REV1 promotes PCNA monoubiquitylation through interacting with ubiquitylated RAD18

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ABSTRACT

Translesion DNA synthesis (TLS) is a mode of DNA damage tolerance which plays an important role in genome mutagenesis and chromatin integrity maintenance. Proliferating cell nuclear antigen (PCNA) monoubiquitylation is one of the key factors for TLS pathway choice. So far, it remains unclear how the TLS pathway is elaborately regulated. Here, we report that TLS polymerase REV1 can promote PCNA monoubiquitylation after UV radiation. Further studies revealed that this stimulatory effect is mediated through the enhanced interaction between REV1 and ubiquitylated RAD18, which facilitates the release of nonubiquitylated RAD18 from ubiquitylated RAD18 trapping, after which RAD18 is recruited 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 ubiquitylated RAD18 could not be detected after exposure to MMS.

KEY WORDS: REV1, PCNA, RAD18, Ubiquitylation, Translesion DNA synthesis, UV

INTRODUCTION

Cellular DNA is at risk of damage from 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 this, 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 the 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,b). 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 Polη, 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 monoubiquitylated at Lys164 in mammalian cells following various DNA damage treatments that cause stalling of the replication fork (Kannouche et al., 2004). Monoubiquitylated PCNA (PCNA–mUb) exhibits an enhanced interaction with Y-family polymerases (Guo et al., 2006b, 2008; Parker et al., 2007; Waters et al., 2009), and thus regulates their access to the replicative ensemble stalled at a lesion to execute their roles in lesion bypass. Given that PCNA–mUb promotes the TLS pathway (Hoeger 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 CRL3 (Terai et al., 2010), have been reported to regulate PCNA–mUb. Additionally, many factors, including apoptosis regulatory protein Siva (SIVA1) (Han et al., 2014), Spartan/C1orf124 [protein with sprT-like domain at the N-terminus, also known as DNA damage protein targeting VCP (DVC1)] (Centore et al., 2012), MutS protein homolog 2 (MSH2) (Zlatanou et al., 2011; Lv et al., 2013), breast cancer 1 (BRCA1) (Tian et al., 2013), have also been found to regulate the RAD18-dependent PCNA–mUb. Ubiquitin-specific protease 1 (USP1) also participates in the regulation of PCNA–mUb as a key deubiquitylase (DUB) for deubiquitylating PCNA–mUb (Huang et al., 2006). Recently, it was reported that monoubiquitylated RAD18 (RAD18–mUb) also regulates PCNA–mUb and TLS activity (Zeman et al., 2014). RAD18–mUb not only releases itself from chromatin (Zeman et al., 2014), but also sequesters nonubiquitylated RAD18, preventing it from recruiting to chromatin.

