whether REV1 plays any role in the ICL-induced ATR-Chk1 DDR pathway.

It is pivotal to understand how exactly TLS and DDR pathways regulate each other, as the dependency and regulation between them is a long-standing question in the field of genome integrity. The 9-1-1 complex associates with DinB (yeast homologue of Pol κ) and may regulate the recruitment of DinB to damage sites in fission yeast; however, it has not been tested whether the ATR kinase itself regulates DinB (Kai and Wang, 2003). REV1 phosphorylation by Mec1 (yeast homologue ATR) is important for the Pol ζ-mediated TLS of UV damage in nucleotide excision repair-deficient, but not wild type, budding yeast cells (Pages et al., 2009). Mec1 also mediates the recruitment of REV1 to a DSB site in budding yeast (Hirano and Sugimoto, 2006). However, the putative Mec1 phosphorylation sites of REV1 are lacking in more complex eukaryotic organisms including humans and *Xenopus* (Pustovalova et al., 2013). Although the DDR pathway may regulate the TLS pathway under some circumstances, it was demonstrated in a recent report that Pol κ actually contributes to ATR-Chk1 DDR pathway activation induced by stalled replication forks, suggesting a complicated regulation between TLS and DDR pathways (Betous et al., 2013; Kai and Wang, 2003; Pages et al., 2009). It remains unknown, however, whether or not REV1 and ATR-Chk1 DDR pathways regulate each other and how this regulation might occur in response to ICLs in higher eukaryotes. *Xenopus* egg extract has been demonstrated as an excellent cell-free model system for studies of ICL repair and DDR pathways (Bai et al., 2014; Ben-Yehoyada et al., 2009; Raschle et al., 2008).

In this chapter, we will test the hypothesis that REV1 plays a previously unidentified, but important, role in the activation of MMC-induced ATR-Chk1 DDR

pathway in *Xenopus* egg extract. Furthermore, we will dissect the molecular mechanism of how REV1 contributes to the ATR-Chk1 DDR pathway in DNA damage response.

2.2 Materials and Methods

2.2.1 LSS preparation, immunodepletion, and rescue experiment procedures

Low-speed supernatant (LSS) egg extract is prepared using eggs from *Xenopus laevis*. The protocol for crude extract extraction has been described previously (Willis et al., 2013a). *Xenopus* LSS is optimal for the study of proteins involved in DDR because it provides an ideal cell-free system to achieve DNA replication *in vitro.* Sperm chromatin was added to mock-depleted and REV1-depleted extracts, as well as an ATP energy mixture and hydrogen peroxide at a concentration of 100 mM. Other stressors include UV-damage to sperm chromatin prior to the addition to LSS, as well as Aphidicolin (APH) and Mitomycin C (MMC) at respective concentrations of 100 ng/uL and 0.5 mM.

Rabbit anti-REV1 serum and 1X Phosphate-buffered saline (PBS) as a control were coupled with 80µl of protein-A sepharose beads and incubated via rotation at 4^{o}C overnight to optimize binding affinity. Three separate 45-minute rounds of REV1 immuno-depletion in egg extract were performed to maximize endogenous protein depletion from LSS. The recombinant plasmid was incubated in TnT® SP6 High-Yield Wheat Germ Protein Expression solution (Promega, Inc.™) at 37°C for 90 minutes prior to addition to depleted extracts. The addition of the recombinant protein was used to compare roles of the extract containing “rescued” protein (exogenous REV1) with the “mock” extract (still containing endogenous REV1) can help determine whether or not co-depletion of an interacting protein occurred and determine if the phenotype is dependent upon REV1 presence in the extract. We isolated and examined chromatin-

bound fractions and perform Western blot analyses on the mock- and REV1-depleted extracts after addition of Myc-REV1.

2.2.2 Isolation of chromatin-bound fractions and immunoblotting analysis

Remaining total cytosolic egg extract were centrifugated through two rounds of sugar cushions to separate higher density sperm chromatin from cytosolic fractions (Willis et al., 2013b). The sugar cushions were prepared in a 1:10 dilution of 10 x ELB (Egg lysis buffer) salts to 1M sucrose. Total egg extract (45µl) were resuspended with 200µl ELB and layered on surface of the sucrose cushion, spun at 11,000 rpm for two minutes, 4ºC. The apical layer of sucrose cushion was aspirated until remaining 100µl. Resuspension of the remaining 100µl with 200µl 0.6% TX-100 with ELB, followed by another surface layer addition and second centrifugation (first conditions) was performed. Finally, the apical layer of sucrose cushion was aspirated until ~20µl of chromatin bound fraction remained; this fraction was resuspended with 35µl 2X Laemmli Sample Buffer for Western blot analysis.

2.2.3 Recombinant protein expression and purification and antibodies

His-REV1-NT300, corresponding to *Xenopus laevis* REV1 (MGC: 83743) nucleotide 246-1145, was cloned into the pET28a expression vector. Recombinant His-REV1-NT300 was expressed and purified from DE3 bacteria cells. Anti-REV1 antibodies were raised in rabbit against His-REV1-NT300 (Cocalico Biologicals). Wild-type full length (WT) Myc-tagged REV1 corresponding to REV1 nucleotide 246-3938 was cloned into the pCS2+MT vector.

For protein-specific probing, we used antibodies purchased from several different vendors:

ATR antibodies were provided by Karlene Cimprich (Stanford University) (Cimprich and Cortez, 2008). Antibodies against ATRIP and Rad9 were provided by Howard Lindsay (Lancaster University) (Yan et al., 2006). Chk1 P-Ser344 antibodies (Cell Signaling Technology) have been previously described (Yan et al., 2006). Antibodies against Chk1, Myc, and PCNA were purchased from Santa Cruz Biotechnology. TopBP1 antibody (HU142) was used as described previously (Yan et al., 2006; Yan and Michael, 2009b). Antibodies against RPA32 P-S33 were purchased from Bethyl Laboratories. Anti-Orc2 and anti-RPA32 antibodies were used as described previously and provided by W. Matthew Michael (Yan and Michael, 2009a).

2.2.4 Coimmunoprecipitation assays and Ni-NTA bead pulldown assays

For coimmunoprecipitation assays in Fig. 7A, control antibodies, anti-ATRIP or anti-REV1 antibodies (1 µg) or were coupled to protein A sepharose beads. *Xenopus* LSS was supplemented with sperm chromatin with or without the presence of aphidicolin and incubated for 45 min at room temperature. The extracts were then diluted with equal volume of an Interacting Buffer (100 mM NaCl, 0.05% NP-40, 5 mM $MgCl_2$, 10% glycerol, 20 mM Tris–HCl, pH 8.0), which was added to the protein A sepharose beads coupled with antibodies. After incubation, the beads were washed by the Interaction Buffer, and the bead-bound proteins and input were analyzed via immunoblotting analysis.

To investigate possible interactions with the N-terminal region of REV1, we performed a Ni-NTA Pulldown using His-REV1-NT300 (Fig. 4A). Nickel-nitrilotriacetic acid (Ni-NTA) affinity resin was used to bind His-REV1-NT300 recombinant protein. The beads were then incubated with LSS which was first supplemented with sperm

chromatin and hydrogen peroxide for 45 minutes. The Ni-NTA beads were washed with 1XPBS in order to remove and non-specific proteins from the sample. Sample Buffer was added to the bead-bound fractions and analyzed via immunoblotting analysis.

2.3 Results

REV1 is important for the MMC-induced Chk1 phosphorylation in Xenopus egg extracts

Chk1 phosphorylation at Serine 344 (Chk1 P-S344) is widely used as an indicator of ATR activation (Ben-Yehoyada et al., 2009; Guo et al., 2000). Chk1 phosphorylation was triggered by MMC treatment in *Xenopus* egg extract (Fig. 4B), consistent with our recently published study (Bai et al., 2014). The MMC-induced Chk1 phosphorylation was compromised by the addition of ATR specific inhibitors, VE-822 or NU6027 (Fig. 1B) (Fokas et al., 2012; Peasland et al., 2011). These observations suggest that MMC-induced ICLs trigger Chk1 phosphorylation in an ATR-dependent manner.

Notably, the MMC-induced Chk1 phosphorylation was compromised in REV1-depleted egg extracts (Fig. 4C). Adding back recombinant Myc-tagged REV1 to REV1-depleted egg extract to a concentration similar to endogenous REV1 rescued the Chk1 phosphorylation induced by MMC (Fig. 4D). These findings suggest that REV1 plays an essential role in the ATR-Chk1-mediated DDR pathway activation in response to MMC-induced ICLs.

REV1 plays an important role in the DDR pathway in response to more general DNA damage and DNA replication stress

To test whether REV1's role in the DDR pathway is limited to MMC-induced ICLs or a more general response to DNA damage, we examined the aphidicolin-induced

stalled replication forks, UV damage, and hydrogen peroxide-induced oxidative stress (Fig. 5). Immunodepleting endogenous REV1 caused a significant reduction in Chk1 phosphorylation induced by stalled replication forks and UV damage (Fig. 5A). Furthermore, Chk1 phosphorylation induced by oxidative stress is compromised when REV1 is absent in *Xenopus* egg extracts (Fig. 5B). Total Chk1 is used as a loading control in these experiments. The defects in Chk1 activation when REV1 is absent from egg extract suggest a more general role for REV1 in DNA damage response.

REV1 is dispensable for the recruitment of checkpoint proteins onto DNA damage sites or stalled DNA replication forks

To test whether REV1 affects the recruitment of checkpoint proteins onto DNA damage sites and stalled replication forks, we isolated the chromatin-bound fractions and examined them via immunoblotting analysis. As shown in Fig. 6A, REV1 is dispensable for the recruitment of ATR, ATRIP, TopBP1, Rad9 and RPA32 to MMC-damaged chromatin in *Xenopus* egg extracts. Consistent with this, the recruitment of ATR, ATRIP, TopBP1, Rad9 and RPA32 to stalled replication forks was not noticeably changed after REV1 was depleted in *Xenopus* egg extracts (Fig. 6B). However, Chk1 activation is diminished when REV1 is absent under exposure to both types of DNA damaging agents. These observations suggest that REV1 is dispensable for the recruitment of checkpoint proteins onto DNA damage sites or stalled replication forks.

