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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.

Keywords

ATR; Chk1; DNA damage response; REV1; TLS

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

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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 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 extracts 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 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/ μ L. 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/ μ L, 120 μ M, 10 μ M, and 1 mM, respectively [10,37,38]. Aphidicolin (APH) and Mitomycin C (MMC) were added to egg extracts at final concentrations of 100 ng/ μ L 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 *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. UBM1, UBM2, and CTD 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-mouse IgG (GE Healthcare) were used as secondary antibodies in immunoblotting analysis as appropriate.

3. Results

3.1 Chk1 phosphorylation was triggered in response to MMC-induced ICLs in an ATR-dependent but ATM-independent manner in *Xenopus* egg extracts

Chk1 phosphorylation at Serine 344 (Chk1 P-S344) is widely used as an indicator of ATR activation [18,39]. Chk1 phosphorylation was triggered by MMC treatment in *Xenopus* egg extracts (Fig. 1A), consistent with our recently published study [34]. The MMC-induced Chk1 phosphorylation was compromised by the ATR/ATM inhibitor, caffeine, but not the ATM specific inhibitor, KU55933 (Fig. 1A) [10]. Furthermore, MMC-induced Chk1 phosphorylation was compromised by the ATR specific inhibitors, VE-822 or NU6027 (Fig. 1B) [37,38]. These observations suggest that MMC-induced ICLs trigger Chk1 phosphorylation in an ATR-dependent, but ATM-independent fashion. To compare the MMC-induced ICLs and aphidicolin-induced stalled replication forks, we examined the recruitment of several key checkpoint proteins to chromatin in *Xenopus* egg extracts. Aphidicolin inhibits DNA polymerase activities, leading to stalled DNA replication forks, functional uncoupling of the helicase and polymerase, and the ATR-Chk1 DDR pathway activation [40]. As expected, aphidicolin triggered Chk1 phosphorylation and the assembly of a checkpoint complex containing ATR, ATRIP, and Rad9 in response to stalled replication forks (Fig. 1C) [14]. Orc2 was used as a loading control. Notably, the checkpoint protein complex (ATR, ATRIP, TopBP1, and Rad9) was also assembled onto MMC-damaged chromatin, suggesting DNA replication forks are stalled by MMC-induced ICLs (Fig. 1C). In addition, RPA32 was hyperloaded onto MMC-damaged chromatin, suggesting that long stretches of ssDNA are generated around ICL sites (Fig. 1C). These observations suggest that when DNA replication forks stall at ICLs, ssDNA is generated and bound by RPA, and that a checkpoint protein complex is recruited to ICL sites to trigger the ATR-Chk1 DDR pathway.

3.2 REV1 was preferentially recruited to MMC-induced ICLs in an ATR-Chk1-independent fashion

To study the role of REV1 in the MMC-induced DDR pathway, we expressed and purified His-tagged recombinant N-terminal 300-amino-acid fragment of REV1 (designated as His-REV1-NT300) from bacteria, as shown on SDS-PAGE gel (Fig. 2A). His-REV1-NT300 protein was then used to generate customized antibodies against REV1 in rabbits, which recognized endogenous REV1 in *Xenopus* egg extracts, recombinant His-REV1-NT300, and Myc-tagged wild type REV1 (WT Myc-REV1) (Figs. 2B and 4A). Notably, endogenous REV1 and recombinant WT Myc-REV1 were preferentially recruited to MMC-damaged chromatin in *Xenopus* egg extracts (Figs. 2C and 2D). PCNA was monoubiquitinated after MMC-treatment (Fig. 2C). These observations suggest that REV1 preferentially associates with MMC-induced ICL sites. Although caffeine compromised MMC-induced Chk1 phosphorylation, this ATR/ATM inhibitor had no noticeable effect on the recruitment of Myc-REV1 to MMC-damaged chromatin, suggesting that the ATR-Chk1 DDR pathway is not required for the recruitment of REV1 to MMC-induced ICLs in *Xenopus* at least under our experimental conditions (Fig. 2D).