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

RESULTS

REV1 promotes PCNA monoubiquitylation

As PCNA–mUb has been shown to interact with REV1 and mediate REV1 accumulation at UV-induced stalled replication sites, we wondered whether REV1 also affects UV-induced PCNA monoubiquitylation. To answer this question, we transfected 293T cells with siRNA directed against human REV1 (siREV1) and examined the level of PCNA–mUb in the Triton-X-100-insoluble
fractions. We found that depletion of REV1 decreased the level of PCNA–mUb in the presence and absence of UV radiation (Fig. 1A). Conversely, we found that overexpression 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 amounts of REV1 induced more PCNA to be monoubiquitylated (Fig. 1C). These data indicate that REV1 promotes basal and UV-induced PCNA–mUb, which is closely correlated to the level of REV1 expression. Additionally, we performed PCNA immunofluorescence and detected no obvious change in PCNA focus formation in cells either depleted of or overexpressing REV1 (Fig. S1). This result is to be expected because PCNA–mUb does not seem to be 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), leading us to wonder 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, and found that overexpression of REV1 still promoted PCNA–mUb in the absence of Polη (Fig. 2A, lanes 1 and 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 and 3), which is in line with the previous report (Durando et al., 2013), overexpression of REV1 in the Polη-expressing XPRO30–Polη cells further increased the level of PCNA–mUb after UV damage (Fig. 2A, lanes 2, 3 and 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 deubiquitylase (Huang et al., 2006), we wondered 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, overexpression of REV1 could also not reduce USP1 expression (Fig. 2C). Additionally, we found that overexpression 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 wondered whether REV1 promotes PCNA–mUb in a RAD18-dependent fashion. We first transfected GFP–REV1 into 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 showed an appreciable level of REV1-promoted PCNA–mUb, it was 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 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 monoubiquitylation of PCNA 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 results (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 speculated that the amount of RAD18 released from the chromatin upon REV1 knockdown might not be enough to produce an obvious difference in the distribution of RAD18–mUb/RAD18 in the soluble fraction. Thus, we examined whether REV1 regulates UV-induced RAD18 focus formation. We transfected U2OS cells with two independent siRNAs (siREV1-1 and siREV1-2) and found that knockdown of REV1 led to a clear reduction in RAD18 focus formation after UV damage (Fig. 3D,E), although depletion of REV1 had no obvious effect on RAD18 global nuclear staining (Fig. 3B). Considering that RAD18 is recruited to stalled replication forks by virtue of its affinity for single-stranded DNA (ssDNA) coated with replication protein A (RPA) (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 (Fig. 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 monoubiquitylation of PCNA 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 to chromatin, we first examined the roles of essential REV1 domains in promoting RAD18 chromatin association and monoubiquitylation of PCNA. 293T cells expressing a panel of REV1 peptides (Fig. 4A) were UV-irradiated and the Triton-X-100-insoluble fractions were analyzed. Like wild-type (WT) REV1, REV11-1123 (without the C-terminal PIR) could promote monoubiquitylation of PCNA and RAD18 recruitment to chromatin (Fig. 4C), indicating that REV1-facilitated PCNA–mUb is independent of its PIR. This data is consistent with REV1 promoting PCNA–mUb in a Poly-independent fashion. Moreover, REV1653–1123 (without the BRCT, catalytic core and PIR) could also promote monoubiquitylation of PCNA and RAD18 recruitment to chromatin (Fig. 4C), suggesting 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 monoubiquitylation of PCNA and RAD18 recruitment to chromatin. Given that the UBM domains not only mediate the interaction of REV1 with other ubiquitylated proteins but also its monoubiquitylation (Guo et al., 2006b; Kim et al.,...
2012), we analyzed whether monoubiquitylated REV1 could stimulate PCNA–mUb. Ubiquitin cDNA lacking the C-terminal Gly-Gly codons was cloned into a pEGFP-C3-REV1 UBM* plasmid to make a full-length REV1-ubiquitin chimera (REV1–Ub; a mimic of monoubiquitylated REV1). We found that the REV1–Ub chimera failed to rescue the decreased PCNA–mUb resulting from expression of UBMs* (Fig. 4C). These data suggest that REV1 does not likely facilitate PCNA–mUb through its own monoubiquitylation. Consistent with this result, overexpression of WT REV1, REV1<sub>1-1123</sub> and REV1<sub>653-1123</sub>, 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 recruitment of RAD18. Therefore, the stimulatory effect of REV1 on PCNA–mUb might be mediated through its interaction with a ubiquitylated protein. It is known that REV1 can interact with PCNA–mUb at the stalled replication sites, where REV1 exhibits focal distribution in the nucleus (Guo et al., 2009). We examined whether the REV1 peptides which promote PCNA–mUb demonstrated 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; Fig. S4). Moreover, we also noticed that, unlike WT REV1, REV1<sub>1-1123</sub> and REV1<sub>653-1123</sub> did not display an appreciable difference in the extent of their colocalization with PCNA after UV treatment when 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, these results hint at the possibility that another...
ubiquitylated protein(s) other than PCNA–mUb might be involved in this process.

