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Cell-free *Xenopus* egg extracts for studying DNA damage response pathways

STEVEN CUPELLO, CHRISTINE RICHARDSON and SHAN YAN*

Department of Biological Sciences, University of North Carolina at Charlotte, Charlotte, NC, USA

ABSTRACT In response to a variety of DNA replication stress or DNA damaging agents, the DNA damage response (DDR) pathways are triggered for cells to coordinate DNA repair, cell cycle checkpoints, apoptosis, and senescence. Cell-free *Xenopus* egg extracts, derived from the eggs of African clawed frogs (*Xenopus laevis*), have been widely used for studies concerning DDR pathways. In this review, we focus on how different experimental systems have been established using *Xenopus* egg extracts to investigate the DDR pathways that are activated in response to DNA replication stress, double-strand breaks (DSBs), inter-strand crosslinks (ICLs), and oxidative stress. We summarize how molecular details of DDR pathways are dissected by the mechanistic studies with *Xenopus* egg extracts. We also provide an update on the regulation of translesion DNA synthesis (TLS) polymerases (Pol κ and REV1) in the DDR pathways. A better understanding of DDR pathways using *Xenopus* egg extracts has opened new avenues for future cancer therapeutics. Finally, we offer our perspectives of future directions for studies of DDR pathways with *Xenopus* egg extracts.

KEY WORDS: *Xenopus egg extracts, DNA damage response, ATR-Chk1, ATM-Chk2, TLS*

Introduction

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 (Gandini Attardi *et al.*, 1976). Assembly of SV40 chromatin was reported in a cell-free *Xenopus* egg extract system (Laskey *et al.*, 1977). 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) (Blow *et al.*, 1987; MacDougall *et al.*, 2007; Raschle *et al.*, 2015; Williams *et al.*, 2012; Yan and Michael, 2009b).

The genomes of all cells are exposed to a variety of insults from endogenous and exogenous sources (Ciccia and Elledge, 2010; Yan *et al.*, 2014), leading to DNA replication stress, double-strand breaks (DSBs), inter-strand crosslinks (ICLs), and oxidative stress (Fig. 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 (Jackson and Bartek, 2009). In addition, cells develop a tolerance to DNA damaging agents, possibly through the translesion DNA synthesis (TLS) pathway that include Y-family DNA polymerases (REV1, Pol η, Pol κ and Pol ι) and a B-family DNA polymerase Pol ζ (Ho and Scharer, 2010). 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 (Chang and Cimprich, 2009). Although we have acquired a better understanding of DDR pathways in the last 20 years or so (Ciccia and Elledge, 2010), 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 reach other in cellular responses to DNA damage or replication stress.

Xenopus egg extracts are an excellent cell-free model system to investigate critical questions in the field of DDR pathways (Willis *et al.*, 2012). In this review article, we will summarize how experimental systems are established using *Xenopus* egg extracts to better understand the molecular mechanisms underlying DDR pathways in response to DNA damage or replication stress. We also provide an update on the positive regulation of TLS polymerases (Pol κ and REV1) in the DDR pathways as well as our perspectives of future directions using *Xenopus* egg extracts as

Abbreviations used in this paper: DDR, DNA damage response; DSB, double-strand break; ICL, inter-strand crosslink; TLS, translesion DNA synthesis.

***Address correspondence to:** Shan Yan. Department of Biological Sciences, University of North Carolina at Charlotte, Charlotte, NC 28223, USA. Tel: +1-704-687-8528. Fax: +1-704-687-1488. E-mail: shan.yan@uncc.edu - web: https://clas-pages.uncc.edu/shan-yan

