REV1 promotes PCNA monoubiquitination through interacting with ubiquitinated RAD18

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Summary Statement
Monoubiquitination of PCNA is a critical event coordinating DNA damage tolerance pathways. We here report that translesion synthesis polymerase REV1 can promote PCNA-mUb via its preferential association with monoubiquitinated RAD18.

Keywords
REV1; PCNA; RAD18; Ubiquitination; Translesion DNA synthesis; UV
Abstract

Translesion DNA synthesis (TLS) is one mode of DNA damage tolerance, which plays an important role in genome mutagenesis and chromatin integrity maintenance. PCNA monoubiquitination is one of the key factors for TLS pathway choice. So far, it remains unclear how TLS pathway is elaborately regulated. Here, we report that TLS polymerase REV1 can promote PCNA monoubiquitination after UV radiation. Further studies revealed that this stimulatory effect is mediated through the enhanced interaction between REV1 and ubiquitinated RAD18, which facilitates the release of nonubiquitinated RAD18 from ubiquitinated RAD18 trapping followed by more RAD18 recruiting to chromatin for its TLS function. Furthermore, we found that this stimulatory effect could also be detected after exposure to hydroxyurea or mitomycin C, but not methyl methanesulfonate (MMS), which is in line with the fact that ubiquitinated RAD18 could not be detected after exposure to MMS.

Introduction

Cellular DNA is endangered by a range of endogenous and exogenous substances, which can result in genome instability and eventually lead to cancer or cell death. When DNA damage occurs during replication, it will stall the replication fork, ultimately causing fork collapse and genome rearrangements (Jackson and Bartek, 2009; Ciccia and Elledge, 2010). To avoid that, cells have evolved a translesion DNA synthesis (TLS) system to replicate damaged DNA templates (Friedberg et al., 2005). Various specialized DNA polymerases, which include Polκ, Polη and REV1, are utilized in TLS pathway (Ohmori et al., 2001; Friedberg, 2005; Sale et al., 2012). After UV radiation, REV1 can be recruited to chromatin through the unique N-terminal BRCA1 C terminus (BRCT) domain and its ubiquitin binding motifs (UBMs) (Guo et al., 2006a; Guo et al., 2006b). Although the dCMP transferase activity of REV1 is conserved throughout eukaryotic evolution (Nelson et al., 1996; Zhang et al., 2002), this activity does not account for its role in UV-induced mutagenesis. REV1 is found to interact with other Y-family polymerases, including
Polκ, Polη and Polι, through its C-terminal polymerase interacting region (PIR) (Guo et al., 2003). Therefore, it is likely that REV1 can coordinate the activity of specialized DNA polymerases, possibly by providing a scaffold to facilitate polymerase switching at lesion sites (Guo et al., 2009).

Proliferating cell nuclear antigen (PCNA) is a replicative polymerase clamp, which becomes monoubiquitinated at Lys164 in mammalian cells following various DNA damage treatments that cause the replication fork stalling (Kannouche et al., 2004). Monoubiquitinated PCNA (PCNA-mUb) exhibits an enhanced interaction with Y-family polymerases (Guo et al., 2006b; Parker et al., 2007; Guo et al., 2008; Waters et al., 2009), and thus regulates them to access the replicative ensemble stalled at a lesion to execute their roles in lesion bypass. Given that PCNA-mUb promotes TLS pathway (Hoege et al., 2002; Moldovan et al., 2007; Chen et al., 2011), many studies have been performed to understand how this modification happens in vivo (Hedglin and Benkovic, 2015). In addition to the major E3 ubiquitin ligase RAD18, several other E3 ligases, like RNF8 (Zhang et al., 2008) and CRL_{cdt2} (Terai et al., 2010), have been reported to regulate PCNA-mUb. Additionally, many factors, including SIV A1 (Han et al., 2014), Spartan/C1orf124 (protein with sprT-like domain at the N terminus, also known as DVC1 [DNA damage protein targeting VCP]) (Centore et al., 2012), MSH2 (MutS protein homolog 2) (Zlatanou et al., 2011; Lv et al., 2013), BRCA1 (breast cancer 1) (Tian et al., 2013), have also been found to regulate the RAD18-dependent PCNA-mUb. USP1 (ubiquitin-specific protease-1) also participates in the regulation of PCNA-mUb. It is a key deubiquitinase (DUB) for deubiquitinating PCNA-mUb (Huang et al., 2006). Recently, it was reported that monoubiquitinated RAD18 (RAD18-Ub) also regulates PCNA-mUb and TLS activity (Zeman et al., 2014). RAD18-Ub not only releases itself from chromatin (Zeman et al., 2014), but also sequesters nonubiquitinated RAD18 from recruiting to chromatin.

During studying REV1’s function(s) in TLS, we accidentally observed that REV1 affects PCNA-mUb. We found that REV1 can promote RAD18 accumulating at chromatin and PCNA-mUb after UV radiation. Further studies indicate that this stimulatory effect is mediated through the enhanced interaction between REV1 and
RAD18-Ub, which facilitates the release of nonubiquitinated RAD18 from RAD18-Ub trapping followed by more RAD18 recruiting to chromatin for its TLS function. Interestingly, this stimulatory effect could also be detected after treatments with hydroxyurea (HU) or mitomycin C (MMC), but not with methyl methanesulfonate (MMS) which leads to the loss of RAD18-Ub.

