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A Transcriptional Repressor ZBTB1 Promotes Chromatin Remodeling and Translesion DNA Synthesis

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SUMMARY

Timely DNA replication across damaged DNA is critical for maintaining genomic integrity. Translesion DNA synthesis (TLS) allows bypass of DNA lesions using error-prone TLS polymerases. The E3 ligase RAD18 is necessary for PCNA monoubiquitination and TLS polymerase recruitment; however, the regulatory steps upstream of RAD18 activation are less understood. Here, we show that the UBZ4 domain-containing transcriptional repressor ZBTB1 is a critical upstream regulator of TLS. The UBZ4 motif is required for PCNA monoubiquitination and survival after UV damage. ZBTB1 associates with KAP-1, a transcriptional repressor whose phosphorylation relaxes chromatin after DNA damage. ZBTB1 depletion impairs formation of phospho-KAP-1 at UV damage sites and reduces RAD18 recruitment. Furthermore, phosphorylation of KAP-1 is necessary for efficient PCNA modification. We propose that ZBTB1 is required for PCNA monoubiquitination, by localizing phospho-KAP-1 to chromatin and enhancing RAD18 accessibility. Collectively, our study implicates a new ubiquitin-binding protein in orchestrating chromatin remodeling during DNA repair.

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SUPPLEMENTAL INFORMATION

Supplemental information includes six figures, two tables, supplemental experimental procedures, and supplemental references, and can be found with this article online.

Keywords

Ubiquitin Binding Zinc Finger 4 (UBZ4); Ubiquitination; Translesion DNA Synthesis; KAP-1; Fanconi anemia

INTRODUCTION

The faithful replication of DNA ensures accurate transmission of genomic information across generations, which is supported by highly complex DNA repair systems to counter a variety of genotoxic stress that leads to DNA damage (Ciccia and Elledge, 2010). In order to prevent replication fork stalling and collapse, which can give rise to strand breaks during DNA replication, cells utilize damage-avoidance mechanisms to temporarily tolerate DNA damage without repairing lesions. One strategy, called translesion DNA synthesis (TLS) allows bypass of DNA lesions such as ultraviolet (UV)-induced DNA adducts that impede replication fork progression using specialized DNA polymerases (Chang and Cimprich, 2009). In this process, the E3 ubiquitin ligase, RAD18, is recruited to RPA-coated single-stranded DNA (ssDNA) generated by uncoupling of polymerase and helicase activities at stalled replication forks (Chang et al., 2006; Davies et al., 2008). Several regulatory factors, such as DVC1 (also known as Spartan/C1orf124) and NBS1, enhance RAD18 recruitment and promote TLS (Centore et al., 2012; Ghosal et al., 2012; Yanagihara et al., 2011). RAD18 monoubiquitinates the DNA polymerase processivity factor, Proliferating Cell Nuclear Antigen (PCNA), at damage sites, and monoubiquitinated PCNA in turn recruits a TLS polymerase, such as Pol κ or Pol η(Lehmann et al., 2007; Moldovan et al., 2007). A TLS polymerase is recruited via its PIP (PCNA-interacting protein) box and its ubiquitin-binding domain, which binds to the monoubiquitin on PCNA (Sale et al., 2012).

TLS polymerases are error-prone, and the mutagenic effect of TLS has been implicated in the acquisition of anti-cancer drug resistance (Xie et al., 2010). Thus, eukaryotic cells have evolved several mechanisms to limit their activity and to restrict mutagenesis. First, the protease complex USP1-UAF1 deubiquitinates monoubiquitinated PCNA (Cohn et al., 2007; Huang et al., 2006). Also, the DNA damage-targeting p97 adaptor DVC1 displaces TLS Pol η from PCNA (Davis et al., 2012; Mosbech et al., 2012). Finally, ATAD5 promotes PCNA unloading from the replication factory (Lee et al., 2013). While many of these downstream regulatory events for TLS are known, the upstream events, such as the recognition of the stalled replication fork and the regulation of RAD18 in the context of chromatin, are less clear.