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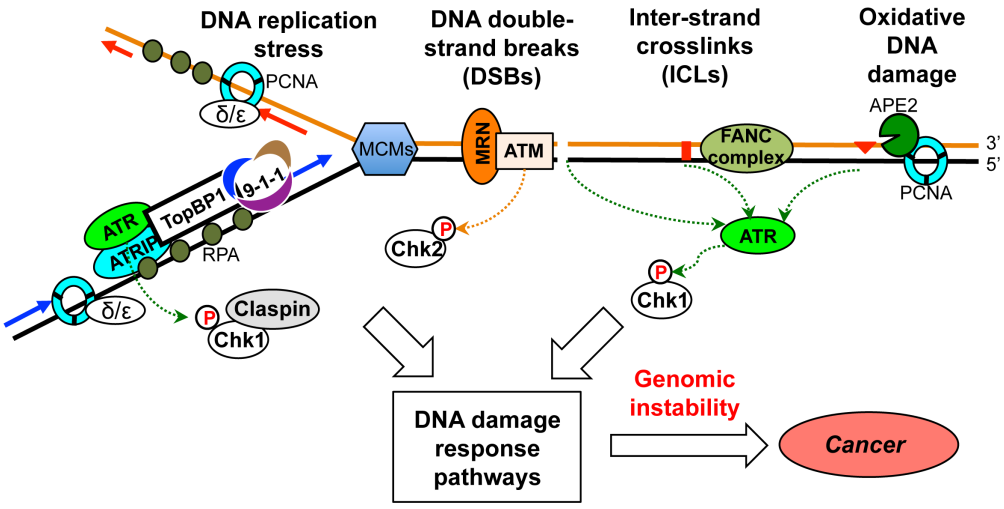

Fig. 1. Schematic diagram of DNA damage response (DDR) pathways in response to DNA replication stress, DNA double-strand breaks, inter-strand crosslinks, and oxidative DNA damage. *The black and orange lines represent two strands of DNA that is unwounded by DNA helicase during DNA replication. The blue 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.*

a model system for mechanistic studies of the DDR pathways.

DNA damage response pathways in cancer biology

The DDR pathways include the ATR-Chk1 and ATM-Chk2 checkpoint signaling cascades, coordinating DNA repair with cell cycle progression and apoptosis/senescence (Branzei and Foiani, 2010; Ciccia and Elledge, 2010; Cimprich and Cortez, 2008; Harrison and Haber, 2006). Defects in DDR pathways lead to genomic instability, which is a hallmark of cancer (Hanahan and Weinberg, 2011). 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 (Byun *et al.*, 2005; Ciccia and Elledge, 2010; Cimprich and Cortez, 2008). The 5'-3' end resection of DSBs mediated by CtIP nuclease also activates the ATR-Chk1 pathway (Kousholt *et al.*, 2012; Sartori *et al.*, 2007). ATR is recruited to RPA-coated ssDNA via direct interaction between RPA and ATRIP (Zou and Elledge, 2003). ATR activation requires several mediator proteins including its interacting protein ATRIP, TopBP1 and the 9-1-1 (Rad9-Rad1-Hus1) complex (Delacroix *et al.*, 2007; Kumagai *et al.*, 2006; Yan and Michael, 2009b; Zou and Elledge, 2003). Activated ATR phosphorylates multiple substrates including Chk1 (Matsuoka *et al.*, 2007). Chk1 is activated upon phosphorylation, serving as an indicator of ATR activation (Chen and Sanchez, 2004). The ATR-Chk1 pathway can also be activated in response to oxidative stress, inter-strand crosslinks (ICLs), and ultraviolet light (UV) (Ben-Yehoyada *et al.*, 2009; Guo *et al.*, 2000; Willis *et al.*, 2013). In addition, ATR is reported to be autophosphorylated after DNA damage at its Thr 1989 residue (Liu *et al.*, 2011).

In response to DSBs, ATM can be activated by autophosphorylation and dimer dissociation (Bakkenist and Kastan, 2003; Lee and Paull, 2005). This ATM kinase activation requires the Mre11-Rad50-Nbs1 (MRN) complex as well as other factors (Paull, 2015). Once activated, ATM kinase phosphorylates a number of substrates including Chk2 and p53 (Rotman and Shiloh, 1999; Smith *et al.*, 2010). Defective ATM kinase is associated with neurodegenerative disease ataxia-telangiectasia (Lavin, 2008). 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 (Lavin *et al.*, 2015; Paull, 2015). Accumulating evidence suggests that ATM is activated by conformational change during oxidative stress, which is independent of the MRN complex (Guo *et al.*, 2010; Khoronenkova and Dianov, 2015).