Results

REV1 promotes PCNA monoubiquitination

PCNA-mUb has been shown to interact with REV1 and mediated the latter to accumulate at UV-induced stalled replication sites. We are wondering whether REV1 conversely affects UV-induced PCNA monoubiquitination. To answer that question, we transfected 293T cells with siRNA directed against human REV1 (siREV1) and examined the level of PCNA-mUb in the triton-insoluble fractions. We found that depletion of REV1 obviously decreased the level of PCNA-mUb in the presence and absence of UV radiation (Fig.1A). Conversely, we found that over-expression of GFP-REV1 in 293T cells could increase the level of PCNA-mUb in the presence and absence of UV radiation (Fig.1B). Interestingly, we found that the stimulatory effect of REV1 on PCNA-mUb is closely related to the level of REV1 expression. Higher amount of REV1 induced more PCNA to be monoubiquitinated (Fig. 1C). These data indicate that REV1 could promote basal and UV-induced PCNA-mUb, which is intimately related to the level of REV1 expression. Additionally, we performed PCNA immunofluorescence and detected no obvious change in PCNA focus formation in cells either depletion or overexpression of REV1 (Fig. S1). This result is expectable because PCNA-mUb seems not necessary for its focus formation in cells either at S-phase or after damage treatments (Essers et al., 2005).

REV1 promotes PCNA-mUb independent of Polη and USP1

Previous studies have shown that REV1 interacts with Polη (Guo et al., 2003) and Polη regulates PCNA-mUb (Durando et al., 2013), we are wondering whether the stimulatory effect of REV1 on PCNA-mUb is mediated by Polη. We transfected GFP-REV1 into XP30RO-Polη cells and examined the level of PCNA-mUb in the
presence and absence of Polη. We found that over-expression of REV1 still promoted PCNA-mUb in the absence of Polη (Fig. 2A, Lanes 1 & 2). In addition, although UV-induced PCNA-mUb was remarkably increased in XPRO30-Polη cells after adding doxycycline (Dox) to induce Polη expression (Fig. 2A, Lanes 1 & 3), which is in line with the previous report (Durando et al., 2013), over-expression of REV1 in the Polη-expressing XPRO30-Polη cells further increased the level of PCNA-mUb after UV damage (Fig. 2A, Lanes 3 & 4). These data indicate that the stimulatory effect of REV1 on PCNA-mUb is Polη-independent. Given that the level of PCNA-mUb is negatively regulated by the USP1 deubiquitinase (Huang et al., 2006), we are wondering whether REV1 reduces the expression of USP1. We compared the level of USP1 in REV1-depleted and control 293T cells in the presence and absence of UV radiation. REV1 depletion did not cause appreciable alterations in USP1 expression (Fig. 2B). Moreover, over-expression of REV1 could not reduce USP1 expression, either (Fig. 2C). Additionally, we found that over-expression of REV1 still significantly promoted PCNA-mUb in the USP1-depleted cells (Fig. 2D). These results indicate that the stimulatory effect of REV1 on PCNA-mUb is USP1-independent.

REV1 promotes PCNA-mUb via a RAD18-dependent manner

It is well established that the level of PCNA-mUb is positively regulated by the RAD6/RAD18 ubiquitin ligase complex (Hoege et al., 2002; Kannouche et al., 2004; Ulrich, 2009; Hedglin and Benkovic, 2015). We are wondering whether REV1 promotes PCNA-mUb via a RAD18-dependent fashion. We first transfected GFP-REV1 into the control or RAD18 stable knockdown U2OS cells (Zhang et al., 2013). We found that depletion of RAD18 significantly inhibited the stimulatory effect of REV1 on UV-induced PCNA-mUb (Fig. 2E). However, considering that the RAD18 stable knockdown cells manifested an appreciable REV1-promoted PCNA-mUb, it is necessary to determine whether REV1 might also stimulate PCNA-mUb in a RAD18-independent manner. We then established a RAD18 knockout 293T cell line based on the TALEN technology. We found that the
stimulatory effect of REV1 on UV-induced PCNA-mUb was completely abrogated in RAD18 knockout cells (Fig. 2F). These results indicate that RAD18 mediates the REV1-promoted PCNA-mUb in the absence and presence of UV damage.

**REV1 facilitates RAD18 but not RPA32 accumulation on chromatin**

We then determined how RAD18 mediates REV1-promoted PCNA-mUb. We first compared the level of RAD18 in the control and REV1-depleted cells and found that REV1 depletion does not affect RAD18 expression (Fig. 3A). However, depletion of REV1 resulted in a decreased chromatin association of RAD18 (Fig. 3B). To avoid the off-target effect of siRNA, we repeated the experiment with another individual siRNA (siREV1-2) and obtained similar result (Fig. 3C). Additionally, depletion of REV1 did not produce an obvious difference in the distribution of RAD18-mUb/RAD18 in the soluble fraction (Fig. 3C). Considering that only a small fraction of total RAD18 associated with chromatin even after UV radiation in our system (Fig. S2A), we speculate that the amount of RAD18 released from the chromatin upon REV1 knockdown might be not enough to produce an obvious difference in the distribution of RAD18-mUb/RAD18 in the soluble fraction. Then, we examined whether REV1 regulates UV-induced RAD18 focus formation. We transfected U2OS cells with two independent siRNAs (siREV1-1 & siREV1-2) and found that knockdown of REV1 led to an obvious reduction in RAD18 focus formation after UV damage (Fig. 3D & E), although depletion of REV1 had no obvious effect on the RAD18 global nuclear staining (Fig. S2B). Considering that RAD18 is recruited to stalled replication forks by virtue of its affinity for replication protein A (RPA)-coated single-stranded DNA (ssDNA) (Davies et al., 2008; Huttner and Ulrich, 2008), we therefore measured the level of chromatin-bound RPA in REV1-depleted cells and observed no appreciable reduction compared with that in siNC-transfected cells (Figure 3B). We also examined the proportion of RPA positive cells in UV-irradiated REV1-depleted cells. At 4 h after irradiation, the cells were treated to remove soluble RPA and were processed for immunofluorescence to reveal chromatin-bound RPA (Lv et al., 2013). As shown in Fig S3, depletion of REV1 had
no obvious effect on RPA focus formation. These data indicate that REV1 promotes PCNA-mUb by facilitating the binding of RAD18 but not RPA on chromatin.