The reorganization of chromatin after DNA damage is an important component of DNA repair process as it ensures accessibility of damaged chromatin by DNA repair factors, which requires a concerted action of chromatin remodeling complexes and histone modifiers (Soria et al., 2012). For instance, maintenance of heterochromatin should be transiently perturbed to initiate DNA repair upon genotoxic stress, and Krüppel-associated box (KRAB)-associated protein (KAP)-1, a universal corepressor of gene transcription by binding a subclass of zinc finger-containing proteins, is one of the factors targeted by ATM-dependent phosphorylation to allow local chromatin relaxation (Goodarzi et al., 2008).

Accordingly, a S824A (non-phosphorylated) mutant of KAP-1 suppresses chromatin relaxation and renders cells hypersensitive to ionizing irradiation (IR) (Ziv et al., 2006). Mechanistically, disruption of interaction between the SUMO conjugate of KAP-1 and the SUMO-interacting motif (SIM) of CHD3 following KAP-1 phosphorylation leads to the dispersal of CHD3 from the chromatin, resulting in local chromatin relaxation (Goodarzi et al., 2011). However, the functional connection between chromatin remodeling and TLS is less understood.

Zinc finger and BTB containing 1 (ZBTB1) is a transcriptional repressor protein and a member of the mammalian ZBTB gene family, consisting of at least forty-nine members (Liu et al., 2011). ZBTB1 is 713 amino acids in length and contains an N-terminal BTB (Broad Complex, Tramtrack, and Bric a brac) domain and eight zinc finger motifs. The BTB domain is an oligomerization domain, required for ZBTB1 autosumoylation (Matic et al., 2010). The BTB domain also functions as a substrate-specific adaptor for a CUL-3 E3 ubiquitin ligase, targeting proteins for proteasome-mediated degradation (Xu et al., 2003). ZBTB1 forms constitutive transcriptional repression foci in mammalian nuclei (Liu et al., 2011; Matic et al., 2010). The protein appears to have a specific role in the transcriptional regulation of T lymphocyte development, and a homozygous murine knockout of *Zbtb1* results in severe combined immunodeficiency (Punwani et al., 2012).

In the current study, we demonstrate that one of the zinc fingers of ZBTB1 is a specialized ubiquitin-binding zinc-finger 4 (UBZ4) domain. UBZ4 domains are frequently found in DNA repair proteins and are essential for targeting to sites of DNA damage by recognizing ubiquitinated proteins (Hofmann, 2009). Knockdown of ZBTB1 in human cells, or mutation of the UBZ4 domain, blocked UV-inducible PCNA-monoubiquitination and conferred cellular hypersensitivity to UV, suggesting that ZBTB1 is required upstream in the TLS DNA repair pathway. Moreover, ZBTB1 is recruited to sites of UV-induced cyclobutane pyrimidine dimers (CPDs) where it enables local chromatin remodeling by pKAP-1. Our results suggest that ZBTB1 plays a specific role in the maintenance of pKAP-1 formation at UV damage sites. Chromatin relaxation allows recruitment of RAD18, thus facilitating PCNA monoubiquitination and TLS. ZBTB1 is therefore another UBZ4-containing protein specializing in TLS regulation, such as RAD18 itself, Pol κ, and DVC1. Our study provides new insights into how ubiquitin signaling is coordinated with chromatin remodeling to promote DNA repair.

RESULTS

ZBTB1 is required for UV-inducible PCNA monoubiquitination

We previously performed a bioinformatic search and identified proteins which contain UBZ4 domains and are therefore likely to function in DNA repair (Kim et al., 2012). Since DNA repair defects often underlie genome instability and tumorigenesis, we determined whether mutations in any of these proteins are enriched in primary human tumor samples from published Cancer Genome Atlas (TCGA) datasets (Table S1). Strikingly, one of these UBZ4-containing proteins, ZBTB1, exhibited nonsense and frameshift mutations, predicted to eliminate the UBZ4 motif and therefore disrupt DNA repair activity (Figure 1A and S1A). Therefore, we hypothesized that, in addition to its known transcriptional regulation, ZBTB1

may directly participate in DNA repair pathways and mutations of this gene may contribute to carcinogenesis.