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

RAD18 in cells exists in at least two different forms: an inactive, monoubiquitylated form (RAD18–mUb) and an active, nonubiquitylated form (Miyase et al., 2005; Zeman et al., 2014). Recently, RAD18–mUb was reported to preferentially interact with nonubiquitylated RAD18 and thus prevent the latter from being recruited to damaged DNA (Zeman et al., 2014). We wondered whether REV1 interacts with RAD18–mUb and thus releases RAD18 from the 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. 5B).
To further confirm this finding, we purified His-tagged REV1* and examined their interactions with either GST–UB–RAD18 or GST–RAD18. Like REV1 protein, His–REV1 displayed much stronger binding with GST–UB–RAD18 relative to its affinity to GST–RAD18 (Fig. 5B). Mutation of UBMs in His–REV1 largely eliminated the preferential binding of His–REV1 to GST–UB–RAD18 (Fig. 5B). Considering that a L8A point mutation 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) chimeras to check their interactions with REV1. Consistently, we found that mutation of L8A in ubiquitin significantly inhibited the binding between REV1 and ubiquitin or RAD18–Ub (Fig. 5C,D), further confirming that the enhanced interaction between REV1 and RAD18–Ub is mediated by the ubiquitin on RAD18–Ub and REV1. 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. By contrast, substantially more RAD18–mUb was comammunoprecipitated with WT REV1 but not with the REV1 UBMs* mutant (Fig. 5E), further supporting preferential association of REV1 with ubiquitylated RAD18 than nonubiquitylated RAD18. We then determined whether REV1 affects the interaction between RAD18–mUb and RAD18. We co-transfected SFB–RAD18 and GPF–Ub–RAD18 into 293T cells and immunoprecipitated SFB–RAD18 with anti-FLAG M2 beads. Then the beads were aliquted and further incubated with an increased amount of expressed GPF–REV1. Finally, the bead-bound proteins were analyzed through western blotting. We noted that the levels of coimmunoprecipitated GPF–Ub–RAD18 were negatively correlated with the amounts of supplemented GPF–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 aliquted 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 aliquted and incubated with an equal amount of purified His–SUMO–RAD18 and increased amount of purified His–REV1. In line with the result shown in Fig. 5F, as more His–REV1 was supplemented, there was an increase in His–SUMO–RAD18 binding and a decrease in His–REV1 binding to GST–UB–RAD18 (Fig. 5H). Compared with His–REV1, His–REV1–UBMs* exhibited a weaker association with GST–UB–RAD18, concomitantly with a lower 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, nonubiquitylated RAD18 in RAD18–mUb complex is released, which allows more RAD18 to be recruited to chromatin for monoubiquitylation of PCNA.

**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 thus checked whether the stimulatory effect of REV1 on PCNA–mUb still occurs after exposure to other damage agents causing replication fork stalling. Hydroxyurea, 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, hydroxyurea stalls replication through 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 293T cells to hydroxyurea, MMC or MMS, and examined the effect of REV1 on PCNA–mUb. We found that expression of REV1 still promoted PCNA–mUb after hydroxyurea and MMC treatments, whereas 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 hydroxyurea and MMC, but not MMS, which was recently reported to induce RAD18–mUb degradation (Zeman et al., 2014). These data further support the conclusion that the stimulatory effect of REV1 on PCNA–mUb is dependent on RAD18–mUb.

**DISCUSSION**

PCNA–mUb plays an important role in recruiting Y-family polymerases to stalled replication forks to facilitate the 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 the monoubiquitylation of PCNA is regulated in vivo (Hedglin and Benkovic, 2015). So far, a number of factors have been identified which 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 study, we report that REV1 also modulates PCNA–mUb in the absence of DNA damage, after exposure to UV, radiation, or treatment with hydroxyurea and MMC.

Because 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 overexpression of REV1 still promoted PCNA–mUb in the absence of Polη. In addition, we determined whether REV1 promoted PCNA–mUb through downregulation of USP1. We found that depletion of REV1 did not affect USP1 expression. Additionally, REV1 still enhanced PCNA–mUb in 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 irradiation required its UBMs, indicating that this process might be mediated by a ubiquitylated protein. Because the stimulatory effect of REV1 on PCNA–mUb is not correlated with increased REV1 focus formation or its colocalization with PCNA upon UV irradiation, PCNA–mUb is not a plausible candidate for this mediation. Intriguingly, we detected reduced RAD18 but not RPA recruitment to chromatin in Triton-X-100-insoluble fractions in REV1-depleted cells. It has been reported that RAD18 can be monoubiquitylated in several different mammalian cell lines (Miyase et al., 2005; Zeman et al., 2014). Unlike nonubiquitylated RAD18, the ubiquitylated form of RAD18 does not bind SNF2 histone linker protein 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 nonubiquitylated RAD18 molecules, potentially inhibiting RAD18 function in trans
(Zeman et al., 2014). Notably, unlike exposure to MMS, UV radiation does not cause obvious RAD18 deubiquitylation (Zeman et al., 2014). Considering RAD18–mUb can sequester nonubiquitylated RAD18 from recruiting to chromatin and thus regulates PCNA–mUb (Zeman et al., 2014), we speculate that RAD18–mUb might be the ubiquitylated protein which mediates the effect of REV1 on PCNA–mUb after UV radiation. We demonstrate that REV1 displays an enhanced interaction with RAD18–Ub (a mimic of RAD18–mUb) compared with RAD18, whereas mutation of UBMs in REV1 significantly eliminated this preferential binding. Furthermore, WT, but not UBMs* REV1, competes with nonubiquitylated RAD18 for binding to RAD18–Ub, suggesting that REV1 likely facilitates the release of nonubiquitylated RAD18 from ubiquitylated RAD18 trapping followed by further RAD18 recruited to chromatin for its TLS function.

