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REV1 is important for the ATR-Chk1 DNA damage response pathway in *Xenopus* egg extracts

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ABSTRACT

The translesion DNA synthesis (TLS) polymerase REV1 is implicated in the bypass of the irreparable DNA damage such as interstrand crosslinks (ICLs). However, the potential role of REV1 in DNA damage response (DDR) pathway has not been determined. In this research communication, we provide evidence to demonstrate that REV1 plays a previously unidentified but important role in the ATR-Chk1 checkpoint activation in response to mitomycin C (MMC)-induced ICLs in *Xenopus* egg extracts. We further pinpointed that REV1 plays a downstream role of a checkpoint protein complex assembly including ATR, ATRIP, TopBP1 and the Rad9-Rad1-Hus1 complex to MMC-induced ICLs on chromatin in the DDR pathway. Notably, domain dissection analysis demonstrates that a C-terminal domain, but not the individual ubiquitin binding motifs, of REV1 is important for the binding of REV1 to MMC-damaged chromatin and the MMC-induced Chk1 phosphorylation. Yet, the ATR-Chk1 DDR pathway appears to be dispensable for the preferential association of REV1 to MMC-damaged chromatin. Taken together, REV1 is important for the DDR pathway in *Xenopus* egg extracts.

1. Introduction

The genome of organisms is exposed to a variety of insults from cellular metabolism byproducts and environmental toxins, leading to several different types of DNA damage, including interstrand crosslinks (ICLs), double-strand breaks (DSBs), and single-strand breaks (SSBs) [1,2]. Lesions generated from ICLs are extremely cytotoxic, as irreparable ICLs prevent DNA replication and transcription, thereby threatening genome stability. 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 [3]. The DNA damage response (DDR) pathway is a surveillance mechanism used by cells to coordinate cell cycle progression, transcription activation, and activation of apoptosis and senescence [1,4]. ATM-Chk2 and ATR-Chk1 pathways are the two major DDR pathways in response to DNA damage or replication stress from endogenous or exogenous sources [5]. ATM is activated with the help of other mediator proteins in response to DSBs [6–8]. ATR activation requires RPA-bound single-strand DNA (ssDNA) and 5-primed ssDNA/dsDNA (double-strand DNA) junctions, which are derived from the functional uncoupling of MCM helicase and DNA polymerase activities, DSB end resection in the 5′-3′ direction, or SSB end resection in the 3′-5′ direction [4,9–11]. ATR activation also requires several mediator proteins including ATRIP (ATR-interaction protein), TopBP1 and the Rad9-Rad1-Hus1 (9-1-1) complex [12–15]. A variety of substrates including Chk1 are phosphorylated by the activated ATR kinase [16]. Our understanding of ICL repair and signaling pathways has progressed with the use of a defined plasmid-based ICL in *Xenopus* egg extracts [17,18]. However, it is still not completely understood how exactly ICLs activate the ATR-Chk1-mediated DDR pathway.

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 [19,20]. TLS polymerases include the Y-family DNA polymerases (REV1, Pol η, Pol κ and Pol ι) and a B-family DNA polymerase Pol ζ [21,22]. 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 [23,24]. As REV1

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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) [25,26]. Together with Pol ζ, REV1 facilitates various DNA repair programs including ICL repair and homologous recombination of DSBs, promoting or preventing genome instability [27,28]. However, it has not been determined whether REV1 plays 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 [29]. 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 [30]. Mec1 also mediates the recruitment of REV1 to a DSB site in budding yeast [31]. However, the putative Mec1 phosphorylation sites of REV1 are lacking in higher eukaryotic organisms including humans and *Xenopus* [32]. 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 [29,30,33]. 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 [17,18,34]. In this communication, our compelling evidence suggests that REV1 plays a previously unidentified, but important, role in the activation of MMC-induced ATR-Chk1 DDR pathway in *Xenopus* egg extracts. Furthermore, we pinpointed the step of REV1 in the ATR-Chk1 DDR pathway and dissected the necessary domain within responsible REV1 for such an important function. In contrast, ATR-Chk1 plays a negligible role for the recruitment of REV1 to ICLs on chromatin in *Xenopus* egg extracts.