3.3 REV1 was important for the MMC-induced ATR-Chk1 DDR pathway

To test the potential role of REV1 in the MMC-induced Chk1 phosphorylation, we successfully immunodepleted endogenous REV1 from egg extract with customized anti-REV1 antibodies (Fig. 3A). Notably, the MMC-induced Chk1 phosphorylation was compromised significantly in the REV1-depleted egg extract, suggesting that REV1 plays an important role in the activation of the ATR-Chk1 DDR pathway (Fig. 3A). Interestingly, Chk1 phosphorylation induced by stalled replication forks was also compromised in the REV1-depleted egg extract, suggesting the novel role of REV1 in the ATR-Chk1 DDR pathway in response to replication stress (Fig. 3B). These observations suggest that REV1 is important for the ATR-Chk1 DDR pathway activation in response to both ICLs and stalled DNA replication forks.

To determine the precise role of REV1 in the ATR-Chk1 DDR pathway, we examined the assembly of the checkpoint protein complex onto MMC-damaged chromatin. The recruitment of ATR, ATRIP, TopBP1, and Rad9 to MMC-damaged chromatin had no noticeable change in REV1-depleted egg extract in comparison with mock-depleted egg extract (Fig. 3A). RPA32 was hyperloaded to MMC-damaged chromatin in REV1-depleted egg extract, suggesting that REV1 has no effect on ssDNA generation. The recruitment of ATR, ATRIP, TopBP1, and Rad9 to aphidicolin-induced stalled replication forks demonstrated no noticeable change in REV1-depleted egg extract when compared to mock-depleted egg extract (Fig. 3B). Histone 3 was used as a loading control in these experiments. Similarly, REV1 depletion had no noticeable effect on RPA32 hyperloading to stalled replication forks (Fig. 3B). To confirm that the Chk1 phosphorylation defect by REV1 depletion is because of the absence of REV1, we added WT Myc-REV1 to REV1-depleted egg extract at a similar concentration as the endogenous REV1 and found that MMC-induced Chk1 phosphorylation was partially rescued (Figs. 4A and 4B). These observations suggest that REV1 may play a role downstream of the checkpoint protein complex assembly, but before Chk1 is phosphorylated by ATR in the DDR pathway and that the role of REV1 in the DDR pathway is not limited to MMC-induced ICLs.

3.4 A C-terminal domain (CTD) of REV1 was required for the recruitment of REV1 to MMC-damaged chromatin and the MMC-induced Chk1 phosphorylation

REV1 contains several distinct domains: BRCT (aa 54-122), dCMP transferase (aa 347-812), UBM1 (aa 916-947), UBM2 (aa 994-1027), and CTD (aa 1090-1193) (Fig. 4A). Through its dCMP transferase domain, REV1 protein transfers a dCMP opposite a damaged nucleotide [23,41]. The UBM1 and UBM2 are important for the binding of REV1 to the ubiquitin moiety of monoubiquitinated PCNA [42]. The CTD fragment of REV1 associates with other TLS proteins, such as Pol η , Pol ζ , and Pol κ [43,44]. To directly test the role of the UBMs and CTD region of REV1 for the DDR pathway, several deletion mutant versions of REV1 were added to REV1-depleted egg extract (Fig. 4A). Surprisingly, UBM1 REV1 and UBM2 REV1 retained binding capacity to MMC-damaged chromatin similar to that of WT REV1. Furthermore, WT REV1, UBM1 REV1 and UBM2 REV1 rescued the MMC-induced Chk1 phosphorylation in the REV1-depleted egg extract (Fig. 4C). However, CTD REV1 failed to rescue MMC-induced Chk1 phosphorylation in the REV1-depleted egg extract (Fig. 4C). Notably, the binding of CTD REV1 to MMC-damaged chromatin was

also compromised (Fig. 4C). These observations suggest that recruitment of REV1 to MMC-damaged chromatin requires its CTD domain, but not UBMs, and that CTD of REV1 is essential for the activation of the ATR-Chk1 pathway in response to MMC-induced ICLs in *Xenopus* egg extracts.