Fig. 6. Rad18–mUb is required for the stimulatory effect of REV1 on PCNA–mUb. HEK293T cells were transfected with GFP–REV1 for 30 h, followed by treatment with the following DNA damage agents: UVC (15 J/m², recovered for 4 h), hydroxyurea (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 PCNA, PCNA–mUb, GFP–REV1 and ubiquitylated RAD18 were determined. (A) REV1-promoted PCNA–mUb in response to hydroxyurea and MMC. (B) REV1 did not promote PCNA–mUb in response to MMS. 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-ubiquitylated RAD18 competitively bind to ubiquitylated RAD18, which facilitates the release of non-ubiquitylated RAD18 from ubiquitylated RAD18 trapping followed by further RAD18 recruited to chromatin for its TLS function.

REV1-mediated TLS is known to play a crucial 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, hydroxyurea and MMC, whose biological function(s) await further studies. The multiple regulatory roles of REV1 in the error-prone TLS pathway make it a promising target for chemotherapy. In support of this, 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 into pEFGP-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 pEFGP-C3. The constructs with mutations in mREV1 UBM domains were generated as described previously (Guo et al., 2006b). Vectors expressing REV1UBM* modification were constructed through PCR amplification. The plasmids of pSFB-RAD18 (FLAG–RAD18) and His–SUMO–RAD18
Rabbit polyclonal antiserum against REV1872-1150 was made by Covance Innovation (Beijing, China). Alexa-Fluor-488-labeled goat anti-mouse-β was purchased from Bethyl Laboratories (Montgomery, TX). Antibody against RAD18 (A301-340A; 1:3000) for immunofluorescent staining was from Santa Cruz Technology. Antibodies against PCNA (PC10) (sc-56; 1:1000), His (H3) (sc-8036; 1:1000) and GFP (FL) (sc-8334; 1:500) were from Santa Cruz Technology. Antibodies against FLAG (M2) (F3165; 1:1000) were purchased from Sigma-Aldrich. Antibody against USP1 (D37B4; 1:1000) was from Cell Signaling Technology. Antibodies against PCNA (PC10) (sc-56; 1:1000), His (H3) (sc-8036; 1:1000) and GFP (FL) (sc-8334; 1:500) were from Santa Cruz Biotechnology. Antibodies against RAD18 (ab57447; 1:1000) for western blotting and RPA32 (9H8) (ab2175; 1:1000) were from Abcam. Antibody against RAD18 (A301-340A; 1:3000) for immunofluorescence was purchased from Bethyl Laboratories (Montgomery, TX). Antibody against β-tubulin (ab69505-7BB-P; 1:4000) was from Beijing Protein Innovation (Beijing, China). Alexa-Fluor-488-labeled goat anti-mouse-IgG (A-11001; 1:1000) and Alexa-Fluor-555-labeled donkey anti-rabbit-IgG (A-31572; 1:1000) antibodies were from Invitrogen – Molecular Probes. Rabbit polyclonal antiseraum against REV1872-1150 was made by Covance (Yang et al., 2015).

Cell culture and reagents
Human U2OS and HEK293T cells were obtained from the American Type Culture Collection (Rockville, MD). The XP30RO-Polβ cell line, which is a Polβ-deficient XP30RO cell line engineered to express SFB-tagged Polβ, 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, China) 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 REV1 and USP1 were obtained from GenePharma (Shanghai, China). The gene-specific target sequences were as follows: REV1-1 (GAACAGUGACGC-AGGAAUA) (Akagi et al., 2009), REV1-2 (AAGCAUAAGGCGGA-CGACU) (Hicks et al., 2010), USP1 (GAAGAUACAGGCAAAGUAUU) (Han et al., 2014). The negative control siNC sequence (UUCUCCGAG-GGUUGACGU) was obtained from GenePharma.

Establishment of RAD18 knockout cell lines
The RAD18 knockout cell lines (RAD18KO) were established using TALEN as described previously (Sanjana et al., 2012) with some modifications. Briefly, the paired RAD18 TALEN arms were designed to target exon 1 of RAD18. The sequences targeting RAD18 (L: gcgcctgctgcgcga; R: cccctctgctgcgcga) were constructed into the backbone of pTALEN-L and pTALEN-R, respectively, by one-step ligation using the Fast TALE™ TALEN Assembly kit (SIDANSAI Biotechnology, China). To get 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 9:2:1 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, the cells were incubated with anti-RAD18 or anti-RPA32 antibodies 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-conjugated secondary antibody (Invitrogen – Molecular Probes) for 30 min. The cells were further counterstained with DAPI to visualize nuclear DNA. Images were taken with equal exposure time. The immunofluorescence studies for PCNA were done as described previously (Kannouche et al., 2001).