**The UBM domains of REV1 are required for its stimulatory effect on PCNA-mUb**

To understand how REV1 facilitates RAD18 binding on chromatin, we first examined the essential domains in REV1 for promoting RAD18 chromatin association and PCNA-mUb. 293T cells expressing a panel of REV1 peptides (Fig. 4A) were UV irradiated and the triton-insoluble fractions were analyzed. Like WT REV1, REV1_{1-1123} (without the C-terminal PIR) could promote PCNA-mUb and RAD18 recruitment to chromatin (Fig. 4C), indicating that REV1-facilitated PCNA-mUb is independent of its PIR. This data is consistent with that REV1 promotes PCNA-mUb via a Polη-independent fashion. Moreover, REV1_{653-1123} (without the BRCT, catalytic core and PIR) could also promote PCNA-mUb and RAD18 recruitment to chromatin (Fig. 4C), hinting that the BRCT and catalytic core of REV1 are not necessary for REV1-facilitated PCNA-mUb. Notably, unlike WT REV1, UBM* REV1 could not promote PCNA-mUb and RAD18 recruitment to chromatin. Given that the UBMs not only mediate REV1 interacting with other ubiquitinated proteins but also its monoubiquitination (Guo et al., 2006b; Kim et al., 2012), we analyzed whether monoubiquitinated REV1 could stimulate PCNA-mUb. The ubiquitin cDNA lacking the C-terminal Gly-Gly codons was cloned to pEGFP-C3-REV1 UBM* plasmid to make a full-length REV1-ubiquitin chimera (REV1-Ub) (a mimic of monoubiquitinated REV1). We found that the REV1-Ub chimera failed to rescue the decreased PCNA-mUb resulting from UBMs* (Fig. 4C). This data suggest that REV1 does not likely facilitate PCNA-mUb via its monoubiquitination. Consistently, overexpression of REV1 WT, REV1_{1-1123} and REV1_{653-1123} but not REV1 UBMs* and REV1-Ub, significantly increase UV-induced RAD18 focus formation (Fig. 4D & E). All the above results indicate that the UBMs of REV1 are required for its stimulatory effect on PCNA-mUb and RAD18 recruitment. Therefore, the stimulatory effect of REV1 on PCNA-mUb might be
mediated through its interaction with an ubiquitinated protein. It is known that REV1 can interact with PCNA-mUb at the stalled replication sites, where REV1 exhibits focal distribution in nucleus (Guo et al., 2009). We examined whether the REV1 peptides which promote PCNA-mUb manifested increased REV1 focus formation after UV irradiation. Unlike WT REV1, REV1<sub>1-1123</sub> and REV1<sub>653-1123</sub> did not exhibit increased REV1 focus formation (Fig. 4B, S4). Meanwhile, we also noticed that, unlike WT REV1, REV1<sub>1-1123</sub> and REV1<sub>653-1123</sub> did not manifest an appreciable difference in the extent of their colocalization with PCNA after UV treatment compared with REV1 UBMs* (Fig. S1C), suggesting that the stimulatory effect of REV1 on PCNA-mUb is not likely mediated by its interaction with PCNA-mUb at stalled replication sites. Collectively, it hints at a possibility that another ubiquitinated protein(s) other than PCNA-mUb might be involved in this process.

**REV1 binds RAD18-Ub to release RAD18 from RAD18-Ub/RAD18 complex**

RAD18 in cells exhibits at least two different forms: an inactive, monoubiquitinated form (RAD18-mUb) and an active, nonubiquitinated form (Miyase et al., 2005; Zeman et al., 2014). Recently, RAD18-mUb was reported to preferentially interact with nonubiquitinated RAD18 and thus prevent the latter from being recruited to the damaged DNA (Zeman et al., 2014). We are wondering whether REV1 interacts with RAD18-mUb and thus releases RAD18 from RAD18-mUb/RAD18 complex. To test this hypothesis, we first examined the interaction between Flag-REV1 and GST-Ub-RAD18 or GST-RAD18. We found that REV1 bound more strongly to GST-Ub-RAD18 than to GST-RAD18 (Fig. 5A). Additionally, mutation of UBMs in REV1 significantly inhibited the binding of REV1 with GST-Ub-RAD18 (Fig. 5A). To further confirm the finding, we purified His-tagged REV1<sub>653-1123</sub> in both WT and UBMs* forms and examined their interactions with either GST-Ub-RAD18 or GST-RAD18. Like REV1 protein, His-REV1<sub>653-1123</sub> WT manifested a much stronger binding with GST-Ub-RAD18 relative to its affinity to GST-RAD18 (Fig. 5B). Mutation of UBMs in His-REV1<sub>653-1123</sub> mostly eliminated the preferential binding of His-REV1<sub>653-1123</sub> to
GST-Ub-RAD18 (Fig. 5B). Considering that mutation of L8A (with a leucine-to-alanine point mutation at L8) in ubiquitin could disrupt its association with the UBMs in REV1 (Bienko et al., 2005; Bomar et al., 2010), we generated GST-Ub(L8A) and GST-Ub(L8A)-RAD18 chimera to check their interactions with REV1<sub>653-1123</sub>. Consistently, we found that mutation of L8A in ubiquitin significantly inhibited the binding between REV1<sub>653-1123</sub> and ubiquitin or RAD18-Ub (Fig. 5C and D), further confirming that the enhanced interaction between REV1 and RAD18-Ub is mediated by the ubiquitin on RAD18-Ub and REV1 UBMs. Additionally, we transfected WT and UBMs* Flag-REV1 into 293T cells followed by crosslinking and immunoprecipitation using anti-Flag M2 beads. Western blot analysis of the immunoprecipitated fractions showed that unmodified RAD18 associated weakly with both WT and UBMs* REV1. In contrast, substantially more RAD18-mUb was coimmunoprecipitated with WT REV1 but not with the REV1 UBMs* mutant (Fig. 5E), further supporting preferential association of REV1 with ubiquitinated RAD18 than nonubiquitinated RAD18. We then determined whether REV1 affects the interaction between RAD18-mUb and RAD18. We co-transfected SFB-RAD18 and GFP-Ub-RAD18 into 293T cells and immunoprecipitated SFB-RAD18 with anti-Flag M2 beads. Then the beads were aliquoted and further incubated with increased amount of expressed GFP-REV1. Finally, the beads-bound proteins were analyzed through western blotting. We noted that the levels of coimmunoprecipitated GFP-Ub-RAD18 were negatively correlated with the amounts of supplemented GFP-REV1 (Fig. 5F), suggesting that REV1 inhibits the interaction between RAD18 and RAD18-Ub. Furthermore, we examined whether this inhibitory effect requires the UBMs of REV1. We performed the similar competitive binding experiment as above, by incubating the aliquoted beads with cell lysates expressing WT or UBMs* REV1. We found that mutation of UBMs in REV1 abrogated its inhibitory effect on the interaction between RAD18 and RAD18-Ub (Fig. 5G). To confirm these results, GST-Ub-RAD18 beads were aliquoted and incubated with equal amount of purified His-SUMO-RAD18 and increased amount of purified His-REV1<sub>653-1123</sub>. In line with the result shown in Fig. 5F, as more His-REV1<sub>653-1123</sub> was supplemented, there were
an increase in His-REV1_{653-1123} binding and a decrease in His-SUMO-RAD18 binding to GST-Ub-RAD18 (Fig. 5H). Compared to WT His-REV1_{653-1123}, UBM* His-REV1_{653-1123} exhibited a weaker association to GST-Ub-RAD18 concomitantly with a less inhibitory effect on the interaction between GST-Ub-RAD18 and His-SUMO-RAD18 (Fig. 5I). These results demonstrate that, through its UBMs, REV1 competes with RAD18 for binding to RAD18-mUb. As a corollary to this, nonubiquitinated RAD18 in RAD18-mUb/RAD18 complex is released, which allows more RAD18 to be recruited to chromatin for PCNA-mUb.