4. Discussion

It is important to investigate the regulatory mechanisms between the DDR and TLS pathways. In this research communication, we report that the ATR-Chk1 DDR pathway is activated by MMC-induced ICLs on chromatin and that REV1 is important for the MMC-induced DDR pathway in *Xenopus* egg extracts. In addition, ATR-Chk1 pathway is dispensable for the preferential binding of REV1 to MMC-damaged chromatin. We further demonstrate that REV1 may function downstream of the assembly of the checkpoint protein complex containing ATR, ATRIP, and the 9-1-1 complex onto MMC-induced ICLs. Domain dissection analysis reveals that the CTD of REV1 is important for its recruitment onto MMC-induced ICLs and for its role in the ATR-Chk1 DDR pathway.

It was recently reported that Pol κ , but not Pol η , is important for the ATR-Chk1 DDR pathway induced by stalled DNA replication forks in both humans and *Xenopus* [33]. Furthermore, it was shown that Pol κ is implicated in the synthesis of short DNA intermediates at stalled replication forks, facilitating the recruitment of the 9-1-1 complex [33]. Here we show that another TLS member REV1 is important for the ATR-Chk1 DDR pathway in response to MMC-induced ICLs and stalled DNA replication forks (Fig. 3). The association of the 9-1-1 complex to MMC-induced ICLs and stalled replication forks was not compromised in the absence of REV1, suggesting that REV1 plays a role downstream of the assembly of the checkpoint protein complex. More studies are needed to clarify the potential regulation between Pol κ and REV1 in the DDR pathway.

It is important to determine how exactly REV1 is recruited to damaged sites. Since REV1 lacks a PIP box motif for PCNA association, it has been suggested that REV1 may associate with a ubiquitin moiety in monoubiquitinated PCNA via its UBMs [42]. Our observation in Figure 4 does not rule out the possibility that REV1 may have a defect in MMC-induced Chk1 phosphorylation when both UBM1 and UBM2 are removed. The CTD fragment of REV1 associates with other TLS polymerases such as Pol η , Pol ζ , and Pol κ [43,44]. Thus, it is also possible that REV1 is recruited by other TLS polymerases via its CTD fragment. In addition, it was recently reported that REV1 CTD forms a quaternary complex consisting of REV1, Pol ζ , and Pol κ [45]. The requirement of CTD for REV1 binding to MMC-damaged chromatin further supports the significance of the CTD of REV1 in the DDR pathway.

More investigation is necessary to test whether the important role of REV1 in the DDR pathway is conserved in humans or other model organisms. COSMIC (Catalogue of Somatic Mutations in Cancer) analysis reveals 100 missense, 7 nonsense, and 44 synonymous substitutions, as well as 3 insertion/deletion frame shifts in REV1 in various cancer patients including breast, ovary, large intestine, endometrium, lung, and pancreas carcinoma patients. These findings further highlight the significance of REV1 in the DDR pathway. Our findings from this work may elucidate novel avenues for cancer treatment through

manipulation of REV1's distinct role in the DDR pathway. Taken together, our presented evidence suggests that REV1 is important for the ATR-Chk1 DDR pathway in response to MMC-induced ICLs in *Xenopus* egg extracts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. REV1 preferentially associates with MMC-damaged chromatin in *Xenopus*.
2. REV1 is important for the Chk1 phosphorylation in response to MMC-induced ICLs.
3. A C-terminal motif of REV1 is important for ICL binding and Chk1 phosphorylation.