2. Materials and methods

2.1. Xenopus egg extract and related procedures

The use and care of *Xenopus laevis* were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Charlotte. Low-speed supernatant egg extract was prepared according to our previously published approach [10,35,36]. Sperm chromatin was added to egg extracts at a concentration of approximately 4000 sperm/μL. Chromatin fractions were isolated and examined as previously described [14]. Caffeine, KU55933, VE-822, and NU6027 were added to egg extracts at final concentrations of 1 ng/μL, 120 μM, 10 μM, and 1 mM, respectively [10,37,38]. Aphidicolin (APH) and Mitomycin C (MMC) were added to egg extracts at final concentrations of 100 ng/μL and 0.5 mM as previously described [14,34]. Immunodepletion of REV1 was performed in a similar fashion as previously described [14].

2.2. Expression vectors, recombinant proteins, and antibodies

His-REV1-NT300, corresponding to *X. 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 nucleotides 246-3938 was cloned into the pCS2+MT vector. ΔUBM1, ΔUBM2, and ΔCTD Myc-tagged REV1 variants were derived from WT Myc-REV1, in which nucleotides 2991-3086, 3225-3327, and 3513-3834 were removed, respectively. Recombinant WT and deletion versions of Myc-tagged REV1 were expressed in SP6 TnT transcription/translation coupled quick master mix kit (Promega). Antibodies against *Xenopus* ATR, ATRIP, TopBP1, Rad9, RPA32, and Orc2 have been described previously [10,14]. Additionally, antibodies against Chk1 P–S344 (Cell Signaling), Chk1 (Santa Cruz), c-Myc (Santa Cruz), GST (Santa Cruz), Histone 3 (Abcam), and PCNA (Santa Cruz) were purchased from respective vendors. Peroxidase-conjugated monoclonal mouse anti-rabbit IgG light chain specific (Jackson ImmunoResearch), peroxidase-conjugated goat anti-rabbit IgG (Thermo), and peroxidase-conjugated sheep anti-

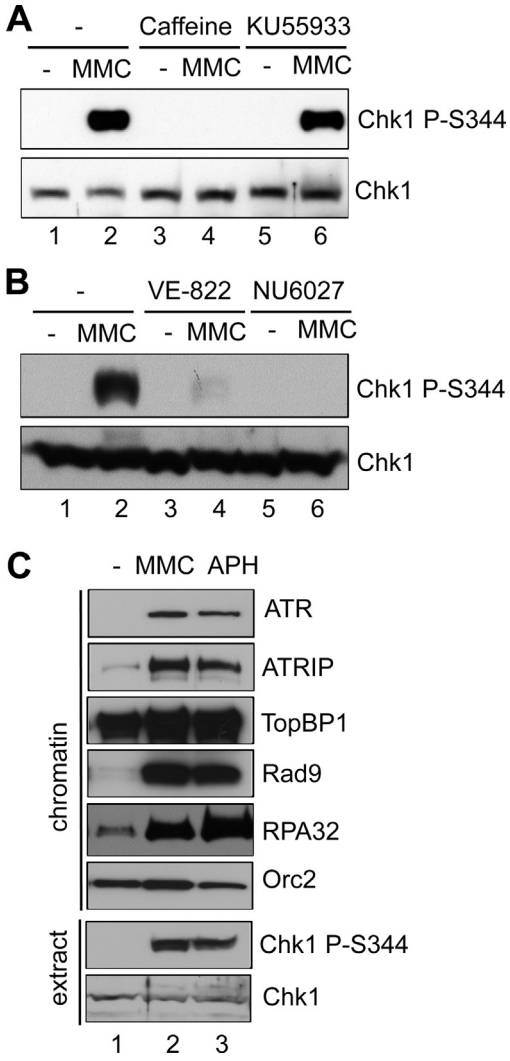

Fig. 1. Chk1 phosphorylation and a checkpoint protein complex assembly in response to MMC-induced ICLs in *Xenopus* egg extracts. (A) Caffeine or KU55933 was incubated in egg extracts supplemented with sperm chromatin and mitomycin C (MMC). After 1-hr incubation, Chk1 phosphorylation (Chk1 P–S344) and total Chk1 in extracts were examined via immunoblotting analysis. (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 aphidicolin (APH) was incubated in egg extracts supplemented with sperm chromatin. After 1-hr incubation, chromatin fractions ("chromatin") and total extract ("extract") were isolated and examined via immunoblotting analysis as indicated. Orc2 was used as a loading control.