**REV1 does not promote PCNA-mUb after exposure to MMS**

In addition to UV radiation, multiple DNA damage treatments can also induce PCNA-mUb at stalled replication forks in mammalian cells (Kannouche et al., 2004; Niimi et al., 2008). We then checked whether the stimulatory effect of REV1 on PCNA-mUb still occurs after exposure to other damage agents causing replication fork stalling. HU, MMC and MMS are commonly used DNA damage agents to induce replication stress and PCNA-mUb (Lin et al., 2011; Mailand et al., 2013; Hedglin and Benkovic, 2015). MMC is a potent DNA crosslinker. HU stalls replication from depletion of nucleotide pools without eliciting DNA lesions, whereas MMS causes multiple DNA alkylation adducts that cannot be bypassed by the replicative DNA polymerases (Friedberg, 2006). We exposed the 293T cells to HU, MMC or MMS, and examined the effect of REV1 on PCNA-mUb. We found that expression of REV1 still promoted PCNA-mUb after HU and MMC treatments, while it failed to stimulate PCNA-mUb after exposure to MMS (Fig. 6A & B). Additionally, we noticed that RAD18-mUb could be detected after exposure to HU and MMC, but not MMS, which was recently reported to induce RAD18-mUb degradation (Zeman et al., 2014). These data further support that the stimulatory effect of REV1 on PCNA-mUb is dependent on the RAD18-mUb.
Discussions

PCNA-mUb plays an important role in recruiting Y-family polymerases to stalled replication forks to facilitate TLS process. Given its key role in TLS and genome mutagenesis (Hoege et al., 2002; Moldovan et al., 2007; Chen et al., 2011), multiple studies have been performed to elucidate how this modification is regulated in vivo (Hedglin and Benkovic, 2015). So far, many factors have been identified to regulate PCNA-mUb, including the RAD6-RAD18 ubiquitin ligase complex (Hoege et al., 2002; Kannouche et al., 2004), USP1 (Huang et al., 2006) and Polη (Durando et al., 2013). In this work, we report that REV1 also modulates PCNA-mUb in the absence of DNA damage or after exposure to UVC, HU and MMC.

Considering that REV1 interacts with Polη (Guo et al., 2003; Tissier et al., 2004) and Polη regulates PCNA-mUb (Durando et al., 2013), we examined whether the stimulatory effect of REV1 on PCNA-mUb was mediated by Polη. We found that over-expression of REV1 still promoted PCNA-mUb in the absence of Polη. In addition, we determined whether REV1 promoted PCNA-mUb through downregulating of USP1. We found that depletion of REV1 did not affect USP1 expression. Additionally, REV1 still enhanced PCNA-mUb in the USP1-depleted cells. These data suggest that the stimulatory effect of REV1 on PCNA-mUb is not mediated by Polη or USP1.