REV1 interacts with ATR, ATRIP, and PCNA in Xenopus egg extracts

To determine whether REV1 interacts with ATR and ATRIP in *Xenopus* egg extracts, we performed coimmunopricipitation assays with anti-REV1 and anti-ATRIP

antibodies with or without aphidicolin treatment in LSS. As expected, REV1 was in the immune complex using anti-REV1 antibodies but not in the immune complex using control antibodies (Fig. 7A). ATR and ATRIP was in the immune complex using anti-REV1 antibodies but not in the immune complex using control antibodies regardless of the presence of aphidicolin, suggesting a weak interaction between REV1 and ATR-ATRIP (Fig. 7A). In addition, to determine which domain of REV1 may be responsible for its role in the ATR-Chk1 pathway, we performed a Ni-NTA Pulldown analysis using His-REV1-NT300, which contains the BRCT domain. Consistent with current literature (Waters et al., 2009), the BRCT domain within REV1 appears to associate with PCNA regardless of the presence of hydrogen peroxide (Fig. 7B). These observations suggest that unlike the Pol kappa, REV1 interacts with ATR-ATRIP and promotes the Chk1 phosphorylation by activated ATR, which is a downstream event of the ATR-Chk1 DDR pathway (Fig. 8).

CHAPTER 3: DETERMINE THE ROLE OF POLη IN THE DNA DAMAGE RESPONSE PATHWAY IN OXIDATIVE STRESS

3.1 Rationale and Hypothesis

Yeast studies have linked the NER pathway to the DDR pathway activation. In particular, XPA-mediated DNA damage processing is necessary for the activation of Mec1/ATR in *Saccharomyces cerevisiae* (Giannattasio et al., 2004; Neecke et al., 1999). It has been shown that ATR signaling in response to UV damage was enhanced in XPV cells in which Pol η is deficient (Bomgarden et al., 2006). Although Pol η was found to preferentially associate with UV-damaged chromatin at low nuclear/cytoplasm ratio, it remains unknown whether the preferential binding of Pol η to UV damage sites has a biological significance (Kermi et al., 2015). Recently, our laboratory has demonstrated that the ATR-Chk1 DDR pathway is activated in oxidative stress and a BER protein APE2 plays an essential role in the DDR pathway in response to oxidative stress but not stalled DNA replication forks (Willis et al., 2013). Thus, we hypothesize that the TLS polymerase Pol η may play a distinct role in the activation of ATR-Chk1DDR pathway in oxidative stress.

In this chapter, we aimed to determine whether Chk1 phosphorylation induced by hydrogen peroxide and stalled replication forks is compromised when Pol η is removed. If so, we will determine whether the recruitment of checkpoint protein complex including ATR, ATRIP, 9-1-1 complex is reduced with the absence of Pol η. We also ask whether

Pol η interacts with ATR and ATRIP as well as APE1 and/or APE2- two emerging crucial players in oxidative stress response. In addition, we examined whether small molecular inhibitors DL-selforaphane (DLS) and celastrol affect the ATR-Chk1 DDR pathway in oxidative stress and pinpointed the steps in which DLS and celastrol play a role for the DDR pathway. Last, we have established in vitro biochemical assays with defined DNA structures including biotin-coupled ssDNA and biotin-coupled dsDNA with a SSB structure at a defined location.

3.2 Materials and Methods

3.2.1 Xenopus egg extracts and related experimental procedures

Xenopus

LSS and sperm chromatin were prepared as previously described (Willis et al., 2012). HSS was also prepared as previously described (Lebofsky et al., 2009). Different stressful conditions (i.e. oxidative stressors) were described in Chapter 2. Pol η immunodepletion with customized anti- Pol η antibodies (UNC 398 serum, which was raised against Xenopus Pol η) was performed in a similar way to REV1 depletion described in Chapter 2. Chromatin-bound fractions isolation and examination were performed as previously described (Willis et al., 2013).

3.2.2 Recombinant protein expression and purification and anti-Pol η antibody generation

PCR amplification of full-length Pol eta (2011nt) was performed using designed forward 5' and reverse 3' primers and full length *Xenopus* Pol eta cDNA template. The restriction endonucleases EcoRI and NotI (NEB) were used to produce complementary 3' overhangs flanking the gene sequence of interest (insert). These same endonucleases

were simultaneously used for the double enzyme cut of plasmid vector, pGEX-4T-1; additional vector incubation with CIP was used to prevent self-ligation. Both insert and plasmid were incubated with EcoRI and NotI at 37ºC for two hours. Gene insert and plasmid vector were then ligated in a 5:1 ratio at room temperature for three hours (NEB, Ligase IV). Five microliters (µl) of the ligated product (SY152) were transformed into 100µl of DH5α *E. coli*. The bacterial cells were then spread on a 1:1000 Ampicillin (75mg/ml) agar plate and incubated at 37ºC for 16 hours. Antibiotic resistant colonies were selected and grown overnight in LB media with 1:1000 dilution Ampicillin (75mg/ml). Bacterial cell lysis and isolation of recombinant plasmid were performed with Qiagen® Miniprep kit.

Recombinant GST-Pol η was expressed in DE3 cells. After verification via restriction enzyme EcoRI and NotI, 1µl of the recombinant plasmid SY152 (i.e., pGEX-4T1-Pol eta) was inoculated into 100µl of DE3 (BL21) E. coli. which contains a T7 promoter system for bacterial transformation. The E. coli was then spread on a 1:1000 ampicillin antibiotic agar plate and incubated at 37ºC for 16 hours. Antibiotic resistant colonies were selected and inoculated into 2X YT media (1:1000 ampicillin) with OD600 (0.5-0.9). Induction with 1mM IPTG occurred at 37ºC for 6 hours. After centrifugation, bacteria were resuspended with 1XPBS and centrifuged again for 10 minutes at 5,000 rpm and 4ºC.

Bacterial pellets containing expressed GST-Pol eta were resuspended with 1XPBS and TEN buffers. Lysozyme and 10% NP-40 were added to resuspended pellets and flash frozen with liquid nitrogen for one hour. A 1:1000 dilution of protease inhibitor A/L was added to the thawed resuspension immediately before bacterial lysis

with three rounds of 100% amplitude sonication (30 seconds alternation of sonication and rest). The bacterial lysate was centrifugated again and supernatant was immediately added to 1.5ml glutathione beads (prewashed with 1XPBS). Overnight incubation at 4°C with rotation was used to optimize GST-tagged Pol eta recombinant protein binding to beads. After incubation, the glutathione beads were column washed three times with 1XPBS buffer to remove non-specific proteins. After the final bead rinse, the recombinant protein was eluted and collected with 125mM glutathione buffer. Overnight dialysis was performed on collected eluate that tested positive for protein with Bradford dye.

We sent purified GST-Pol η (~2mg) to Cocalico Biologicals, Inc.™ in order to create specific polyclonal rabbit serum antibodies (UNC398 and UNC399) for use in Western blot and other immuno-assays.

3.2.3 Coimmunoprecipitation assays

For coimmunoprecipitation assays, control antibodies or anti-Pol eta antibodies (1 µg) or were coupled to protein-A sepharose beads. *Xenopus* LSS was supplemented with sperm chromatin with or without the presence of hydrogen peroxide and incubated for 45 min at room temperature. The extracts were then diluted with equal volume of an Interacting Buffer (100 mM NaCl, 0.05% NP-40, 5 mM $MgCl_2$, 10% glycerol, 20 mM Tris–HCl, pH 8.0), which was added to the protein A sepharose beads coupled with antibodies. After incubation, the beads were washed by the Interaction Buffer, and the bead-bound proteins and input were analyzed via immunoblotting analysis.

3.2.4 Biotinylated DNA structures as a model for oxidative DNA damage

To create a working model for ATR-CHK1 DDR study, we tested the length-

dependent recruitment of checkpoint pathway proteins onto Biotin-coupled ssDNA. We incubated the 200ng/ml of biotin-labeled oligonucleotides with HSS for 1 hour at room temperature. Prior to incubation, 5μl of HSS was removed from each reaction to run as "input" samples on the gel. The lengths were 10, 20, 40, 60, and 80 nucleotides with a single biotin molecule covalently attached to the 5' end. The oligonucleotide sequence was derived from the plasmid used to test SSB response *in vitro,* pUC19-derived SY92.

In order to create a testable assay mimicking oxidatively generated SSBs *in vitro,* we used a nicking enzyme, Nt. BstNB1 to generate a SSB on a dsDNA with biotin-labeled from both sides (Fig. 14A). First, PCR was performed using the plasmid SY92 as a template. The primers were as follows:

F-SY177: (28mer, nt 36-63)

5'-Biotin- CACATGCAGCTCCCGGAGACGGTCACAG-3'

R-SY177: (25mer, nt 611-635)

5'-Biotin- TGCAGCTGGCACGACAGGTTTCCCG-3'

The PCR product is 600 bp long (dsDNA). After purification on 1% agarose gel, this Biotin-WT-dsDNA was further treated with nicking enzyme Nt.BstNBI at 55 degree for 2 hours first in NEB Buffer 3, and then followed by 1hr treatment with CIP at 37 degrees C. The cut Biotin-dsDNA purified from agarose gel, labeled "Biotin-SSB-dsDNA" is the final product containing a defined SSB (600bp in total and the SSB is right at the 2/3 position in this structure).

3.3 Results

Generation of recombinant GST-tagged Pol eta and customized anti-Pol eta antibodies

To generate customized anti- Pol η antiserum, we subcloned a recombinant GST-tagged wild type full length Pol η and expressed this recombinant GST- Pol η protein in *E.coli* DE3 cells. The purified GST- Pol η protein was examined on a SDS-PAGE gel and stained with coomassie blue. As shown in Fig. 9A, the purified GST- Pol η protein was shown in a band at an expected size, suggesting that the GST- Pol η protein is expressed and purified (Fig. 9A). Two anti-GST- Pol η antiserum were generated in rabbits, UNC398 and UNC 399. The anti-Pol η antibodies was able to detect endogenous Pol η in *Xenopus* egg extract via immunoblotting analysis, and to effectively remove endogenous Pol η from *Xenopus* egg extract via immunodepletion procedure (Fig. 9B). Importantly, the Chk1 phosphorylation induced by hydrogen peroxide was significantly compromised when Pol η was depleted from *Xenopus* egg extract (Fig. 9B).

Pol η is important for Chk1 phosphorylation induced by oxidative stress but not by stalled replication forks in Xenopus egg extracts

Several checkpoint proteins including ATR and ATRIP in the ATR-Chk1 DDR machinery are hyperloaded onto hydrogen peroxide-damaged chromatin in LSS, compared to normal conditions (Fig. 10A). When Pol η is absent from the extract, however, there is reduced binding of ATR and ATRIP to hydrogen peroxide-damaged chromatin (chromatin panel, Fig. 10A). It was also noticed that ATR and ATRIP were not co-depleted with anti-Pol η antibodies (extract panel, Fig. 10A). Interestingly, the modification on PCNA seems to shift from ubiquitination to sumoylation in Pol η-depleted extract, suggesting that Pol η may be necessary for the formation of ubiquitination on PCNA in oxidative stress in a positive feedback mechanism (Fig. 10A).

It was noticed that the binding of Rad9 and Pol delta but not Pol epsilon were slightly enhanced in Pol η-depleted egg extracts (Fig. 10A).