ZBTB1 contains a highly conserved BTB/POZ domain at its amino-terminus, and eight C2H2-type zinc fingers, the third of which constitutes an evolutionary conserved C2HC-type UBZ4 motif (Figures 1A, 1B, and S1B). Interestingly, depletion of ZBTB1 in HeLa cells, using multiple independent siRNAs, decreased PCNA monoubiquitination following UV damage. A similar decrease was observed following siRNA knockdown of RAD18, an E3 ubiquitin ligase responsible for monoubiquitinating PCNA (Figures 1C, 1D, and S1C). The cell-cycle distribution was not markedly affected by ZBTB1 knockdown (Figure S1D), and reintroduction of an siRNA-resistant ZBTB1 restored PCNA monoubiquitination, arguing against an off-target effect (Figures 1E, lane 4). Furthermore, the decrease in PCNA monoubiquitination following ZBTB1 depletion resulted from an upstream defect and not from an increase in the deubiquitination of PCNA-Ub by USP1 (Figure S1E). The siRNA to ZBTB1 also caused an accumulation of γH2AX and phospho-RPA2, denoting spontaneous DNA damage (Figures 1C, 1D, S1F, and S1G). Furthermore, the enhanced γH2AX signal caused by ZBTB1 knockdown could be reduced by co-depleting MUS81, a structure-specific endonuclease responsible for cleaving stalled replication forks (Figure 1F and S1H). This result suggests that ZBTB1 suppresses the formation of replication-associated double-strand breaks (DSBs) following UV damage, perhaps by enhancing TLS and promoting fork progression. Taken together, these results suggest that ZBTB1 may play a role in preserving genomic integrity during DNA replication.

ZBTB1 is required for Pol η recruitment, point mutagenesis, and TLS-mediated UV resistance

PCNA-Ub provides a landing pad for the recruitment TLS polymerases to DNA lesions, allowing bypass of replication-blocking lesions such as CPDs and pyrimidine-pyrimidone [6-4] photoproducts (6-4 PPs) caused by UV damage (Ulrich, 2009). The recruitment of TLS polymerases is essential for the timely completion of DNA replication and cellular survival. Therefore, we examined the effect of ZBTB1 depletion on TLS activity, regulated by PCNA-Ub. ZBTB1 knockdown, like RAD18 depletion, significantly decreased the assembly of TLS polymerase η(Pol η) foci. Pol η is a TLS polymerase with a UBZ3 domain and a binding partner of ubiquitin-modified PCNA (Figures 2A and 2B). The UBZ4-containing DVC1 protein was recently shown to regulate RAD18 in the TLS pathway (Centore et al., 2012; Davis et al., 2012; Ghosal et al., 2012; Mosbech et al., 2012). However, whether the UBZ4 motif of DVC1 specifically recognizes PCNA-Ub is uncertain. In our experiments, neither RAD18 nor ZBTB1 knockdown substantially affected the recruitment of DVC1 to UV-damage sites, indicating that ZBTB1 does not regulate DVC1 localization (Figures S2A and S2B).

Knockdown of ZBTB1 decreased the frequency of UV-inducible mutagenesis (Figure 2C), consistent with its putative role in TLS. Accordingly, cells exposed to ZBTB1 siRNA were hypersensitive to UV damage (Figure 2D). Moreover, *ZBTB1* and *POLH*(encoding TLS polymerase η) were epistatic in conferring UV resistance (Figure S2C). In contrast, ZBTB1-depleted cells were not hypersensitive to PARP inhibitors, suggesting that ZBTB1 is not

directly involved in HR repair (Figure 2E), or to the cross-linking agent MMC (Figure S2D). However, ZBTB1 may also play a minor role in HR since ZBTB1 knockdown leads to some reduction in DSB-induced HR, comparable to RAD18 depletion (Figure S2E). How ZBTB1 contributes to the HR process is currently unknown. Taken together, these data suggest that ZBTB1 plays a confined role in regulating the cellular response to UV damage via the PCNA-dependent TLS pathway.

A conserved UBZ4 domain is required for ZBTB1 localization and PCNA monoubiquitination

We next determined whether the UBZ4 motif of ZBTB1 is a bonafide ubiquitin-binding domain and whether this activity is essential for its function. GST (Glutathione-S-transferase) tagged-ubiquitin pulled down wild-type ZBTB1, while mutation of two conserved cysteines in the UBZ4 domain abolished this interaction, confirming that ubiquitin-binding is mediated by the UBZ4 motif (Figure 3A). The assembly of Lys63-linked polyubiquitin chains is a specific signal for DNA repair pathways (Ciccia and Elledge, 2010). The bacterially expressed GST-UBZ4 domain of ZBTB1 could bind to Lys63-linked polyubiquitin chains *in vitro* (Figures S3A and S3B). Again, binding was abrogated by inactivating mutations of the UBZ4 domain, suggesting that the UBZ4 of ZBTB1 may bind to a protein modified by Lys63-linked polyubiquitin chains at sites of UV-mediated DNA damage *in vivo* (Figure S3B).