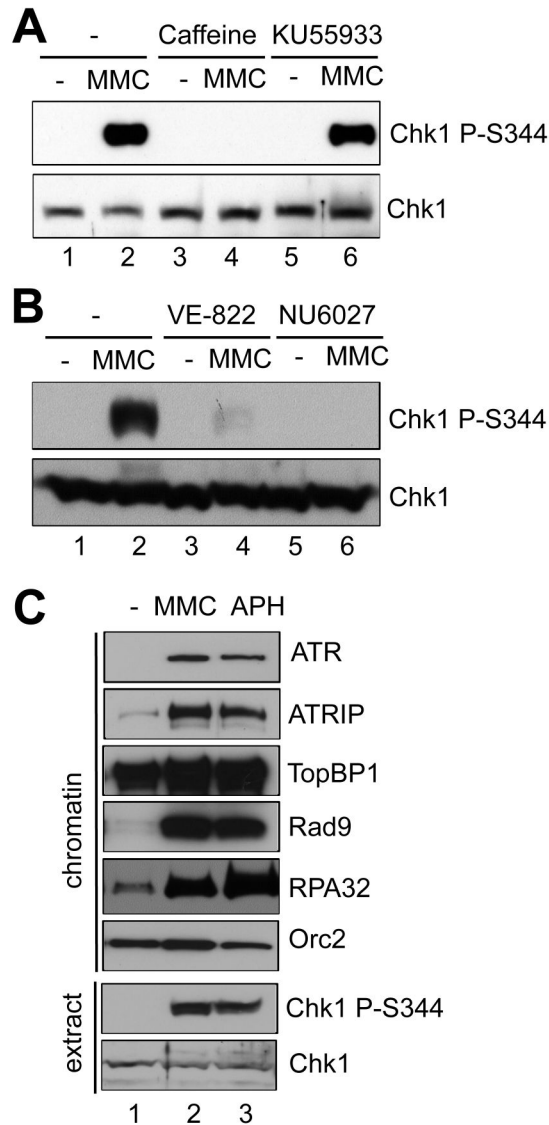
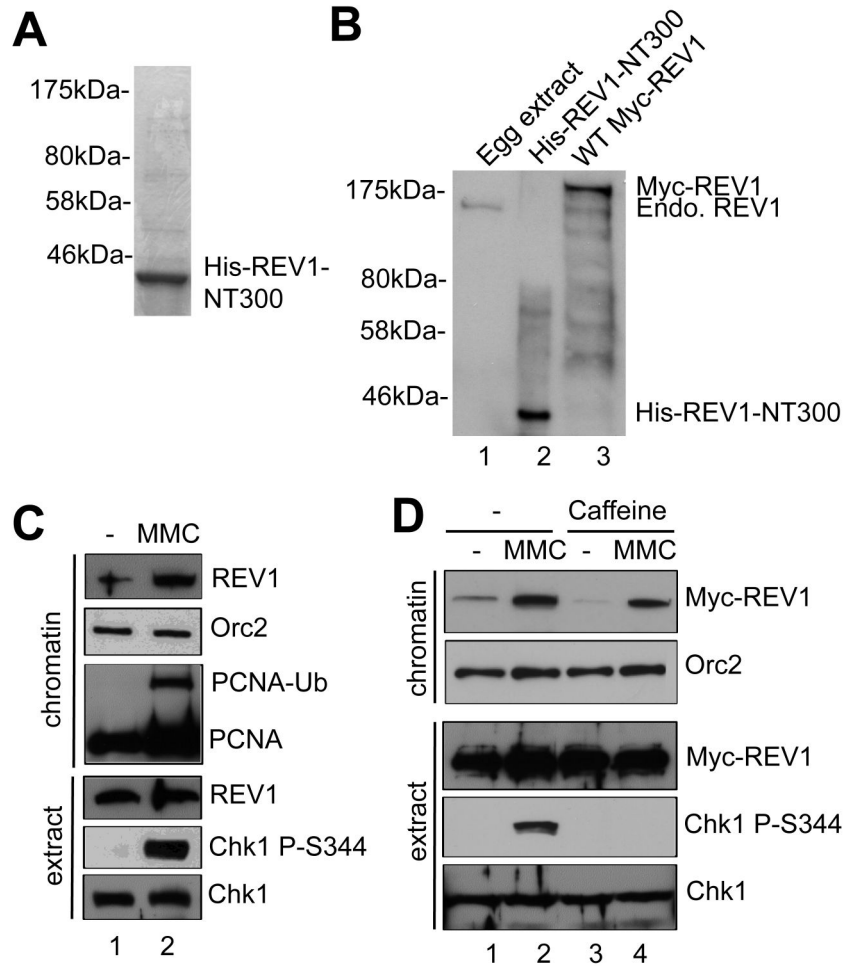


Fig. 1. Chk1 phosphorylation and a checkpoint protein complex assembly in response to MMC-induced ICLs in *Xenopus* egg extract. (A) Caffeine or KU55933 was incubated in egg extracts supplemented with sperm chromatin and mitomycin C (MMC). After 1-hr incubation, Chk1 phosphorylation (Chk1 P-S344) and 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.