Dysfunctions in DDR signaling pathways are implicated in cancer development and characterized in primary patient tumors (Charames and Bapat, 2003; Curtin, 2012). Importantly, multiple DDR proteins are potent therapeutic targets for anti-cancer therapy in preclinical and clinical studies (Fokas *et al.*, 2014; Hosoya and Miyagawa, 2014; Kastan and Bartek, 2004). For example, ATR, Chk1, ATM, and Chk2 are targets for anti-cancer therapy via inhibiting their kinase activities (Antoni *et al.*, 2007; Fokas *et al.*, 2012; Toledo *et al.*, 2011; Weber and Ryan, 2015). Studies of pharmacological inhibitors targeting DDR pathways provide evidence of improved efficacy in chemotherapeutic drugs (Bouwman and Jonkers, 2012). 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.

Cell-free *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 (Costanzo and Gautier, 2004; Karpinka *et al.*, 2015; Kumagai and Dunphy, 2000; Lupardus *et al.*, 2002; Michael *et al.*, 2000; Philpott and Yew, 2008; Raschle *et al.*, 2008; Willis *et al.*, 2013). 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) (Fig. 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*) (Fig. 2). The approaches of how these different *Xenopus* egg extracts are made have been described previously (Lebofsky *et al.*, 2009).

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 (Blow and Laskey, 1986; Newport, 1987). 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 (Fig. 3). Chromatin bound fractions can be isolated through sucrose cushion and analyzed via immunoblotting analysis (Fig. 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) (Fig. 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 (Fig. 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 (Fig. 3). In addition, *Xenopus* egg extracts can be aliquoted, frozen and stored in freezers at -80°C for multiple experiments.

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 (O'Donnell *et al.*, 2013; Schekman *et al.*, 1974). 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 (Branzei and Foiani, 2010; Mazouzi *et al.*, 2014; Zeman and Cimprich, 2013). 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 a ssDNA gap (Lopes *et al.*, 2006; Yan and Michael, 2009a). The ssDNA gaps then are filled via DNA damage tolerance mechanisms such as lesion bypass or template switching (Chang and Cimprich, 2009). 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 (Zeman and Cimprich, 2013), and DNA replication stress is now accepted as a hallmark of cancer (Macheret and Halazonetis, 2015).

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) (Michael *et al.*, 2000; Van *et al.*, 2010). However, Chk1 phosphorylation is compromised when Pol α is inhibited by aphidicolin at a higher concentration (~300ng/μL) (Byun *et al.*, 2005). More mechanistic studies have elucidated molecular details of the ATR-Chk1 pathway in DNA replication stress response from various research laboratories (Michael *et al.*, 2000; Trenz *et al.*, 2008; Van *et al.*, 2010; Willis *et al.*, 2012; Yoo *et al.*, 2004). A primed M13-derived ssDNA mimics the ATR-activating structure and activates the ATR-dependent DDR pathway activation in the *Xenopus* HSS/NPE system (MacDougall *et al.*, 2007). This primed ssDNA structure was further used to demonstrate that the MRN complex recruits TopBP1 for ATR activation (Duursma *et al.*, 2013).

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 (Jackson and Bartek, 2009). ATM can be activated in response to DSBs and