DNA repair factors are recruited to DNA lesions, as exemplified by the targeting of TLS polymerases, Rev1 and Pol η, to PCNA-Ub upon UV damage (Figure S3C). We hypothesized that ZBTB1 might also be recruited to UV-induced DNA lesions, thereby regulating RAD18 and the PCNA monoubiquitination process. Indeed, in addition to the constitutive ZBTB1 foci previously described (Matic et al., 2010), inducible ZBTB1 foci colocalized with PCNA following UV damage (Figure S3C). It is difficult, however, to identify the DNA damage-inducible ZBTB1 foci, due to the high background of constitutive foci. Therefore, we employed a UVC microfilter technique to generate localized DNA lesions, detectable with an antibody against CPDs (Katsumi et al., 2001) (Figure S3D). Under these experimental conditions, GFP-tagged DVC1 was recruited to CPD-marked DNA lesions as previously described (Centore et al., 2012) (Figure S3E), and the UV-inducible colocalization of ZBTB1 with both PCNA and CPDs was observed (Figures 3B and 3C). In contrast, mutation of the UBZ4 domain abolished the recruitment of ZBTB1 to CPD-marked sites (Figure 3C). Since the UBZ4 mutation destabilizes ZBTB1 in a proteasome-dependent manner, we constructed an additional deletion of the BTB domain in this mutant to stabilize the protein, as shown by Western blot and fluorescence microscopy (Figure S3F). The ZBTB1 UBZ4/ΔBTB double mutant protein, although it is well expressed, failed to localize to UV-induced CPD foci, indicating that the UBZ4 domain is required for damage-inducible ZBTB1 recruitment (Figures 3C and 3D). Interestingly, the BTB domain was also required for UV-inducible recruitment to CPD foci (Figure 3C). Both the siRNA-resistant UBZ4 and ΔBTB mutants failed to restore the level of PCNA-Ub caused by ZBTB1 knockdown, demonstrating that the recruitment of ZBTB1 to DNA lesions is required for the promotion of PCNA monoubiquitination (Figure 3E). The UV

damage-inducible recruitment of ZBTB1 further indicates that the protein has an additional role in DNA repair, extending beyond its role in transcriptional repression.

UV damage is also repaired by the nucleotide excision repair (NER) pathway (Kamileri et al., 2012). However, the recruitment of the xeroderma pigmentosum complementation group C (XPC) protein, a component of global genome (GG) repair in the NER pathway, to CPDs was not affected by ZBTB1 knockdown. This result indicates that ZBTB1 plays a specific role in regulating the TLS pathway (Figure S3G). Depletion of DDB2 (XPE), a damage sensor that localizes key GG-NER proteins to UV damage sites and modifies histones and XPC by ubiquitination (Li et al., 2006), also failed to affect ZBTB1 recruitment (Figure S3H). Also, unlike cells lacking DDB2, ZBTB1-depleted cells exhibited normal kinetics of CPD repair, comparable to the repair kinetics observed in wild-type cells (Figures S3I and S3J). Taken together, these data indicate that the NER pathway is not directly involved in ZBTB1 targeting to DNA lesions, and ZBTB1 does not affect the removal of CPDs by the NER pathway.