Preparation of triton-insoluble fractions for western blotting
Triton-X-100-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, 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 units of RNase-free DNase I) containing a cocktail of protease inhibitors overnight. The supernatants were harvested following 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). His–REV1553.1123–UBM5 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 mM 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.

Comunoprecipitation and western blotting
HEK293T cells transfected with WT and UBMs* 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 two washes with PBS, the cells were harvested and lysed with CSK100 buffer for 30 min at 4°C. After centrifugation at 21,000 g the supernatants were incubated with anti-FLAG M2 beads overnight. The bead-associated proteins were separated by SDS-PAGE followed by immunoblotting with either anti-FLAG or anti-RAD18 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, 10 μM ZnCl2, 25 mM NaF) for 1 h at 4°C and then clarified by centrifugation at 21,000 g. 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 ubiquitlated RAD18, we transfected GFP–REV1 or SFB–RAD18 and GFP–UB–RAD18 into 293T cells. The cell lysates were harvested. SFB–RAD18 and associated GFP–UB–RAD18 were communoprecipitated using anti-FLAG M2 beads in HEPEs buffer, then an equal amount of beads were further incubated with different amount of REV1-expressing lysates. To directly determine how the REV1 C-terminus affects the interaction between RAD18 and ubiquitlated RAD18, an equal amount of GST–UB–RAD18 protein was incubated with His–SUMO–RAD18 and increasing amounts of His–REV1 in 1232.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
C.G. and T.T. conceived the study and designed experiments; Z.W., T.T. and C.G. constructed several expression vectors. Z.W., T.T. and C.G. analyzed the data and interpreted the results. M.H and H.L. established the RAD18-knockout cell line. X.M., C.G. and T.T. wrote the manuscript. All authors read and approved the manuscript for publication.

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Supplementary information
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References


**Supplemental figures**

**Fig. S1. Depletion of REV1 has no obvious effects on PCNA focus formation.** U2OS cells were transfected with two 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 PCNA immunofluorescence. The proportion of cells with greater than 30 foci was determined. (A) Representative PCNA foci were shown. (B) Quantification results were the mean of three independent experiments and were presented as means ± SEM. More than 100 cells were counted in each experiment. (C,D) REV1 promoting PCNA-mUb is independent of its colocalization with PCNA and itself focus formation. U2OS cells were transfected with the indicated GFP-REV1 peptides. 24 h later, cells were treated and PCNA were detected as in Fig.S1A & B. The proportion of cells with greater than 30 foci was determined. (C) Representative PCNA and indicated GFP-REV1 peptides foci were shown. (D) Quantification results were the mean of three independent experiments and were presented as means ± SEM. More than 100 cells were counted in each experiment. Overexpression of REV1 has no obvious effects on PCNA focus formation.
Fig. S2. (A) UV irradiation increases the amount of chromatin-associated RAD18, but fails to cause an obvious change at the level of RAD18 in soluble fraction. 293T Cells exposed to UV radiation (+UV) or not (-UV) were incubated with CSK100 buffer for 20 min at 4°C, the triton-soluble fractions were harvested. The triton-insoluble pellets were treated with buffer 1 (the volume is equivalent to that of CSK100 buffer used as above) overnight to get the chromatin fractions. The levels of RAD18 and PCNA in triton-soluble fractions and chromatin fractions were examined through western blot with antibodies against RAD18 and PCNA. Chromatin-associated RAD18 is only a small fraction of total RAD18 in cells even after UV radiation. (B) Depletion of REV1 had no obvious effect on the RAD18 global nuclear staining. RAD18 immunofluorescence staining was done as in Fig. 3 (D) except without triton X-100 treatment. Representative RAD18 global stainings were shown.
Fig. S3. Depletion of REV1 has no obvious effects on UV-induced RPA focus formation. U2OS cells were transfected with siREV1 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 and processed for RPA immunofluorescence. (A) Representative RPA positive cells were shown. (B) Quantification results were the mean of three independent experiments and were presented as means ± SEM. More than 100 cells were counted in each experiment.
**Fig. S4.** Representative images of REV1 foci for the indicated GFP-REV1 peptides shown in Fig. 4(B).