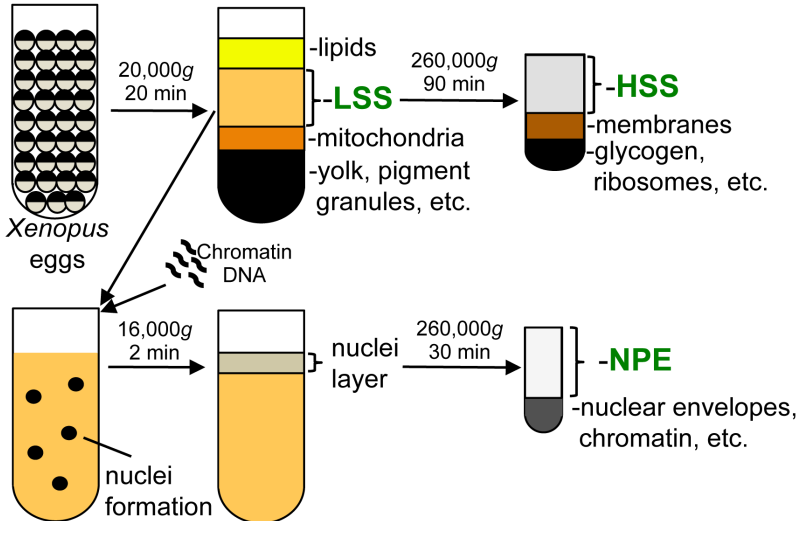

Fig. 2. Diagram of how low-speed supernatant (LSS), high-speed supernatant (HSS) and nucleoplasmic extracts (NPE) are prepared from *Xenopus* eggs. *After PMSG & HCG stimulation,* Xenopus *eggs are collected, processed, and centrifuged at 20,000* g *to prepare the LSS fraction, while the top lipids layer 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 (Lebofsky* et al.*, 2009; Willis* et al.*, 2012).*

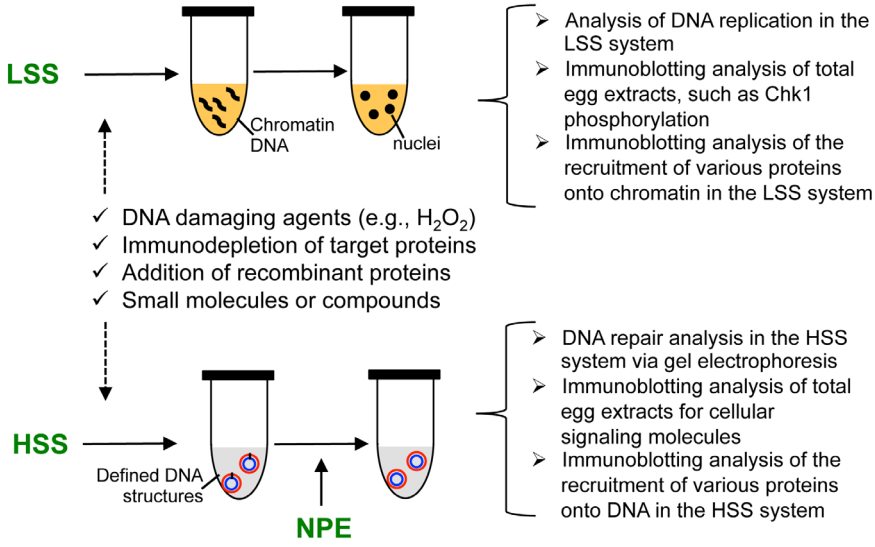

Fig. 3. The *Xenopus* LSS or HSS/NPE system is utilized to study DDR pathways. *LSS, Low-speed supernatant; HSS, high-speed supernatant; NPE, nucleoplasmic extracts. Two approaches are utilized in* Xenopus *system: (I) Chromatin DNA can be added to the LSS, in which chromatin is surrounded with nuclear 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.*

phosphorylates hundreds of substrates including Chk2 (Bakkenist and Kastan, 2003; Daniel *et al.*, 2012). The ATM-Chk2-mediated DDR pathway was suggested as an anti-cancer barrier in early human tumorigenesis (Bartkova *et al.*, 2005). The ATR-Chk1-mediated DDR pathway can also be activated after DSB end resection (Shiotani and Zou, 2009).

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 restriction enzyme (in particular, EcoRI and PflMI) to LSS system, triggering an ATR-mediated checkpoint response (Ramirez-Lugo *et al.*, 2011). EcoRI-treated chromatin DNA can also trigger ATM and Nbs1 phosphorylation in the LSS system (You *et al.*, 2005). After HaeIII treatment, DSB-containing plasmid pBR322 triggers ATM-dependent checkpoint signaling that inhibits chromosomal DNA replication (Costanzo *et al.*, 2000). 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 (You *et al.*, 2007).