UV hypersensitivity of *Zbtb1* knockout DT40 cells

To confirm the cellular phenotype of ZBTB1 deficiency in a different genetic background, we created a *Zbtb1* knockout in the chicken B lymphocyte line, DT40. Intriguingly, the BTB and UBZ4 domains, but not the five C-terminal zinc fingers of ZBTB1, are highly conserved between human and chicken, underscoring the conserved role of these two domains (Figure 4A). Independent knockout clones were obtained by deleting the first exon of *Zbtb1* (Figures 4B and 4C). *Zbtb1*$^{-/-}$ DT40 cells proliferated similarly to wild-type or *Rad18*$^{-/-}$ cells, in contrast to the delayed growth observed in the recently identified anti-recombinase *Pari* knockout DT40 cells (Moldovan et al., 2012) (Figure S4A). Like human cells, the *Zbtb1* knockout avian cells displayed defective PCNA monoubiquitination and increased γH2AX level, while Fancd2 monoubiquitination was not affected (Figure 4D). *Zbtb1* knockout cells were specifically hypersensitive to UV damage or to treatment with the UV-mimetic drug 4-NQO (4-Nitroquinoline 1-oxide) (Figure 4E and S4B), but displayed normal cellular resistance to camptothecin (CPT), an agent known to generate replication-associated DSBs (Figure 4F), and to the cross-linking agents, mitomycin C (MMC) and cisplatin (Figures S4C and S4D). Knockout of *Zbtb1* in a *Rad18*$^{-/-}$ background did not further sensitize *Rad18*$^{-/-}$ cells to UV damage, suggesting that *Rad18* and *Zbtb1* are epistatic in this pathway conferring UV resistance (Figure 4E).

We next reconstituted *Zbtb1*$^{-/-}$ cells with GFP-tagged human wild-type or mutant ZBTB1 proteins (Figure 4G). While wild-type human ZBTB1 rescued the UV hypersensitivity of the chicken cells, neither the UBZ4 mutant nor the BTB mutant complemented the null cells (Figure 4H). Taken together, these data support a model in which ZBTB1 is required for RAD18-dependent PCNA monoubiquitination and survival against UV damage. Recruitment of ZBTB1 to DNA lesions, mediated by the UBZ4 domain, is essential for its function.

ZBTB1 binds to a KAP-1-containing chromatin remodeling complex

In order to determine the mechanism by which ZBTB1 positively regulates RAD18-dependent PCNA monoubiquitination, we next identified proteins in the native ZBTB1 cellular complex. We performed a tandem affinity purification of ZBTB1 from undamaged HeLa cell nuclear extracts expressing the epitope-tagged protein (Figures 5A and 5B). From the mass spectrometric results, we subtracted proteins detected in the context of a negative control. We identified a subset of ZBTB1 interacting proteins that are associated with transcription and the DNA damage response (Table S2). We identified MTA2, a component of the nucleosome-remodeling and histone deacetylation (NuRD) complex and KAP-1 (also known as TRIM28), a protein that mediates gene silencing by recruiting the NuRD complex and SETDB1, a H3K9 methyltransferase to specific gene promoters (Goodarzi et al., 2010). The constitutive interaction of ZBTB1 with KAP-1 and MTA2, a known subunit of the NuRD complex, was confirmed by co-immunoprecipitation of proteins extracted from undamaged cells (Figures 5C and S5A). A reciprocal immunoprecipitation confirmed that myc-tagged KAP-1 interacts with HA-tagged ZBTB1 (Figure 5D). In addition, ZBTB1 associated with KAP-1 after UV damage and interacted with phosphorylated KAP-1 (Figures S5B and 5E). GST pull-downs demonstrated that the interaction is largely mediated by the BTB domain of ZBTB1 (Figure 5F).

ZBTB1 increases the affinity of phospho-KAP-1 for UV damage sites, promoting chromatin remodeling and RAD18 recruitment

Recent studies implicate the NuRD complex in DNA repair (Chou et al., 2010; Larsen et al., 2010; Polo et al., 2010; Smeenk et al., 2010). The complex is rapidly targeted to DNA lesions, facilitating the recruitment of DNA repair factors, and depletion of subunits of the NuRD complex disrupts the DNA damage response. Of note, DNA damage activates ATM-dependent phosphorylation of KAP-1 on Serine 824, which initiates chromatin relaxation during DNA DSB repair (Goodarzi et al., 2008; Ziv et al., 2006). pKAP-1 forms at DNA damage foci, and colocalizes with several DNA damage response and repair proteins (Goodarzi et al., 2011; White et al., 2006). Therefore, we hypothesized that ZBTB1 promotes KAP-1-dependent chromatin remodeling at sites of UV damage, leading to enhanced local accessibility of RAD18. To test this hypothesis, we determined whether chromatin remodeling can affect UV-induced PCNA monoubiquitination. Pretreatment of cells with Trichostatin A (TSA), a histone deacetylase inhibitor, increased histone acetylation and chromatin relaxation, thereby accelerating PCNA monoubiquitination at early time points after UV (Figure 6A; lane 5-8). PCNA monoubiquitination was RAD18-dependent. Interestingly, TSA treatment, or co-depleting HDAC1 & 2, in ZBTB1-knockdown cells increased histone H4 acetylation and restored PCNA monoubiquitination, suggesting that increased histone acetylation and chromatin relaxation can bypass the requirement of upstream ZBTB1 in TLS (Figure 6B).