Interestingly, we found that the stimulatory effect of REV1 on PCNA-mUb after UV radiation required its UBMs, indicating that this process might be mediated by an ubiquitinated protein. Since the stimulatory effect of REV1 on PCNA-mUb is not correlated with increased REV1 focus formation or its colocalization with PCNA upon UV radiation, PCNA-mUb is not likely a plausible candidate. Intriguingly, we detected reduced RAD18 but not RPA recruitment to chromatin in triton-insoluble fractions in REV1-depleted cells. It has been reported that RAD18 can be monoubiquitinated in several different mammalian cell lines (Miyase et al., 2005; Zeman et al., 2014). Unlike nonubiquitinated RAD18, the ubiquitinated form of
RAD18 didn’t bind SNF2 histone linker plant homeodomain RING helicase (SHPRH) or helicase-like transcription factor (HLTF), two downstream E3 ligases needed to carry out error-free bypass of DNA lesions (Lin et al., 2011; Zeman et al., 2014). Instead, RAD18-mUb prefers to dimerize with nonubiquitinated RAD18 molecules, potentially inhibit RAD18 function in trans (Zeman et al., 2014). Notably, unlike exposure to MMS, UV radiation does not cause obvious RAD18 deubiquitination (Zeman et al., 2014). Considering RAD18-mUb can sequester nonubiquitinated RAD18 from recruiting to chromatin and thus regulates PCNA-mUb (Zeman et al., 2014), we speculate that RAD18-mUb might be the ubiquitinated protein which mediates the effect of REV1 on PCNA-mUb after UV radiation. We demonstrate that REV1 manifests an enhanced interaction with RAD18-Ub (a mimic of RAD18-mUb) compared to RAD18, while mutation of UBMs in REV1 significantly eliminated this preferential binding. Furthermore, WT, but not UBMs* REV1, competes with nonubiquitinated RAD18 for binding to RAD18-Ub, suggesting that REV1 likely facilitates the release of RAD18 from RAD18-Ub sequestration and promotes RAD18 to be recruited to chromatin (Fig. 6C). Consistently, the stimulatory effect of REV1 on PCNA-mUb was detected in cells exposed to examined DNA damage agents which do not abrogate RAD18-mUb, but not to MMS, an agent shown to remove RAD18-mUb (Zeman et al., 2014). Together, our data reveal a novel role for REV1 in regulating PCNA-mUb and TLS pathway after certain types of DNA damage. Notably, our mode of regulation of REV1 on PCNA-mUb is distinctively different from that of Polη, which was recently reported to regulate PCNA-mUb depending on its recruitment to damage sites, its PIP boxes and its interaction with RAD18 (Durando et al., 2013; Masuda et al., 2015).

REV1-mediated TLS is known to play a critical role in DNA damage-induced nucleotide substitutions in eukaryotes (Jansen et al., 2015). In addition to functioning as a scaffold protein for polymerase switching at sites of lesions (Guo et al., 2003), our data indicate that REV1 can promote PCNA-mUb in response to UV, HU and MMC, whose biological function(s) await further studies. The multiple regulatory roles of REV1 in error-prone TLS pathway make it a promising target for
chemotherapy. In support of that, recent studies using mouse lymphoma and prostate cancer models have shown that depletion of REV1 can remarkably inhibit drug-induced mutagenesis and sensitize cancer cells to chemotherapy (Xie et al., 2010; Xu et al., 2013).

**Materials and methods**

**Plasmids and reagents**

Mouse Rev1 cDNA was cloned in pEGFP-C3 (Clontech) or p3xFlag-CMV (Sigma) to generate EGFP or Flag tagged proteins (named GFP-REV1 and Flag-REV1, respectively). A series of truncated mREV1 mutants were PCR amplified and cloned into pEGFP-C3. The constructs with mutations in mREV1 UBM domains were generated as described previously (Guo et al., 2006b). WT and UBM* REV1_{653-1123}-pET-16b expressing vectors were constructed through PCR amplification. The plasmids of pSFB-RAD18 (Flag-RAD18) and His-SUMO-RAD18 (His-RAD18) were gifts from Dr. Jun Huang (Zhejiang University, Hangzhou, China). The cDNA of human RAD18 was cloned into pEGFP-C3 (Clontech) to generate the construct for expressing GFP-tagged RAD18 protein (GFP-RAD18). The ubiquitin cDNA lacking the C-terminal Gly-Gly codons was cloned into either pEGFP-C3-REV1 UBM* plasmid for expression of GFP-REV1-Ub chimera or pEGFP-C3-RAD18 plasmid for expression of GFP-Ub-RAD18 as previously described (Bienko et al., 2005; Zeman et al., 2014). The PCR products of RAD18 and Ub-RAD18 were also subcloned into pGEX-4T-2 (GE Healthcare) for expression of GST-RAD18 and GST-Ub-RAD18 in *E.coli*. The mutant of GST-Ub(L8A)-RAD18 was prepared from GST-Ub-RAD18 by site-directed mutagenesis.

Anti-Flag M2 agarose affinity gel (A2220) and mouse monoclonal antibody against Flag (M2) (F3165) were purchased from Sigma-Aldrich (St Louis, MO). Antibody against USP1 (D37B4) was from the Cell Signaling Technology (Danvers, MA). Antibodies against PCNA (PC10) (sc-56), His (H3) (sc-8036) and GFP (FL) (sc-8334) were from Santa Cruz Biotechnology. Antibodies against RAD18 (ab57447)
for western blotting and RPA32 (9H8) (ab2175) were from Abcam. Antibody against RAD18 (A301-340A) for immunofluorescence was purchased from Bethyl Laboratories (Montgomery, TX, USA). Antibody against β-Tubulin (abM59005-37B-PU) was from Beijing Protein Innovation (Beijing, China). Alexa Fluor-conjugated secondary antibodies were from Invitrogen. Rabbit polyclonal antiserum against REV\textsubscript{1872-1150} was made by Covance (Yang et al., 2015).

Cell Culture and Reagents

Human U2OS, HEK293T cells were obtained from the American Type Culture Collection (Rockville, MD). The XP30RO-Polη cell, which is a Polη-deficient XP30RO cell line engineered to express SFB-tagged Polη under the control of a tetracycline-inducible promoter (Han et al., 2014), was a gift from Dr. Jun Huang (Zhejiang University, Hangzhou, China). RAD18 stable knockdown U2OS cells were prepared as described (Zhang et al., 2013). All cell lines were grown in DMEM medium supplemented with 10% fetal bovine serum at 37°C in the presence of 5% CO₂. For transient transfection experiments, cells were transfected with indicated constructs, using VigoFect (Vigorous Biotechnology Beijing Co., Ltd) or Lipofectamin 2000 (Invitrogen) following the manufacturer’s protocols.