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 (Costanzo and Gautier, 2004). 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 (Costanzo *et al.*, 2003; Tsuji *et al.*, 2008). Camptothecin, an inhibitor of topoisomerase I, was used to generate DSBs to study the ATM/ATR-dependent replication restart mechanism in *Xenopus* (Trenz *et al.*, 2006). 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* (Jazayeri *et al.*, 2008; Kumagai and Dunphy, 2000; Yan *et al.*, 2006).

Oxidative stress and oxidative DNA damage

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the capacity of antioxidant defenses (Jones, 2006; Sies, 1997). 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 (Dizdaroglu, 2012; Riley, 1994). 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 (Cadet *et al.*, 2012; Lindahl, 1993). 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 (Berquist and Wilson, 2012; Yan *et al.*, 2014). Oxidative stress has been implicated in the pathogeneses of cancer, aging, and neurodegenerative diseases (Richardson *et al.*, 2015).

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 (Willis *et al.*, 2013). 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 (Willis *et al.*, 2013). This study led to a more general conception that various DNA repair proteins interplay functionally with DDR pathways in oxidative stress (Yan *et al.*, 2014).

Inter-strand crosslinks (ICLs)

ICLs are extremely cytotoxic lesions because irreparable ICLs prevent DNA replication and transcription programs, thereby threatening genome stability (McVey, 2010; Wang *et al.*, 2008). Although DNA crosslinking agents such as mitomycin C (MMC) are widely used in chemotherapy, tumor cells also acquire resistance to such agents (Long and Walter, 2012). 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 (Yan and Willis, 2013). This MMC-induced system has been utilized to elucidate the requirements of nuclear import of TopBP1

and FANC complex for DDR pathway activation (Bai *et al.*, 2014; Wang *et al.*, 2008). 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 (Figs. 2 and 3) (Ben-Yehoyada *et al.*, 2009; Raschle *et al.*, 2008). ICLs activate the DDR pathway, which requires the Fanconi anemia (FANC) complex (Ben-Yehoyada *et al.*, 2009; Knipscheer *et al.*, 2009).

Role of TLS polymerases in the ATR-Chk1 DDR pathway

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, Fig. 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, Fig. 4) (Yan and Michael, 2009a; Yan and Michael, 2009b). 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, Fig. 4) (Van *et al.*, 2010). 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, Fig. 4). Lastly, ATR is directly activated by TopBP1, and Chk1 is then phosphorylated by activated ATR (Step 5, Fig. 4).