Consistent with a previous study (Bomgarden et al., 2006), Chk1 phosphorylation induced by stalled DNA replication forks was not noticeably reduced but rather slightly enhanced in Pol η-depleted LSS in response to aphidicolin-induced stalled replication forks (Fig. 10B). Pol η is dispensable for the recruitment of checkpoint proteins including ATR, ATRIP, Rad9, and RPA32 to stalled replication forks (Fig. 10B). Histone 3 was used as a loading control for chromatin-bound fractions and total Chk1 was used as a loading control for proteins in extract. Immunblotting analysis was used to confirm that Pol η was removed via immunodepletion procedure in these functional analysis (Fig. 10C).

Pol η interacts with ATR, ATRIP, PCNA and APE1 in Xenopus egg extract

In a method similar to Co-IPs with REV1 antibody, we used protein A-sepharose beads coupled to our Pol η antibody to perform a co-immunoprecipitation determining possible binding partners. It appears there is a slight interaction between endogenous Pol η, ATR and ATRIP and this interaction may increase under oxidative stress conditions (Fig. 11A). We also tested for interactions with proteins of the BER pathway and found a possible interaction between Pol η and APE1, a major AP endonuclease in BER pathway (Fig. 11B). Further investigation is needed to determine whether the SSB end resection is affected by Pol η through APE1 interaction (Fig. 15). Interestingly, PCNA was found in the immune complex with anti-Pol η antibodies but not immune complex with control antibodies, suggesting that Pol η may interact with PCNA directly or via another unknown protein indirectly (Fig. 11B).

Small molecule inhibitors of ATR-Chk1 DDR pathways

To test the effectiveness of known ATR inhibitors and have a means by which to compare the TLS polymerase functions in the DDR pathways, we introduced two small-molecule inhibitors into LSS under oxidative stress and APH-induced stalled fork stress. The two small molecules chosen were DL-Sulforaphane (DLS) and Celasterol. Both of these compounds are shown to have potent antioxidant and anti-inflammatory effects (Allison et al., 2001; Zhang et al., 1992). In addition to its anti-cancer effects, DLS is also implicated in protection against neurodegenerative disease (Tarozzi et al., 2013). Chk1 phosphorylation induced by hydrogen peroxide and stalled replication forks was compromised by the addition of DLS in *Xenopus* LSS (Fig. 12A and 12B). Notably, the recruitment of checkpoint proteins including RPA32, ATR, ATRIP, and Rad9 to hydrogen peroxide-damaged chromatin was compromised with the presence of DLS, suggesting that DLS addition potentiates the generation of ssDNA in oxidative stress and the subsequence checkpoint protein recruitment (Fig. 12A). Similarly, DLS addition impairs the recruitment of checkpoint proteins including RPA32, ATR, ATRIP, and Rad9 to stalled replication forks (Fig. 12B).

Interestingly, however, celastrol treatment seemed to have a damage-dependent effect on the ATR-Chk1 DDR pathway. Whereas Chk1 phosphorylation induced by stalled replication forks was not affected by celastrol addition (Fig. 12D), the oxidative stress-induced Chk1 phosphorylation was vastly reduced with the celastrol treatment (Fig. 12C). Preliminary studies have shown slight different effect of DLS and celastrol on the recruitment of checkpoint proteins onto oxidative stress-damaged chromatin and stalled replication forks. More work is needed to validate these observations.

Nevertheless, the phenotype of Celasterol treatment in DDR pathway happens to be strikingly similar to that of the phenotype of lacking Pol η in DDR pathway.

Length-dependent recruitment of checkpoint proteins onto ssDNA in Xenopus HSS

The binding capacity for ATRIP to ssDNA was previously shown to be ~70-80 nt (Zou and Elledge, 2003). We wished to determine the length dependence on ssDNA for the recruitment of checkpoint proteins. Biotin-coupled oligonucleotides with different length were tested *in vitro* to determine checkpoint protein binding partners (Fig. 13A). RPA32 was able to bind to ssDNA as short as 10 nt, although this binding is weak compared to the 20 nt ssDNA (Fig. 13B). Surprisingly, ATR and ATRIP are also able to bind ssDNA as short as 20 nt (Fig. 13B). TopBP1 was not recruited to any of these ssDNA, which is consistent with the recent finding that TopBP1's binding to RPA-coated ssDNA requires a minimum of 150 nt (Acevedo et al., 2016). It is not a surprise that Rad9 was not found on the ssDNA as the 9-1-1 complex prefers the 5' recessed ssDNA/dsDNA junctions but not ssDNA only (Ellison and Stillman, 2003). Pol η was not found on the ssDNA; however, it will be interesting to test whether the binding of ATR and ATRIP onto ssDNA is compromised when Pol η is absent in HSS.

Checkpoint protein recruitment to Biotin-SSB-dsDNA in Xenopus HSS

We generated a SSB at a defined location on a dsDNA (Fig. 14A). As expected, Biotin-WT-dsDNA was cut by SbfI into 400bp and 200bp fragment (Fig. 14B). Biotin-SSB-dsDNA was resistant to the SbfI treatment, suggesting the SSB is generated at the defined location on the dsDNA (Fig. 14B). The checkpoint proteins including ATR, ATRIP, TopBP1, and RPA32 were preferentially recruited to the SSB-dsDNA but not WT-dsDNA in pulldown assays from HSS, suggesting that ATR, ATRIP, RPA32, and

TopBP1 bind to SSB sites in HSS (Fig. 14C). When Pol η is absent from extract, the binding ATR and ATRIP to SSB-dsDNA was slightly reduced (Fig. 14C). Further analysis of the role of Pol η in the recruitment of checkpoint proteins using this assay is crucial.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

In response to DNA damage and replication stress, cells have evolved various DNA repair, DDR, and DDT pathways to maintain genomic stability. However, the functional interplay between these different pathways remains largely unknown. As research into treatment approaches for degenerative disease and cancer is expanding, it is imperative to investigate the possible roles of TLS polymerases in DDR pathways. Recent studies have demonstrated that

Pol κ is important for the ATR-Chk1 DDR pathway activation in response to stalled replication forks (Fig. 3) (Betous et al., 2013). The objectives of this study are to determine roles of two TLS polymerases, REV1 and Pol η, in the ATR-Chk1 DDR pathway in response to DNA damage and replication stress.

As a TLS polymerase REV1 has been suggested to play a potential role in the DDT pathway. We reveal REV1 to be important for DNA damage-induced Chk1 phosphorylation under oxidative stress as well as UV-induced damage, MMC-induced ICLs, and APH-induced stalled forks (Figs. 4-6). REV1 likely plays a downstream role of the checkpoint protein complex assembly including ATR, ATRIP, TopBP1 and the Rad9-Rad1-Hus1 complex to MMC-induced ICLs and stalled replication forks on chromatin in the DDR pathway (Fig. 6). We also have evidence suggesting that REV1 interacts with the ATR-ATRIP complex in *Xenopus* egg extract, and this interaction could be vital for effective activation of Chk1 (Fig. 7). Thus, we propose a working model in which REV1

is important for Chk1 phosphorylation by activated ATR, which is downstream step of the DDR pathway (Fig. 8). The role of REV1 is different from that of Pol κ in the DDR pathway, which is stability of Rad17 and 9-1-1 recruitment (Fig. 3).

Surprisingly, Pol η is important for Chk1 phosporylation induced by oxidative stress but not stalled DNA replication forks (Figs. 9-10). This is possibly due to the presence of bulky adducts on DNA under oxidative stress, which aren't present in arrested replication forks. In addition, oxidatively damaged DNA is processed differently by the BER pathway compared to the stalled replication forks. As Pol η is involved in repair across cyclobutane pyrimidine dimers, it could play a role in repair of the ssDNA once the damaged bases are resected. It is possible that this polymerase plays a role in the BER pathway, as there is evidence suggesting that Pol η functions in several other repair pathways (Mcllwaith et al., 2005; Kawanoto et al., 2005). For example, apart from its catalytic function in TLS, Pol η can promote DNA synthesis in HR-mediated DSB repair (Kawanoto et al., 2005; Rattray et al., 2005).

Other research has implicated two possible mechanisms for DNA lesion bypass via the TLS polymerases, either of which could function in a variety of damage types. The polymerase-switching model suggests that when replicative polymerases encounter a lesion, there is a rapid shift to the TLS polymerase to continue primer extension during replication at the primer-template junction. Once the lesion is efficiently bypassed, the replicative pols will continue synthesis. This polymerase switching might occur via protein modification (i.e. PCNA unbiquitination) or other protein interactions, possibly through a REV1 scaffold (Friedberg et al., 2005). In the gap-filling model, an ssDNA gap opposite a lesion is filled via the TLS polymerases and subsequently resealed with DNA

ligase. Repair of the lesion would occur after the gap has been filled using one of the DNA repair pathways. The aforementioned "gap-filling model" is indicative of the ssDNA intermediate seen in the BER pathway, which is largely responsible for the repair of oxidatively damaged DNA.

We propose several possible mechanisms for Pol η's role in the ATR-Chk1 pathway under oxidative stress (Fig. 15):

(I) Pol η may be an essential interacting partner for the BER enzymes APE1 or APE2 to promote SSB end resection in oxidative stress. When we remove Pol η from extract, our results indicate reduced hyperloading of RPA32 onto chromatin, suggesting that the ssDNA generation via SSB end resection is compromised (Fig. 10A). We have shown that Pol η interacts with APE1 in LSS and may have an oxidative damage-dependent interaction with ATR (Fig. 11). APE1 endonuclease activity is responsible for the initial nicking of the sugar-phosphate backbone adjacent to the DNA lesion. Pol η might potentially increase APE1 affinity for dsDNA and Pol η absence sufficiently reduces damage-dependent DNA exonuclease activity. Previous literature has also supported a role for APE2 in creating the ssDNA gaps required for ATR-Chk1 activation following oxidative stress (Willis et al., 2013). Evidence for a possible interaction with APE2 exists in evidence linking APE2 to ATR-Chk1 pathway activation in response to oxidative damage, but not stalled replication forks (Willis et al., 2013). These observations suggest that the performance or presence of APE1 and/or APE2 and subsequent formation of ssDNA intermediates are compromised when Pol η is absent (Fig. 15). It is crucial to further investigate the exact role of Pol η in the recruitment of APE1 and APE2 to oxidatively damaged DNA. Another possibility is that Pol η may

interact with an unidentified protein responsible for RPA loading onto ssDNA in the ATR-Chk1 DDR pathway.