**Fig. 2.**

REV1 preferentially associates with MMC-damaged chromatin. (A) Purified His-REV1-NT300 was examined via SDS-PAGE and Coomassie blue staining. Molecular weight markers were also labeled. (B) *Xenopus* egg extract, recombinant His-REV1-NT300 and SP6 TnT expressed WT Myc-REV1 were examined via immunoblotting analysis using anti-REV1 antibodies. “Endo. REV1” represents endogenous REV1. (C) MMC was incubated in egg extracts supplemented with sperm chromatin. After 1-hr incubation, chromatin fractions and total extract were isolated and examined via immunoblotting analysis. “PCNA-Ub” denotes monoubiquitinated PCNA. (D) WT Myc-REV1 was incubated in egg extracts at a similar concentration as the endogenous REV1, which was followed by addition of MMC and Caffeine. After 1-hr incubation, chromatin fractions and total extract were analyzed via immunoblotting analysis as indicated. Orc2 was used as a loading control.

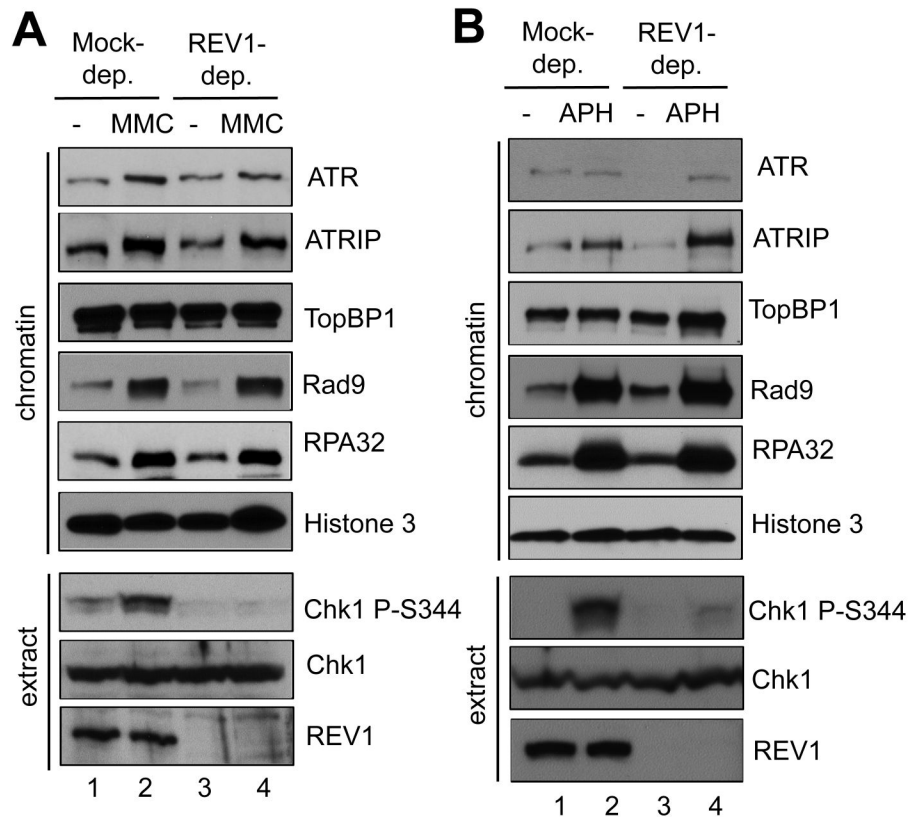


Fig. 3. REV1 is important for Chk1 phosphorylation in response to MMC-induced ICLs and stalled DNA replication forks. (A) MMC was incubated in mock-depleted or REV1-depleted egg extracts supplemented with sperm chromatin. After 1-hr incubation, chromatin fractions and total extracts were examined via immunoblotting analysis as indicated. (B) APH was incubated in mock-depleted or REV1-depleted egg extracts supplemented with sperm chromatin. After 1-hr incubation, chromatin fractions and total extracts were analyzed via immunoblotting analysis. Histone 3 was used as a loading control.