RNA interference

The introduction of small interfering RNA (siRNA) into cells was carried out with RNAiMAX (Invitrogen). siRNAs directed against human REV\textsubscript{1}, USP1 were obtained from GenePharma (Shanghai, China). The gene-specific target sequences were as follows: REV\textsubscript{1-1} (GAACAGUGACGCAGGAAUA) (Akagi et al., 2009), REV\textsubscript{1-2} (AAGCAUCAAAGCUGGACGACU) (Hicks et al., 2010), USP1 (GAAAUACACAGCCAAGUAAUU) (Han et al., 2014). The negative control siNC sequence (UUCUCCGAACGUGUCACGU) was obtained from GenePharma.

Establishment of RAD18 knockout cell lines

The RAD18 knocked out cells (RAD18KO) were established using TALEN-Tech as described previously (Sanjana et al., 2012) with some modifications. Briefly, the paired RAD18 TALEN arms were designed to target exon1 of RAD18. The sequences
targeting \textit{RAD18} (L: ggactccctggccga; R: caccttcagactgccag) were constructed into the backbone of pTALEN-L and pTALEN-R by one-step ligation using the Fast TALE\textsuperscript{TM} TALEN Assembly kit (SIDANSAI Biotechnology, China), respectively. To get \textit{RAD18}-deficient clones, HEK293T cells were transfected with a mixture of plasmids containing pTALEN-Rad18-L, pTALEN-Rad18-R, and pEGFP-C3 at a ratio of 9:9:2 in a 6 cm dish. One day later, the cells were incubated in media containing puromycin (1.2 μg/ml) for 3 days. Individual clones were isolated by limiting dilution and screened for RAD18 expression through western blot. Genomic DNA isolated from the RAD18KO cells was PCR amplified and the targeted exon of RAD18 was confirmed through sequencing.

**Immunofluorescence**

U2OS cells were UV irradiated and processed for immunofluorescence as described previously (Lv et al., 2013). Briefly, before fixing in 4% paraformaldehyde, the cells were treated with 0.5% Triton X-100 for 10 to 30 min. Then the cells were blocked with 5% donkey serum (for RAD18 staining) or 5% BSA and 2% goat serum (for RPA32 staining) for 30 min. After the blocking, cells were incubated with anti-RAD18 or anti-RPA32 for 45 min. Then the samples were washed three times with PBST (0.2% Tween 20 in PBS) and incubated with the appropriate Alexa Fluor dye conjugated secondary antibody (Molecular Probes) for 30 min. The cells were further counterstained with DAPI to visualize nuclear DNA. Images were taken with equal exposure time. The immunofluorescences for PCNA were done as described previously (Kannouche et al., 2001).

For quantitative analysis of UV-induced REV1 focus formation, U2OS cells transfected with GFP-REV1 were treated with UVC (15 J/m\textsuperscript{2}) and fixed with 4% paraformaldehyde 4 h later after UV irradiation as described previously (Guo et al., 2006a). The cells with more than 40 REV1 foci were counted.
**Preparation of triton-insoluble fractions for western blotting**

Triton-insoluble fractions were prepared as described previously (Kannouche et al., 2004) with modifications. Briefly, harvested cells were incubated with CSK100 buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM PIPES [pH 6.8], 1 mM EGTA, 0.2% Triton X-100) containing a cocktail of protease inhibitors for 20 min at 4°C. The pellets were lysed with buffer 1 (50 mM HEPES [pH 7.5], 50 mM NaCl, 0.05% SDS, 2 mM MgCl₂, 10% Glycerol, 0.1% Triton X-100, 10 unit of RNase-free DNase I) containing a cocktail of protease inhibitors overnight. The supernatants were harvested followed by western blotting.

**Proteins expression and purification in E. coli**

GST fusion proteins (GST-RAD18, GST-Ub-RAD18 and GST-Ub(L8A)-RAD18) were expressed and purified as described previously (Zeman et al., 2014). His-SUMO-RAD18 was expressed and purified as described previously (Han et al., 2014). WT and UBM* His-REV1₁₆₅₃-₁₁₂₃ were expressed in *E. coli* BL21 at 16°C overnight. Bacterial pellets were incubated with lysis buffer (50 mM Tris [pH 6.8], 300 mM NaCl, 1% Triton X-100, 10 mM imidazole, 1 mM PMSF, and 1 mM DTT) containing 1 mg/ml lysozyme (Sigma-Aldrich) for 1 h. After sonication, the lysates were clarified by centrifugation (12 000 g, 4°C, 30 min). The supernatant was incubated with Ni-NTA Agarose (Qiagen) for 2.5 h at 4°C. After washing with buffer (50 mM Tris [pH 6.8], 1 M NaCl, 1% Triton X-100, 10 mM imidazole), the proteins were eluted with buffer (50 mM Tris [pH 6.8], 300 mM NaCl, 1% Triton X-100, 250 mM imidazole and 1 mM DTT) and frozen at -80°C.

**Coimmunoprecipitation and Western blotting**

HEK293T cells transfected with WT and UBM* Flag-REV1 were incubated with 1% formaldehyde for 15 min at room temperature to crosslink proteins. The reaction was stopped by a 5-min incubation with 0.1 M glycine. After twice washes with PBS, the cells were harvested and lysed with CSK100 buffer for 30 min at 4°C. The supernatants after centrifugation were incubated with anti-Flag M2 beads
overnight. The beads-associated proteins were separated by SDS-PAGE followed by
immunoblotting with either anti-Flag or anti-RAD18 (A301-340A) antibodies.