(II) Pol η may play an important role for the recruitment of ATR and ATRIP onto RPA-coated ssDNA at SSB site. Pol η is important for the recruitment of ATR and ATRIP onto RPA-coated ssDNA in oxidative stress but not stalled replication forks (Fig. 10). Functional uncoupling of DNA polymerase and helicase can generate a long stretch of ssDNA, which is estimated to ~ hundreds of nt ssDNA (Byun et la., 2005). However, the ssDNA generated at SSB site in oxidative stress is very small and estimated to be only 10-30 nt. Furthermore, *in vitro* ssDNA binding assays have demonstrated that ATR and ATRIP can bind to RPA-ssDNA with only 20 nt ssDNA (Fig. 13). In addition, the binding of ATR and ATRIP is slightly diminished when Pol η is absent (Fig. 14). These observations suggest that Pol η may play an important role promoting the recruitment of ATR and ATRIP onto ssDNA at SSB sites.

If the catalytic function of Pol η is required for its role in the ATR kinase activation, then this would be through its continued DNA synthesis past primers. This elongation would enable a structure required for 9-1-1 complex loading, a key component to ATR-Chk1 activation. The DNA polymerase α has been shown to be directly required for the loading of the 9-1-1 complex onto damaged replication stress loci (Yan and Michael, 2009b). This occurs via the unique 5'-3' primase activity on on ssDNA, which creates the 5' dsDNA-ssDNA junction, a necessary structure for 9-1-1 loading. TopBP1 is an important sensor protein for ssDNA-RPA and thus, also with Polα, will recruit 9-1-1 complex to ssDNA-dsDNA junctions. In a similar role to that of Pol α, elongation via Pol η DNA extension may be one way in which Pol η contributes to ATR-Chk1 activation,

creating additional ssDNA-dsDNA junctions for which 9-1-1 complex to bind. However, considering the role of Pol η in oxidative stress response but not in replication stress response, it is unlikely that the catalytic function of Pol η is important for the ATR-Chk1 DDR pathway.

The TLS pathway has evolved to provide protection from DNA damaging agents. Although mutagenic, TLS improves genomic integrity enough to avoid larger mutations such as translocations and chromosome alterations, which would prove more lethal to cells. Understanding the exact mechanism for TLS recruitment to damaged DNA and the association with DDR pathway can allow us to re-think the ways in which genomic integrity is maintained. By investigating these TLS polymerases, we hope to contribute an understanding of their function that could lead to improved cancer therapies and knowledge of DNA repair and DDR related diseases. More investigations are needed to further understand how exactly TLS polymerases function in the DDR pathway and how TLS polymerases contribute to genomic stability and cancer development.

Taken together, we have demonstrated that TLS polymerases REV1 and Pol η are important for the ATR-Chk1 DDR pathway.

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APPENDIX A: FIGURES

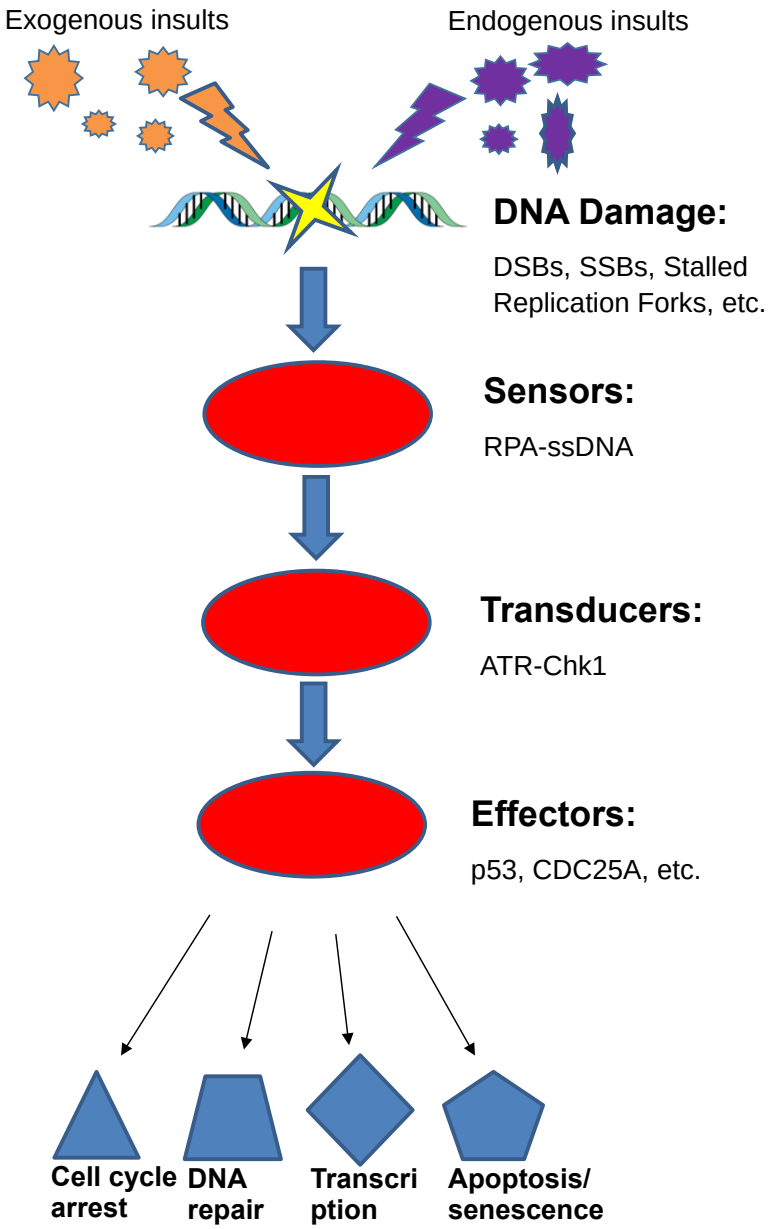


Figure 1: Through ATR, the DDR pathway can transduce the signals from sensors (RPA coated ssDNA) and activate effector proteins to initiate the appropriate cellular response. The phosphatase CDC25a, for instance, is inhibited by Chk1 activation, which ultimately prevents cell-cycle progression.

TLS	Function
REV1	• dCMP insertion • Scaffolding protein for other TLS pols
Pol η (eta)	• Inserts dAMP and dTMP past lesion • Error free
Pol κ (kappa)	• Inserts dGMP or dCMP • Error-prone
Pol ι (iota)	• Inserts dATP, dCTP, or dTTP • Error-prone
Pol ζ (zeta)	• Contains REV3 and REV7 subunits • Extension past mismatched 3' nucleotide

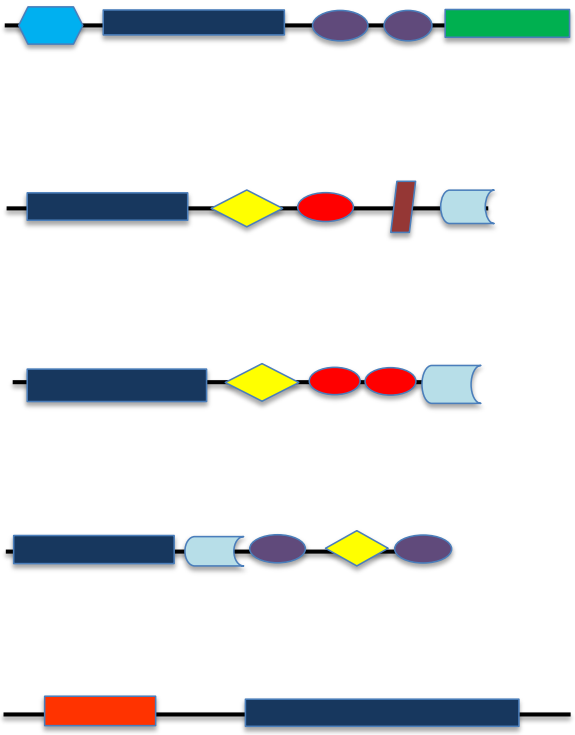


Figure 2: Representation of the structural domains of four human Y-family TLS polymerases and one B-family polymerase (Pol ζ). BRCT domain indicates the Brca1 C-terminal domain, UBM is the ubiquitin binding motif, and UBZ is ubiquitin-binding zinc finger domain.

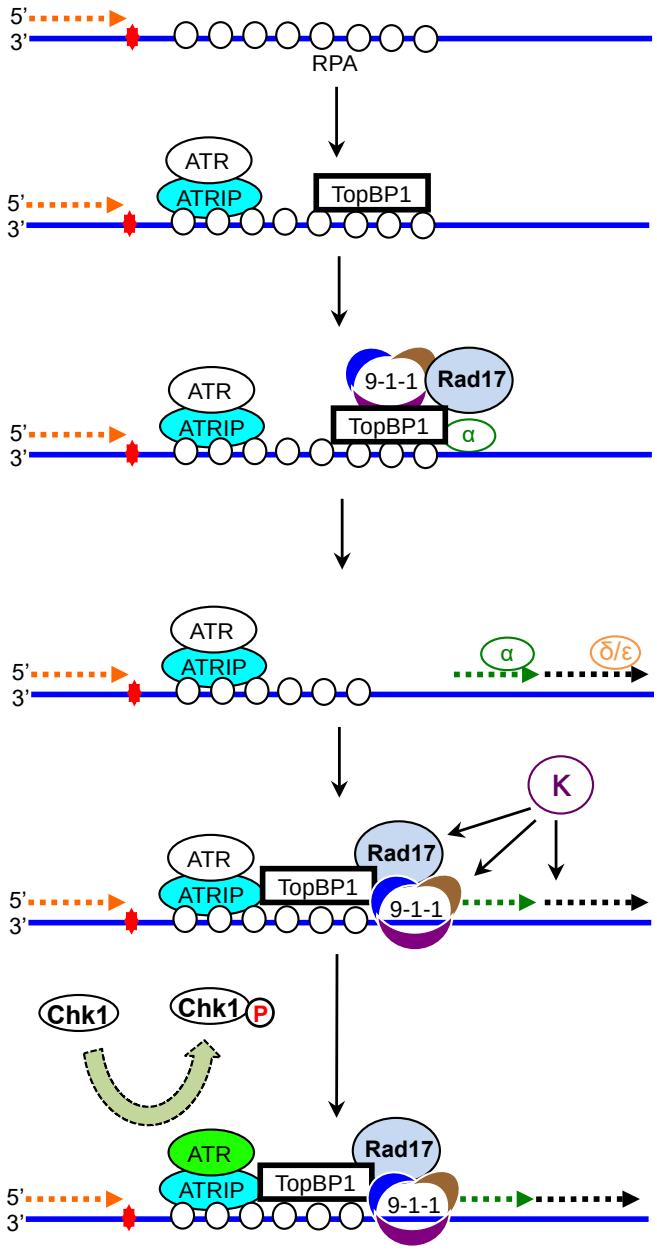


Figure 3: Pol κ requires DNA primer formation via Polα in order to be recruited to the chromatin. It potentially acts on the DDR pathway directly through DNA synthesis i.e. elongation of the nascent strand, through interaction with the Rad9 subunit of the 9-1-1 complex, or through a Rad17 interaction. TopBP1 is required for both 9-1-1 and Polα recruitment, so Pol κ may form a complex with these mediators.