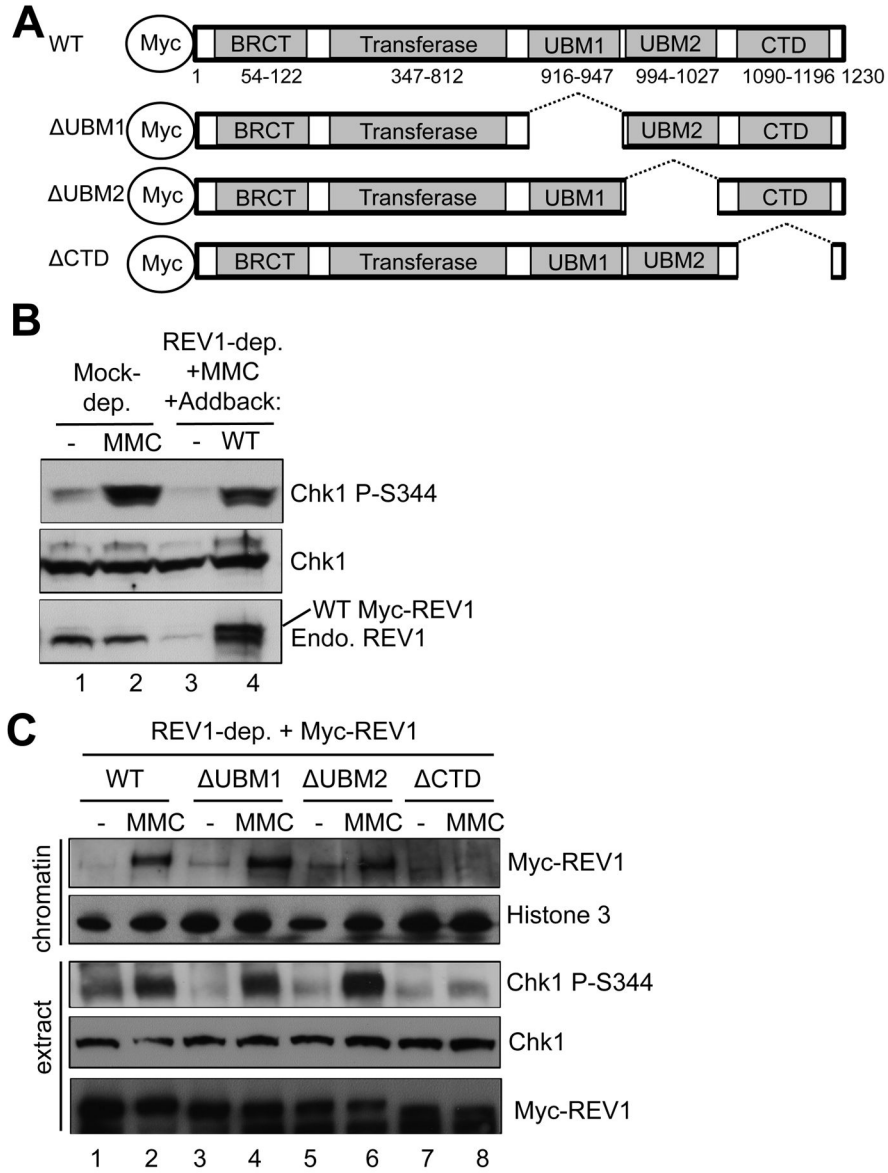


Fig. 4. Domain dissection of REV1 for association with MMC-damaged chromatin and MMC-induced Chk1 phosphorylation. (A) Schematic demonstration of WT REV1 and domain deletion versions of REV1. (B) WT Myc-REV1 (“WT”) or an egg lysis buffer (“-”) was added to REV1-depleted egg extract, which was supplemented with sperm chromatin and MMC. Total extracts were examined for indicated proteins via immunoblotting analysis. “Endo. REV1” represents endogenous REV1. (C) WT REV1 or depletion mutant versions of REV1 were individually added back to REV1-depleted egg extract supplemented with sperm chromatin and MMC. Chromatin fractions and total extracts were analyzed for indicated proteins via immunoblotting analysis. Histone 3 was a loading control.