**GST pull down assay**

For interaction between REV1 and GST-RAD18, REV1 expressing cells were
lysed with HEPES buffer (50 mM Hepes [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1
mM EGTA, 1% Triton X-100, 10% glycerol, 10 uM ZnCl, 25 mM NaF) for 1 h at
4°C and then clarified by centrifugation. The supernatants were incubated with the
indicated GST fusion proteins for 2.5 h at 4°C. For GST pull-down with purified
proteins, the purified proteins were diluted in HEPES buffer and then incubated with
the indicated GST proteins for 2.5 h at 4°C. After washing, the bound proteins were
separated by SDS-PAGE and analyzed by western blot with indicated antibodies.

**Competitive protein binding assays**

To examine how REV1 affects the interaction between RAD18 and ubiquitinated
RAD18, we transfected GFP-REV1 or SFB-RAD18 and GFP-Ub-RAD18 into 293T
cells. The cell lysates were harvested. SFB-RAD18 associated GFP-Ub-RAD18
were coimmunoprecipitated using anti-Flag M2 beads in HEPES buffer, then equal
amount of beads were further incubated with different amount of REV1-expressing
lysates. To directly determine how REV1 C-terminus affects the interaction between
RAD18 and ubiquitinated RAD18, equal amount of GST-Ub-RAD18 protein was
incubated with His-SUMO-RAD18 and increasing amounts of His-REV1653-1123. The
bound proteins were separated by SDS-PAGE followed by immunoblotting with
indicated antibodies. GST proteins were visualized by Ponceau S staining.
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Conflict of interest statement. None declared.
References