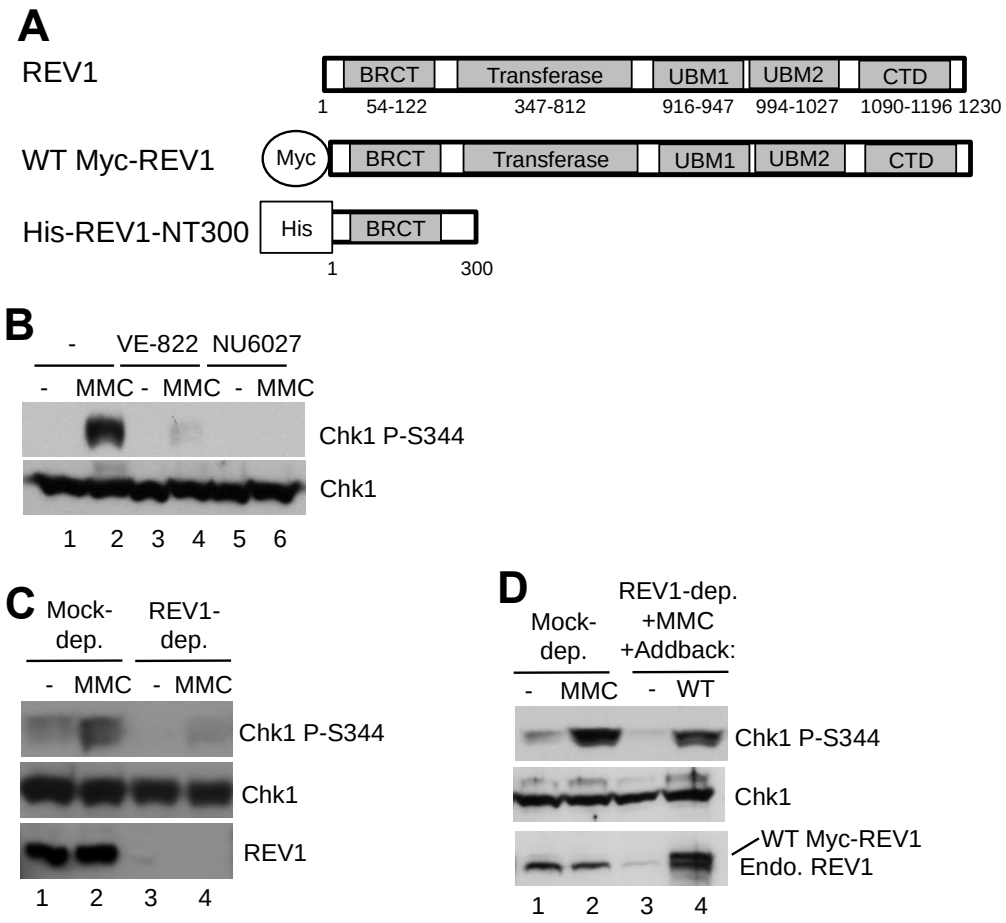


Figure 4: REV1 is important for the MMC-induced Chk1 phosphorylation in Xenopus egg extracts. (A) Schematic demonstration of WT REV1, Myc-tagged FL REV1, and His-tagged NT 300aa REV1 sequence. (B) VE-822 or NU6027 was incubated in egg extracts supplemented with sperm chromatin and MMC. After 1-hr incubation, Chk1 phosphorylation and total Chk1 in extracts were examined via immunoblotting analysis. (C) MMC or was incubated in mock-depleted and REV1-depleted egg extracts supplemented with sperm chromatin. After 1-hr incubation, Chk1 phosphorylation at the Serine 344 residue and total Chk1 in extracts were examined were isolated and examined via immunoblotting analysis as indicated. (D) WT Myc-REV1 ("WT") or an egg lysis buffer ("-") was added to REV1-depleted egg extract, which was supplemented with sperm chromatin and MMC. Total extracts were examined for indicated proteins via immunoblotting analysis. "Endo. REV1" represents endogenous REV1.

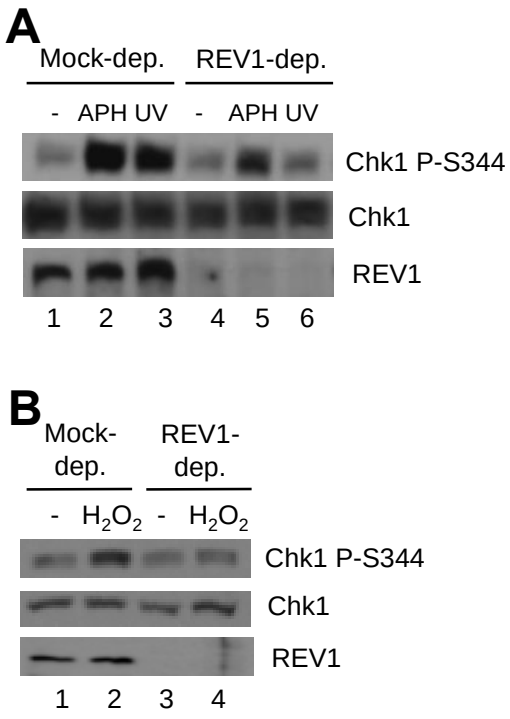


Figure 5: Chk1 phosphorylation induced by stalled replication forks, UV damage, or oxidative stress is compromised when REV1 is absent in Xenopus egg extracts. (A) Sperm chromatin was added to Xenopus egg extracts supplemented with APH, as indicated. Prior to addition for extract, additional sperm chromatin (4000sperm/µl) was treated with UV-light to induce UV damage and added into extract. Both treatments were applied to extract containing REV1 and lacking REV1. Chk1 phosphorylation at S344 and total Chk1 were examined via immunoblotting. (B) Sperm chromatin was added to egg extracts with the presence or absence of H_2O_2 (100 mM) to mock-depleted or REV1-depleted egg extracts.

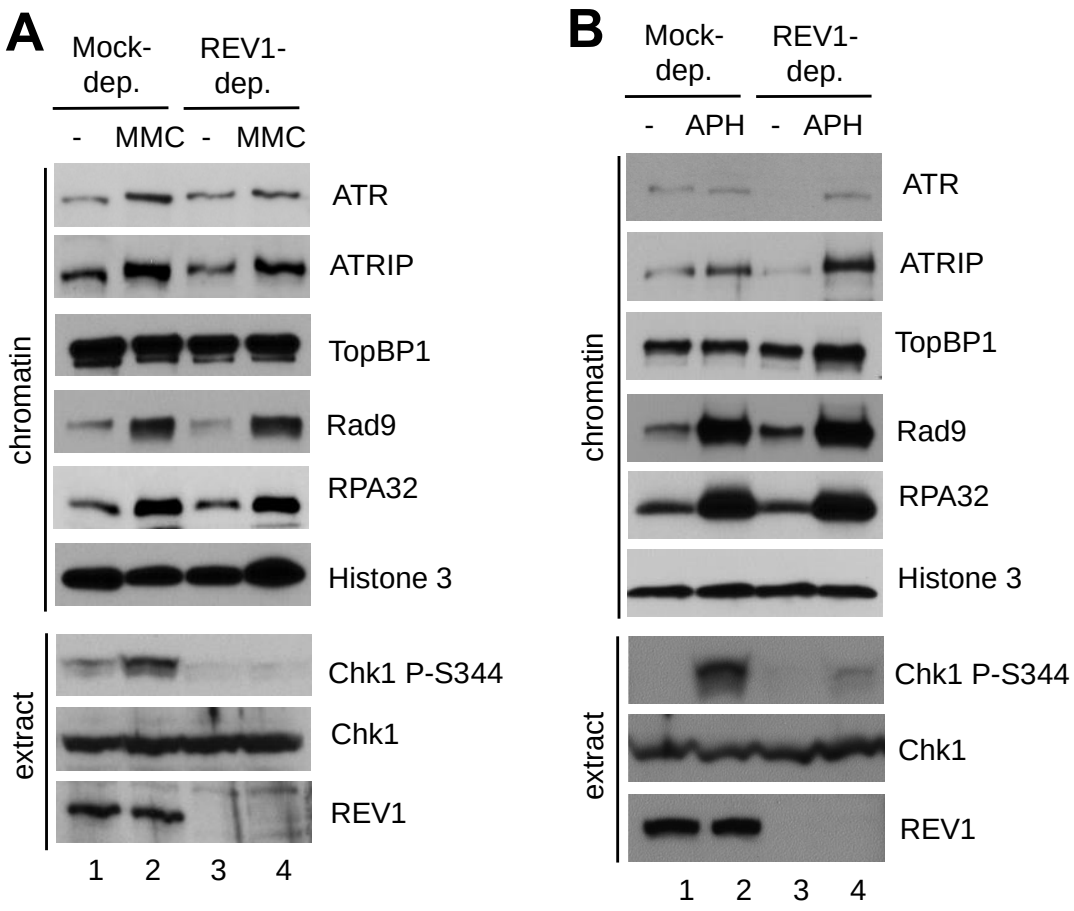


Figure 6: REV1 is dispensable for the recruitment of ATR, ATRIP, TopBP1, Rad9, and RPA32 to interstrand crosslinks and stalled replication forks in *Xenopus* egg extracts. (A) Sperm chromatin was added to Xenopus egg extracts treated with MMC, causing ICLs in CE. The presence or absence of MMC was examined in mock-depleted and REV1-depleted extract. Chk1 phosphorylation at S344 and total Chk1 were examined via immunoblotting. (B) Sperm chromatin was added to egg extracts with the presence or absence of APH, causing replicative polymerase stalling. Chromatin-bound fractions ("chromatin" panel) and total egg extracts ("extract" panel) were analyzed for the indicated proteins via immunoblotting. Histone 3 is used as a loading control for all chromatin-assessed proteins. Total Chk1 is used as a loading control for proteins in extract.