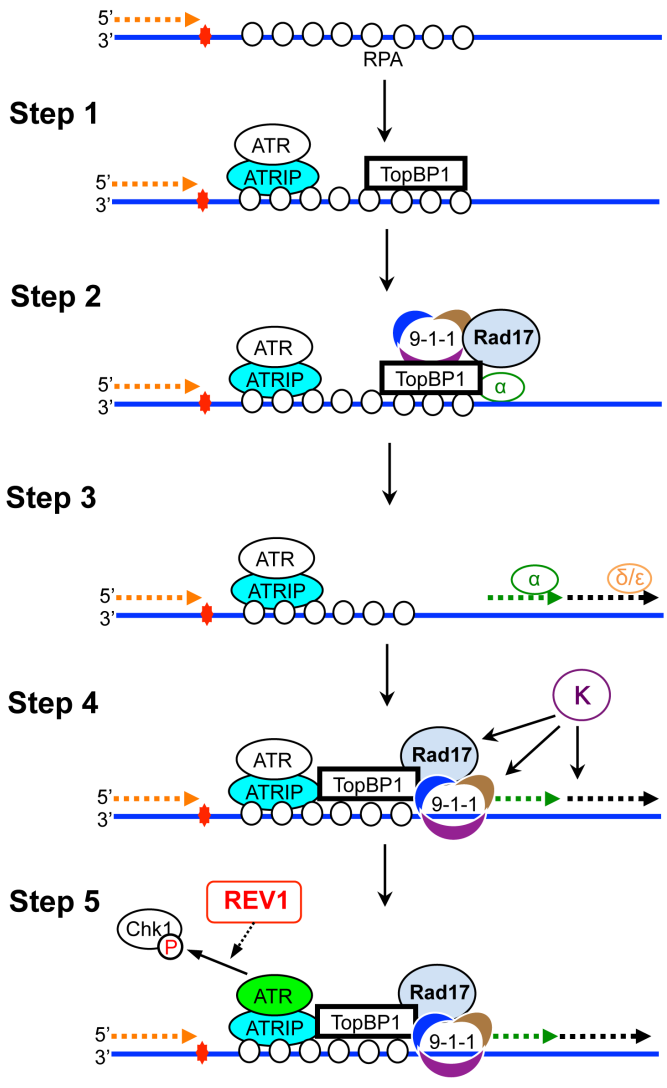

Fig. 4. A model for the role of TLS polymerases Pol κ and REV1 in the ATR-Chk1 DDR pathway. *Pol κ has three mechanisms: primer synthesis, 9-1-1 recruitment, and Rad17 stabilization. REV1 is dispensable for the recruitment of ATR, ATRIP, TopBP1, 9-1-1, and RPA onto stalled forks, but is important for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

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, Fig. 4) (Betous *et al.*, 2013). 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, Fig. 4) (Wang *et al.*, 2016). These findings suggest that TLS polymerases play a previously uncharacterized role in ATR-Chk1 DDR pathway via its catalytic and non-catalytic functions. Importantly, another TLS polymerase REV1 is required for the activation of in 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, Fig. 4) (DeStephanis *et al.*, 2015). 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 also regulate the DDR pathways. Defects in TLS polymerases have been implicated in human tumorigenesis and inhibitors to TLS polymerases such as Pol κ are being developed (Curtin, 2012; Yamanaka *et al.*, 2012).

Future directions

One advantage of the *Xenopus* egg extracts system is the ability to study DDR pathways through removing a target protein via antibody-based immunodepletion procedure and adding back recombinant wild type or mutant protein. These antibody-based approaches in *Xenopus* typically require large amounts of customized antiserum (i.e., in the scale of milliliters), which limits the wide use of this cell-free model system. There is a great demand from the *Xenopus* community to establish a national or international resource center providing antisera targeting specific proteins of interest.

CRISPR/Cas9-mediated targeted gene editing has been successfully utilized in *Xenopus laevis* and *Xenopus tropicalis* (Nakayama *et al.*, 2013; Wang *et al.*, 2015). It will be interesting to determine whether egg extracts from wild type and CRISPR/Cas9-edited *Xenopus laevis* can be compared and analyzed to study DDR pathways. One possible caveat of this CRISPR/

Cas9-mediated knock-out approach is that it may not be feasible to generate viable gene-edited frogs if protein of interest is essential for early embryogenesis. Recently a CRISPR/Cas9-mediated knock-in technology was reported in *Xenopus tropicalis* (Shi *et al.*, 2015). These CRISPR/Cas9-mediated knock-out and knock-in techniques would be applied to investigate the DDR pathways in response to environmental toxins or chemotherapeutic drugs during early embryogenesis and development. Nevertheless, CRISPR/Cas9-mediated gene editing in frogs may offer complementary approach for mechanistic studies of DDR pathways in addition to the cell-free *Xenopus* egg extract system.

Proteomics and mass spectrometry-based approaches can be incorporated into the *Xenopus* system. Proteomics-based analysis has been used to reveal a switch in CDK1-associated proteins upon M-phase exit during the *Xenopus laevis* oocyte to embryo transition (Marteil *et al.*, 2012). This kind of systematic analysis can be applied to DDR pathway research in *Xenopus* egg extracts too. A recent report described a new technique called chromatin mass spectrometry (CHROMASS) to study protein recruitment dynamics on psoralen-damaged chromatin in *Xenopus* egg extracts (Raschle *et al.*, 2015). With this CHROMASS procedure, systematic analysis of assembly and disassembly of DNA repair proteins on ICL-damaged chromatin in *Xenopus* becomes feasible. It remains to be determined whether the proteomics and mass-spectrometry-based approaches will be expanded to study other types of DNA damage and stressful conditions.

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