Fig. 1 REV1 promotes PCNA-mUb. (A) 293T cells were transfected with REV1-targeted siRNA (siREV1) or negative control siRNA (siNC). 48 h later, REV1 expression in whole cell extracts (WCE) was determined using anti-REV1 antibodies.
The REV1-depleted cells were exposed to 15 J/m² UVC radiation (UV) or control (Con), the triton insoluble fractions (CF) were extracted and the level of PCNA or PCNA-mUb was analyzed by western blotting using anti-PCNA antibodies. Tubulin: loading control. (B) 293T cells were transfected with GFP-REV1 or GFP vector. 30 h later, the cells were treated with UVC as in (A). The triton insoluble fractions were extracted and the levels of GFP-REV1, PCNA or PCNA-mUb were detected with anti-GFP or anti-PCNA antibodies, respectively. (C) The stimulatory effect of REV1 on PCNA-mUb is closely related to the level of REV1 expression. 293T cells were transfected with 1 μg (Lane 2) or 2 μg (Lane 3) of GFP-REV1 expressing construct or 2 μg of GFP vector (Lane 1). 30 h later, cells were treated as in (A). The levels of GFP-REV1 in WCE were analyzed with anti-GFP antibodies. The level of PCNA or PCNA-mUb was analyzed by western blotting using anti-PCNA antibodies. Tubulin: loading control. SE: short exposure; LE: long exposure.
Fig. 2 REV1 promotes PCNA-mUb via a RAD18-dependent fashion, but not dependent on Polη and USP1. (A) REV1 promotes PCNA-mUb independent of Polη. XP30RO-Polη cells were transfected with GFP-REV1 or GFP vector. 12 h later, the cells were incubated with media containing DMSO or 1 μg/ml doxycycline (Dox) for 12 h followed by 15 J/m² UVC radiation. 4 h later, the insoluble fractions (CF) were harvested and analyzed by Western Blotting. Expression of GFP-REV1 and SFB-Polη were determined in whole cell extracts (WCE). (B) Depletion of REV1 does not affect the level of USP1 in whole cell extracts. (C) Over-expression of REV1 does not affect the level of USP1 in whole cell extracts. (D) REV1 promotes PCNA-mUb independent of USP1. USP1-depleted (siUSP1) 293T cells were transfected with GFP-REV1 or GFP vector. The CF and WCE were harvested and analyzed through western blotting. Expression of USP1 was determined in WCE. Tubulin: loading
control. (E) RAD18 knocked down (shRAD18) and negative control (shNC) cells were transfected with GFP-REV1 or GFP vector. 30 h later, cells were treated with 15 J/m² UVC irradiation. The triton insoluble fractions were harvested and analyzed by western blotting as in Fig. 1C. The expression of RAD18 was determined by anti-RAD18 antibodies. (F) RAD18 wild-type (WT) and knockout (KO) 293T cells were transfected with GFP-REV1 (lanes 2, 4, 6, 8) or GFP (lanes 1, 3, 5, 7). The cells were treated and analyzed as in (A). Tubulin: loading control. GR: GFP-REV1. SE: short exposure; LE: long exposure.
Fig. 3 REV1 promotes the recruitment of RAD18 to chromatin. (A) 293T cells were transfected with siNC or siREV1. 48 h later, the cells were treated with 15 J/m² UVC radiation or not (Con). The whole cell extracts (WCE) were harvested. The expressions of RAD18 and REV1 were examined through western blotting using antibodies against RAD18 or REV1, respectively. Tubulin: loading control. (B) Depletion of REV1 inhibits the recruitment of RAD18 but not RPA32 to chromatin. The cells were treated as in (A). The triton-insoluble fractions were harvested and analyzed by western blotting. (C) Depletion of REV1 inhibits the recruitment of RAD18 to chromatin. The cells were treated as in (A) and the levels of RAD18 in WCE, triton-insoluble (CF) and soluble fractions were detected. LE: longer exposure; SE: short exposure. (D), (E) & (F) REV1 is required for optimal RAD18 focus formation. U2OS cells were transfected with two independent siRNAs against REV1 (siREV1-1 & siREV1-2) or negative control siNC. 48 h later, the cells were treated with 15 J/m² UVC radiation or not. 4 h later, the cells were permeabilized, fixed and
processed for RAD18 immunofluorescence. (D) Representative RAD18 foci were shown. (E) The proportion of cells with > 50 RAD18 foci was quantified. Quantification results were the mean of three independent experiments and were presented as means ± SEM. More than 100 cells were counted in each experiment. Depletion of REV1 significantly decreases UV-induced RAD18 focus formation as calculated using a student’s t-test (p < 0.01). (F) The level of REV1 in U2OS cells was analyzed by western blotting using anti-REV1 antibodies. Tubulin: loading control.
Fig. 4 REV1 promotes PCNA-mUb via its UBMs. (A) Schematic representation of mouse REV1. PIR: Polymerases interaction region; UBMs*: mutations in REV1 UBMs. (B) U2OS cells transfected with the indicated GFP-REV1 were irradiated with 15 J/m² UVC radiation and further incubated for 4 h. The cells were fixed and the proportion of GFP-REV1 expressing cells with greater than 40 foci was determined. More than 300 cells were counted in each experiment. Quantification results were from three independent experiments and were presented as means ± SEM. The *P values are respectively 0.0083, 0.007, 0.0042 and 0.003 from left to right. (C) 293T cells were transfected with the indicated GFP-REV1 constructs. The cells were treated and analyzed as in Fig. 1B. Expressions of GFP-REV1 were determined in WCE. Tubulin: loading control. (D) & (E) The REV1 peptides containing WT UBMs promote UV-induced RAD18 focus formation. U2OS cells were transfected with the indicated GFP-REV1 peptides by electroporation (LONZA, Amaxa Nucleofector, cell line nucleofector kit V: VCA-1003 for U2OS cell line). 24 h later, cells were UV
treated. 4 h later, cells were triton-pretreated followed by immunofluorescences for RAD18. The RAD18 foci positive cells in the total population were analyzed as in Fig. 3D & E. (D) Representative RAD18 foci were shown. (E) Quantification results were the mean of three independent experiments and were presented as means ± SEM. Overexpression of WT REV1 or REV1_{1-1123} or REV1_{653-1123} significantly increases UV-induced RAD18 focus formation as calculated using a student’s t-test (p < 0.05). More than 100 cells were counted in each experiment.
**Fig. 5** REV1 competes with RAD18 for binding to RAD18-Ub. (A) & (B) REV1 interacts with ubiquitinated RAD18 via its UBMs. Equal amounts of GST fusion proteins (GST-Ub-RAD18 and GST-RAD18) were incubated with cell lysates expressing Flag-REV1 (WT or UBM*) (A) or purified His-REV1<sub>653-1123</sub> (WT or UBMs*) (B). The associated REV1 was detected by immunoblotting with antibodies against Flag or His. (C) Mutation of L8A (with a leucine-to-alanine point mutation at L8) in ubiquitin inhibits its association with His-REV1<sub>653-1123</sub>. Equal amounts of GST fusion proteins (GST-Ub and GST-Ub-L8A) were incubated with purified His-REV1<sub>653-1123</sub>. (D) Mutation of L8A in ubiquitin inhibits RAD18-Ub association with His-REV1<sub>653-1123</sub>. Equal amounts of GST fusion proteins (GST-Ub-RAD18 and GST-Ub(L8A)-RAD18) were incubated with purified His-REV1653-1123. (E) HEK293T cells were transfected with Flag-REV1 WT and UBMs*. 48 h later, the cells were treated with 1% formaldehyde for 15 min at room temperature to crosslink proteins. The triton-soluble fractions were harvested and immunoprecipitated with
anti-Flag M2 beads. The beads-associated RAD18 and REV1 were detected with antibodies against RAD18 and Flag, respectively. (F) & (G) REV1 inhibits the binding of RAD18 and RAD18-Ub through its UBMs. HEK293T cells were transfected with GFP-REV1 or GFP-Ub-RAD18 and SFB-RAD18. 40 h later, the cell lysates were harvested. SFB-RAD18 associated GFP-Ub-RAD18 were coimmunoprecipitated using anti-Flag M2 beads, then equal amount of beads were further incubated with increased amount of REV1-expressing lysates (F) or equal amount of WT or UBM* REV1-expressing lysates (G). The immunoprecipitated GFP-Ub-RAD18 and SFB-RAD18 were detected with antibodies against GFP and Flag, respectively. The amounts of GFP-REV1 used in the competitive binding systems were determined with anti-GFP antibody. (H) & (I) Purified His-REV1_{653-1123} inhibits the binding of RAD18 and ubiquitinated RAD18 via its UBMs. GST-Ub-RAD18 proteins were incubated with equal amounts of His-SUMO-RAD18 and increased amounts of WT His-REV1_{653-1123} (H) or equal amounts of WT or UBM* His-REV1_{653-1123} (I). The His fusion proteins (His-RAD18 and His-REV1_{653-1123}) pulled down by GST-Ub-RAD18 were detected with anti-His antibody. Ponceau staining shows that equal amounts of GST fusion proteins were used for the experiments. WT: wild type; U*: UBMs*.
Fig. 6 Rad18-mUb is required for the stimulatory effect of REV1 on PCNA-mUb. (A) REV1 promoted PCNA-mUb in response to HU and MMC. (B) REV1 did not promote PCNA-mUb in response to MMS. HEK293T cells were transfected with GFP-REV1 for 30 h, treated with the following DNA damage agents: UVC (15 J/m², recovered for 4 h), HU (5 mM for 4 h), MMC (2.5 mg/ml for 4 h), MMS (50 μg/ml for 4 h) or DMSO (Con). The whole cell proteins (WCE) and triton insoluble fractions (CF) were extracted and analyzed. The expression levels of GFP-REV1 and ubiquitinated RAD18 were determined. Tubulin: loading control. SE: short exposure; LE: long exposure. (C) Model of REV1-promoted recruitment of RAD18 to chromatin. In the nucleoplasm, REV1 and non-ubiquitinated RAD18 competitively bind to ubiquitinated RAD18, which facilitates the release of non-ubiquitinated RAD18 from ubiquitinated RAD18 trapping followed by more RAD18 recruited to chromatin for its TLS function.