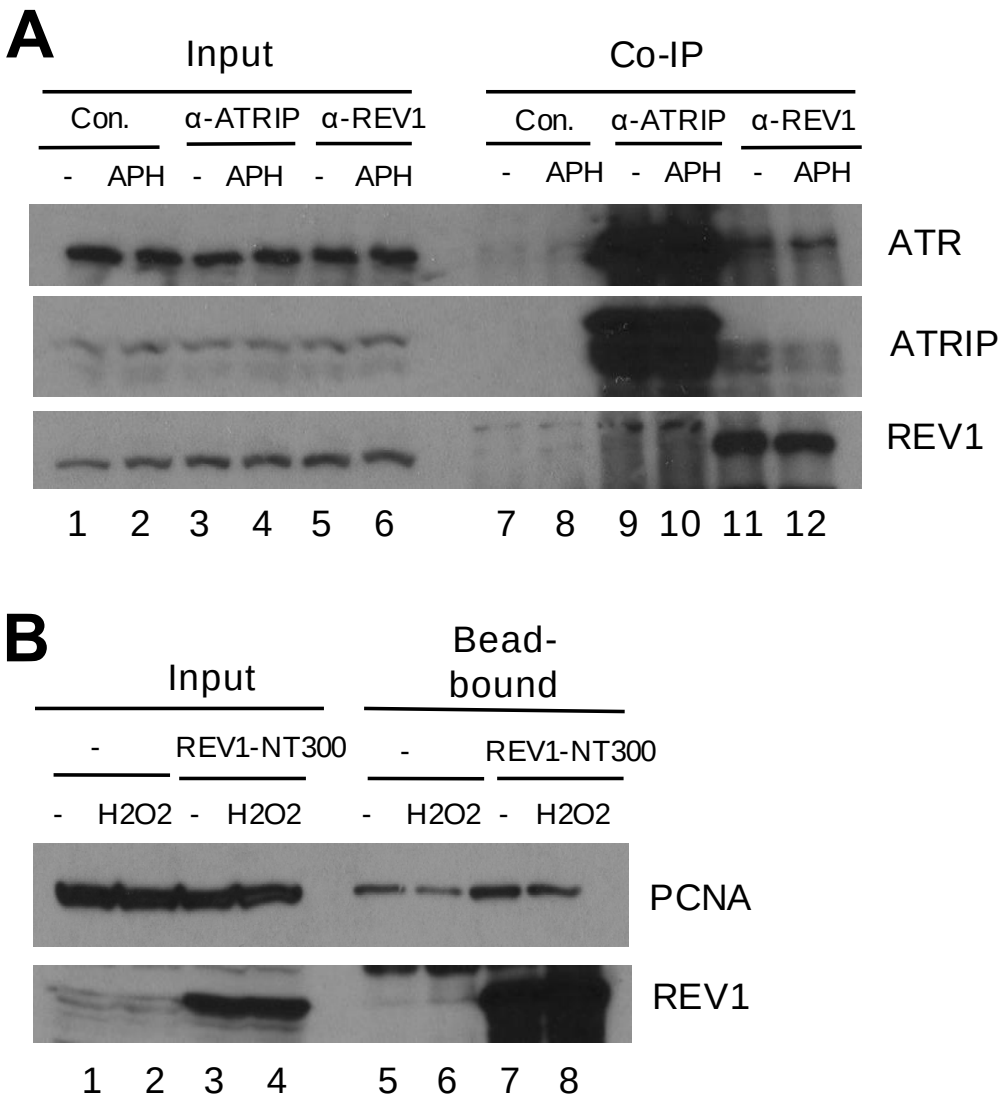


Figure 7: REV1 interacts with the ATR/ATRIP complex to initiate Chk1 activation. (A) anti-REV1 ab. is able to pull down out endogenous REV1 protein. APH is added to extracts following SC treatment. Compared to control Co-IP lanes, anti-REV1 pull-down indicates a possible association with ATR. (B) Ni-NTA beads coupled to REV1-NT300 are able to pull-down PCNA in LSS.

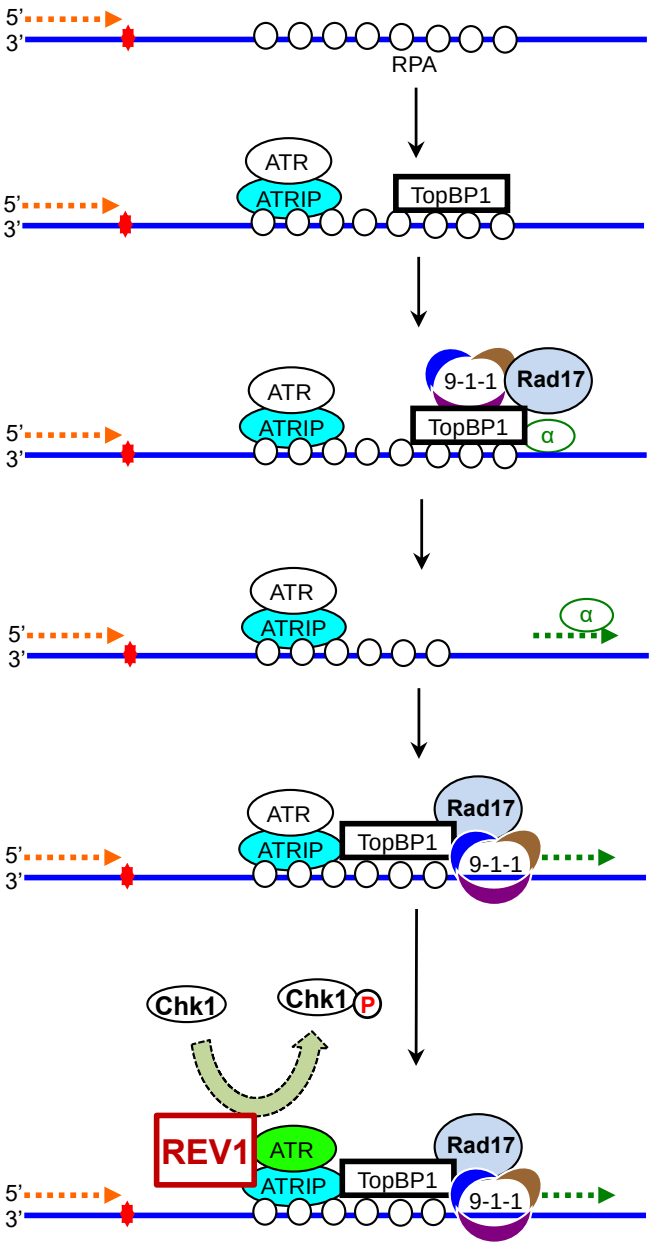


Figure 8: A working model for the role of REV1 in DDR pathway in response to ICLs and stalled replication forks. REV1 interacts with the ATR/ATRIP complex in order to activate ATR-Chk1 kinase. This interaction make function transiently through a complex with Proliferating cell nuclear antigen (PCNA), or independently of PCNA. Additionally, REV1 may have a role in the ATR kinase activity despite any protein-protein interaction with the enzyme.

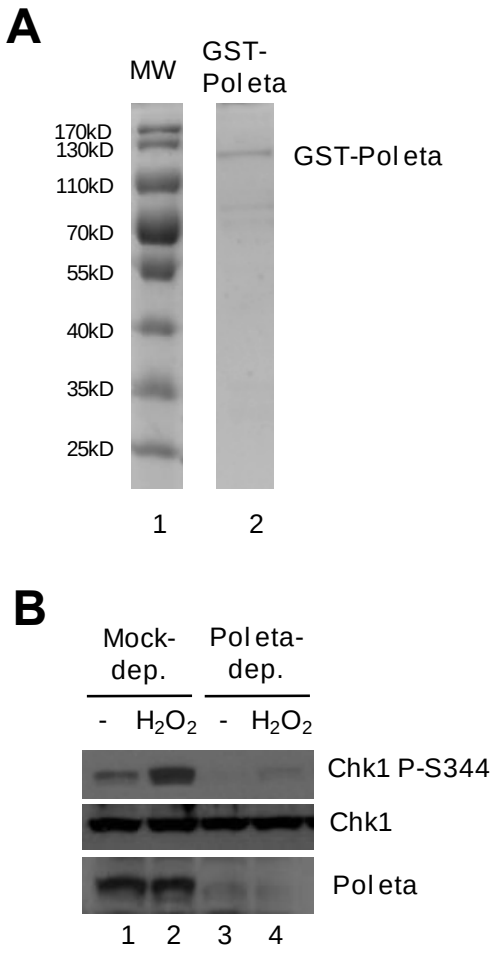


Figure 9: Generation of recombinant GST-tagged Pol eta and customized anti-Pol eta antibodies. (A) Recombinant GST-FL Pol η protein was expressed from the GST-FL Pol η plasmid after bacterial transformation and IPTG induction. Polyclonal antibodies for this protein were generated using two rabbits, UNC398 and UNC399. (B) UNC399 serum was able to efficiently detect endogenous Pol η in mock-depleted LSS. UNC398 serum was able to remove endogenous Pol eta from LSS. Hydrogen peroxide-induced Chk1 phosphorylation was compromised in REV1-depleted LSS. Histone 3 and Chk1 are used as loading controls.

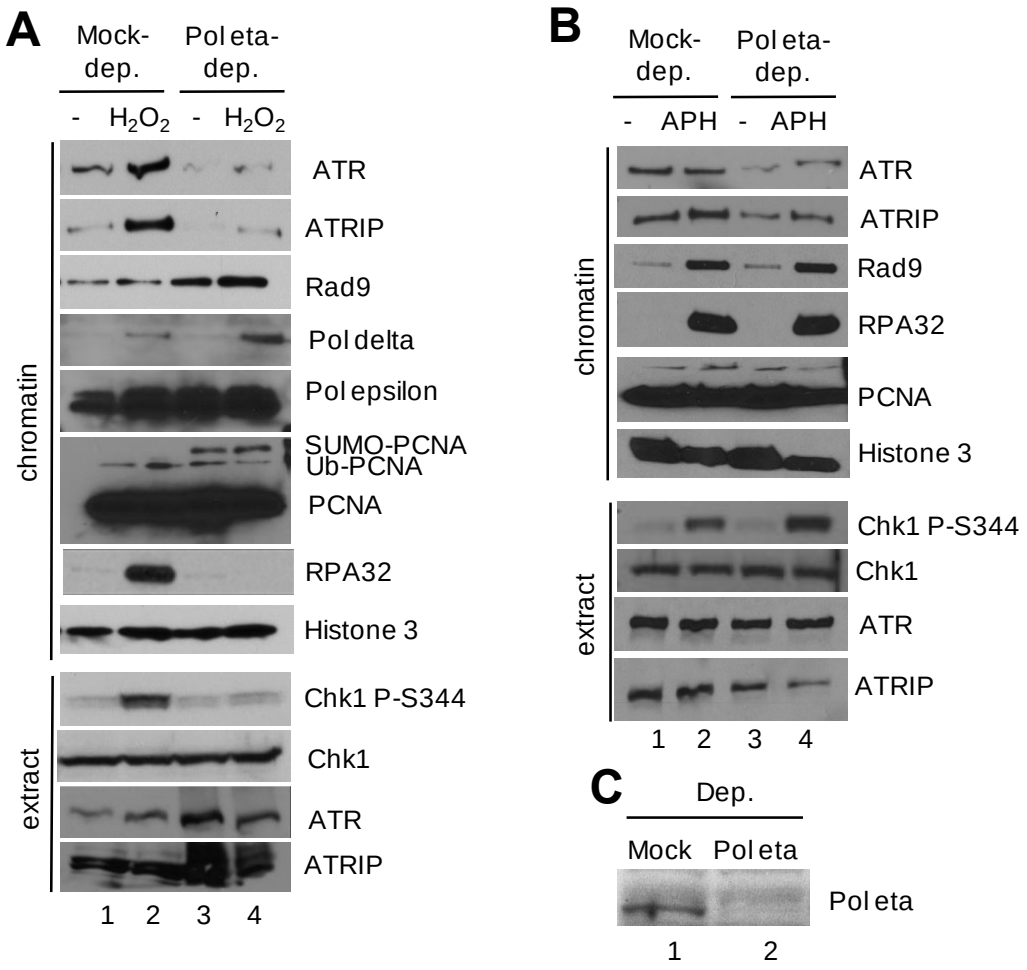


Figure 10: Pol eta is important for Chk1 phosphorylation induced by oxidative stress but not by stalled replication forks in *Xenopus* egg extracts. (A) Sperm chromatin was added to egg extracts supplemented with H2O2 (100mM). The presence or absence of H2O2 was examined in mock-depleted and Polη-depleted extract. Chk1 phosphorylation at S344 and total Chk1 were examined via immunoblotting. (B) Sperm chromatin was added to egg extracts with the presence or absence of APH, causing replicative polymerase stalling. Chromatin-bound fractions ("chromatin" panel) and total egg extracts ("extract" panel) were analyzed for the indicated proteins via immunoblotting. (C) Western blot analysis reveals Polη is sufficiently depleted from the extract.

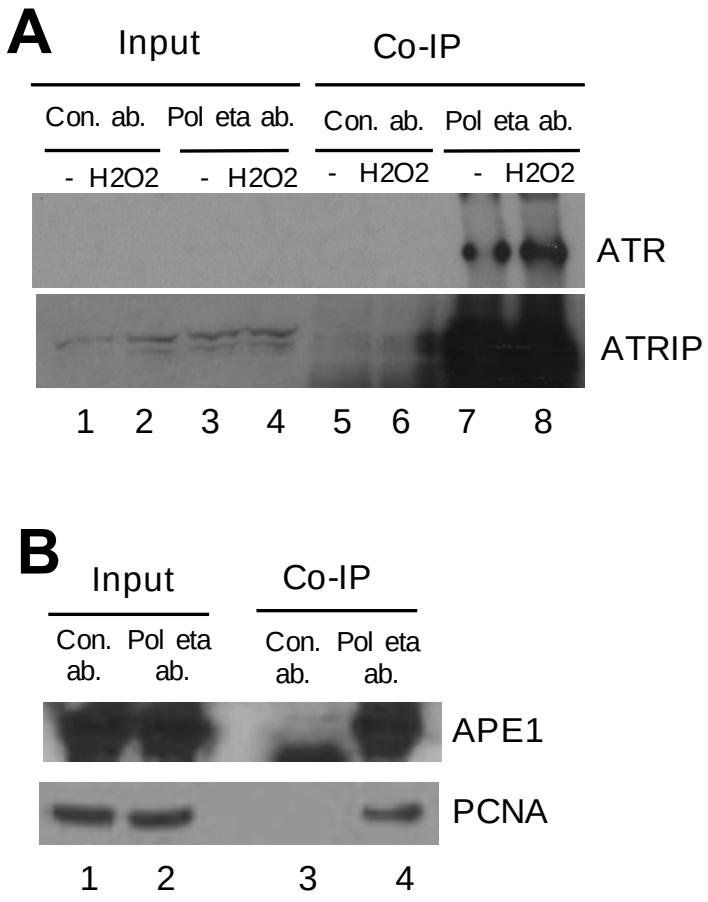
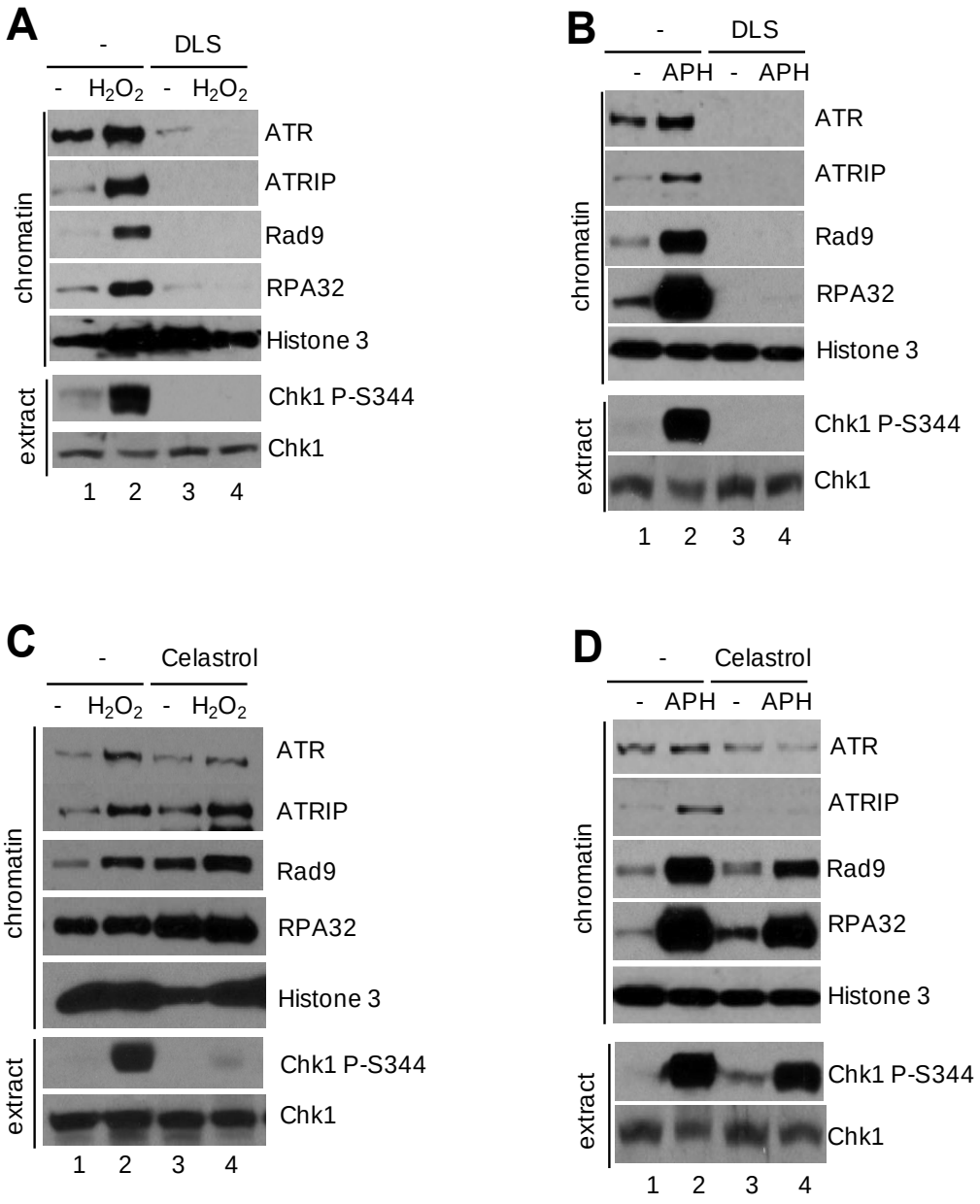
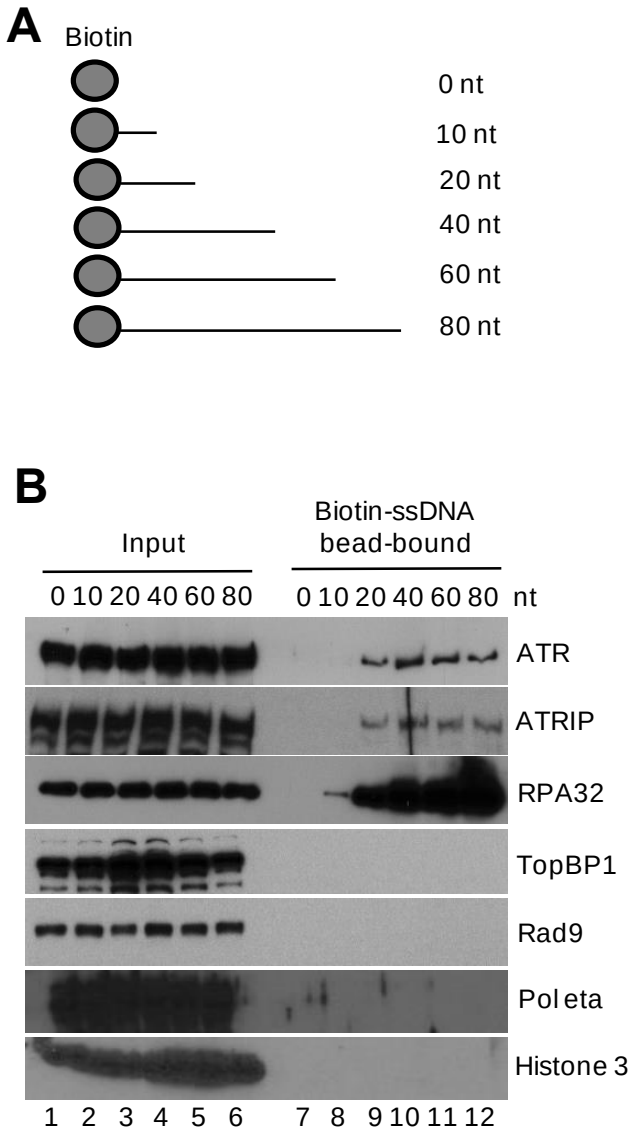


Figure 11: Pol eta interacts with ATR, ATRIP, PCNA, and APE1 in Xenopus egg extracts. (A) Anti-Pol η coupled to protein A-sepharose beads is able to pull down ATR/ATRIP complex. H_2O_2 may increase the affinity of association between Pol eta and ATR-ATRIP. (B) Anti-Pol η antibodies but not control antibodies were able to pull down APE1 and PCNA in LSS.



Figures 12: Small molecular inhibitors of ATR-Chk1 pathways. (A) 1.5 µl DL-Sulforaphane (DLS) is added to which were supplemented with sperm chromatin and H_2O_2. (B) DLS is added to egg extracts which were supplemented with sperm chromatin and aphidicolin (APH). (C) 1.5µl Celasterol is added to egg extracts which were supplemented with sperm chromatin and H_2O_2. (D) Celasterol is added to egg extracts which were supplemented with sperm chromatin and APH. For each experiment, Histone 3 is used as a loading control for chromatin analysis and total Chk1 is the loading control for proteins in extract.



Figures 13: Length-dependent recruitment of checkpoint proteins onto ssDNA in *Xenopus* egg extracts. (A) Six biotin-labeled oligonucleotides with different length were tested *in vitro* to determine checkpoint protein binding partners. The lengths were 10, 20, 40, 60, and 80 nucleotides with a single biotin molecule covalently attached to the 5' end. The oligonucleotide sequence was derived from the same plasmid used to test SSBR in vitro, pUC19. (B) 200ng/ml of biotin-labeled oligonucleotides were incubated with HSS for 1 hour at room temperature. Prior to incubation, 5µl of HSS was removed from each reaction to run as input samples on the gel. After washing, the bead-bound fractions were examined via immunoblotting analysis as indicated.

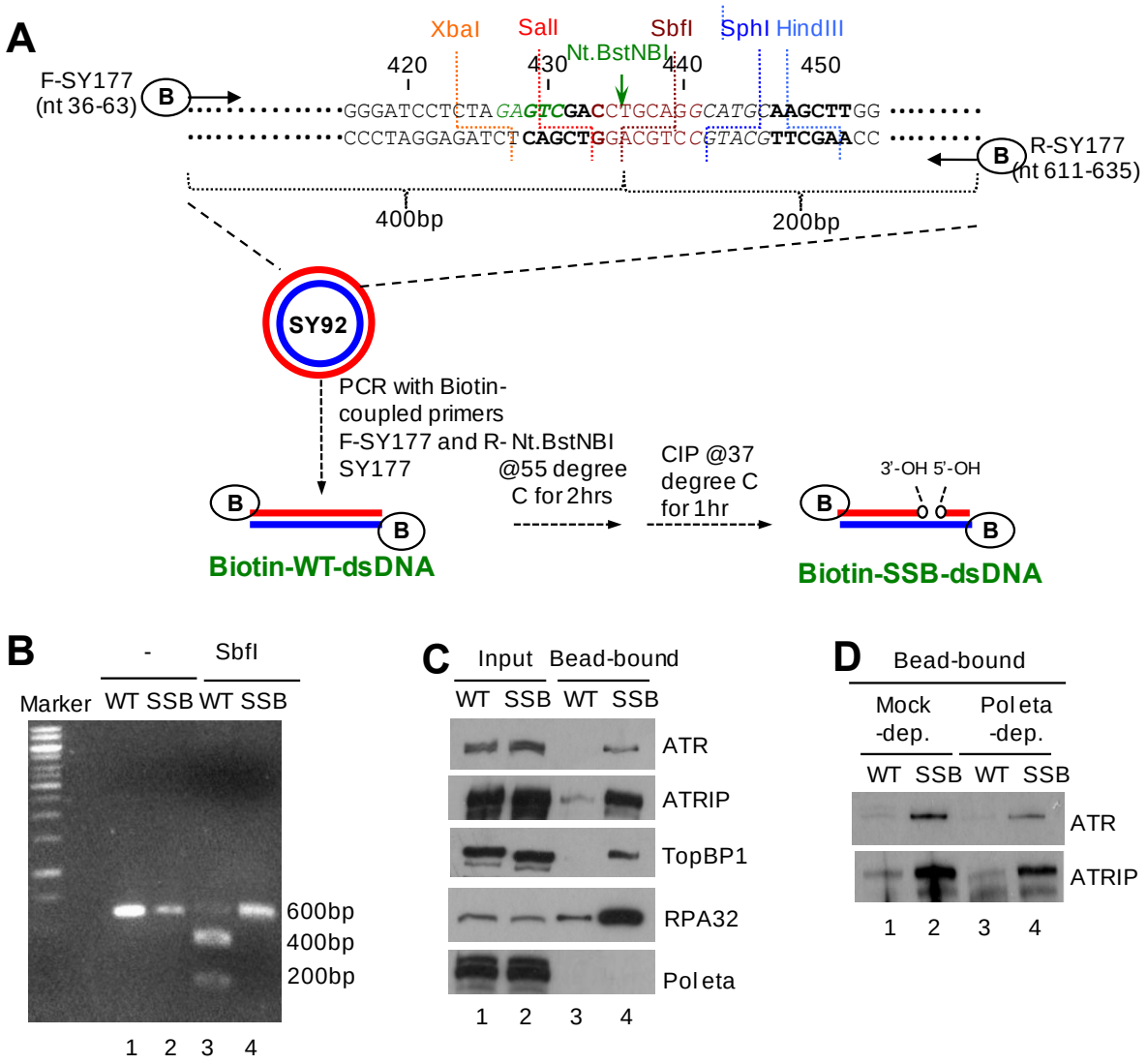


Figure 14: A role for Pol η in the recruitment of ATR and ATRIP onto a defined SSB-dsDNA structure in HSS. (A) Schematic diagram of generating biotin-WT-dsDNA and biotin-SSB-dsDNA. The cut site for the restriction endonuclease Nt.BstNBI occurs between C435 and T436 on the plus strand in the SY92 plasmid. The cut site for Sbf1 is also indicated. (B) The verification of SSB-dsDNA through treatment with restriction enzyme Sbf1. Under Sbf1 treatment, the SSB will not create two dsDNA intermediates as seen in the WT (un-nicked) DNA. (C) Pull-down of Biotinylated dsDNA using Invitrogen Dynabeads™ indicates preferential binding of several key checkpoint proteins to SSB-dsDNA compared to WT-dsDNA control (D) A role for Pol η in checkpoint protein recruitment to the Biotin-SSB-dsDNA structures was determined by Pol η depletion from HSS followed by a pulldown assay.

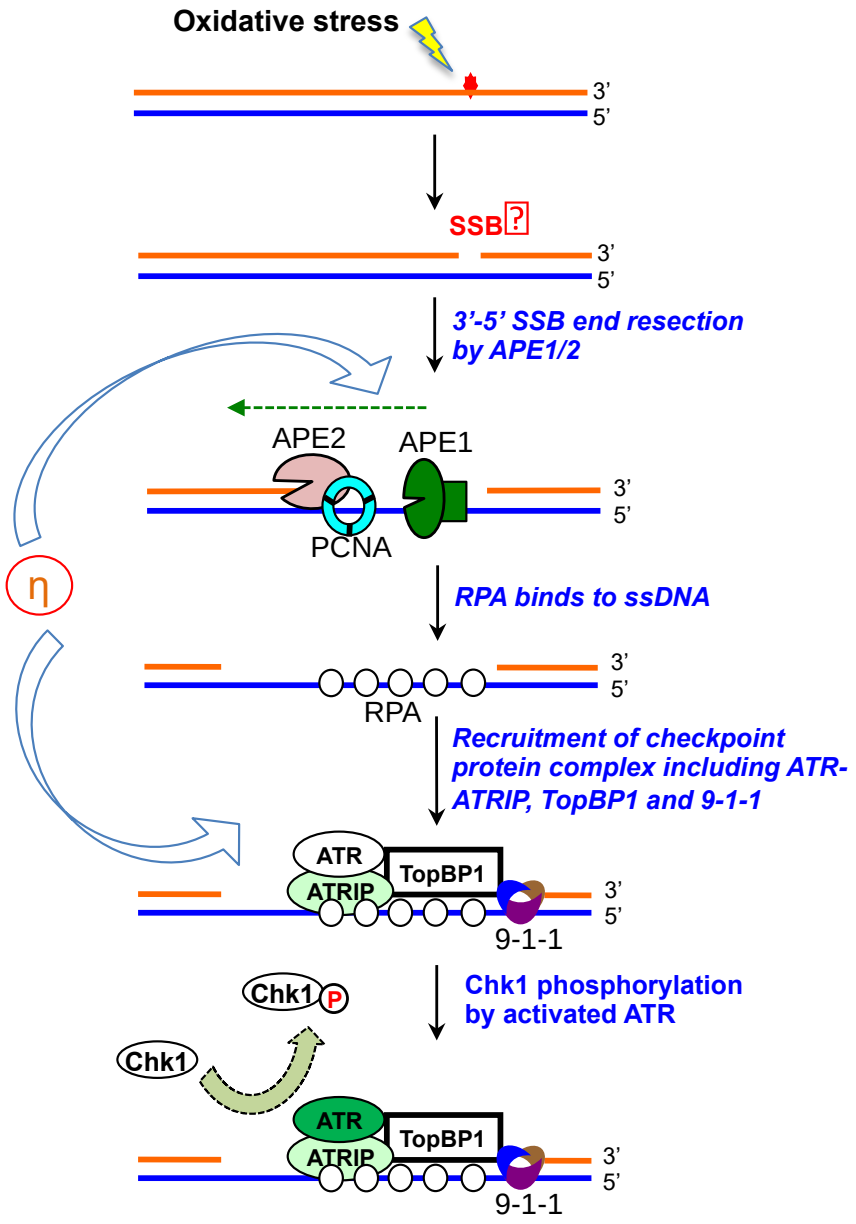


Figure 15: A working model for the role of Pol η in DNA damage response in oxidative stress. Pol η has a potential for recruitment of APE1/2 to damaged loci. This could be either through complex formation with PCNA, or via direct interaction with one of the endonucleases. Alternatively, Pol η may promotes the recruitment of ATR and ATRIP onto RPA-coupled ssDNA via its interaction with ATR-ATRIP.

PUBLICATIONS AND PRESENTATIONS

Publications:

1. **Melissa M McLeod**, Shan Yan. 2017. Pol eta plays an distinct role in the DNA damage response pathway in oxidative stress but not replication stress response. (Manuscript in preparation)
2. Darla DeStephanis*, **Melissa M McLeod***, Shan Yan. 2015. REV1 is important for the ATR-Chk1 DNA damage response pathway in *Xenopus* egg extract. Biochemical and Biophysical Research Communications. 460 (3): 609-615. (* contributed equally to this work) (PMCID: PMC4426025) DOI: http://dx.doi.org/10.1016/j.bbrc.2015.03.077
3. Ryan Y Wong, **Melissa M McLeod**, John Godwin. 2014. Limited sex-biased neural gene expression patterns across strains in Zebrafish (Danio rerio). BMC Genomics. 15 (1): 905. (PMCID: PMC4216363) http://dx.doi.org/10.1186/1471-2164-15-905

Presentations:

1. Darla DeStephanis D, **Melissa M McLeod**, Shan Yan. REV1 is important for the ATR-Chk1 DNA damage response pathway in *Xenopus* egg extract. Center of Biomedical Engineering and Science (CBES) and the Charlotte Research Institute (CRI) Graduate Student Poster Competition, UNC Charlotte. Spring 2015.
2. **Melissa M McLeod**. Presented research to faculty and graduate students regarding the paper "REV1 is important for the ATR-Chk1 DNA damage response pathway in *Xenopus* egg extract." Biology Graduate Student Research Awards, UNC Charlotte. Spring 2015.