KAP-1 was strongly phosphorylated on Serine 824 following UV damage, and to a lesser extent after DNA cross-linking or replication stress (Figure S6A). Depletion of ZBTB1, but not RAD18, increased both the basal level of KAP-1 phosphorylation and its induction, possibly due to increased spontaneous DNA damage (Figure 6C, lane 4). While pKAP-1 showed increased affinity for chromatin after UV damage in wild-type cells, pKAP-1 was

not efficiently associated with chromatin in ZBTB1-depleted cells (Figure 6D, compare lane 2 and 6 vs. 4 and 8) This aberrant pattern of pKAP-1 formation at the chromatin was confirmed by an independent fractionation protocol (Figure S6B). Moreover, RAD18 association in chromatin was diminished in ZBTB1 knockdown cells (Figure 6D; lane 6 and 8). Accordingly, UV-inducible RAD18 foci were reduced following ZBTB1 depletion (Figures 6E and 6F). Of note, pretreatment of TSA partially restored the association of pKAP-1 and RAD18 with chromatin (Figure S6C, lane 7 and 8). Taken together, these data demonstrate that ZBTB1 is required for enhanced affinity of pKAP-1 and RAD18 to DNA damage sites.

We next examined the role of KAP-1 phosphorylation in PCNA monoubiquitination. It is known that heterologous expression of the KAP-1 S824A (non-phosphorylated) mutant blocks chromatin relaxation, leading to defective DNA repair signaling, while expression of the wild-type or S824D (phospho-mimetic) mutant of KAP-1 bypasses this relaxation step (Goodarzi et al., 2008; Ziv et al., 2006). KAP-1 S824A, but not the wild-type or S824D mutant, resulted in reduced PCNA monoubiquitination, further suggesting that chromatin relaxation is required for PCNA monoubiquitination (Figure 6G, lanes 4-6). These results were consistent with the decreased chromatin association and foci formation of RAD18 observed in UV-damaged cells expressing the KAP-1 S824A mutant (Figures S6D and S6E). Therefore, ZBTB1 promotes KAP-1-dependent chromatin relaxation near UV damage, thus increasing the accessibility of RAD18 and enhancing local PCNA monoubiquitination.

Heterochromatin DSB repair requires dispersal of CHD3 from the chromatin by disrupting the CHD3 SIM:KAP-1^{SUMO} interaction upon KAP-1 phosphorylation; therefore CHD3 knockdown or a defect in KAP-1 SUMOylation alleviates the need for pKAP-1 in DSB repair (Goodarzi et al., 2011). However, SUMOylation-defective KAP-1, confirmed in Figure 6H, could not rescue the PCNA monoubiquitination defect caused by the expression of the KAP-1 S824A mutant (Figure 6G, lane 7). Accordingly, CHD3 depletion did not rescue the decreased PCNA monoubiquitination caused by ZBTB1 knockdown (Figure S6F, lane 4). This result suggests that KAP-1 may contribute to chromatin remodeling following UV damage independently of the CHD3 SIM: KAP-1^{SUMO} interaction previously implicated in DSB repair. Interestingly, knockdown of CHD4 did restore PCNA monoubiquitination in ZBTB1-knockdown cells, further arguing for the existence of a distinct chromatin remodeling process in TLS (Figure S6F, lane 5).

DISCUSSION

Multiple functions of UBZ4 proteins in DNA repair

In the current study, we demonstrate that ZBTB1 has a role in translesion DNA synthesis in addition to its known role in transcriptional repression. ZBTB1 is required for UV-inducible RAD18 loading, PCNA monoubiquitination, Pol η recruitment to replication factories, and efficient TLS. Accordingly, knockdown of ZBTB1, either in mammalian cells or in avian DT40 cells, results in UV hypersensitivity. Moreover, ZBTB1 contains a conserved UBZ4 domain, necessary for its recruitment to UV damage sites. ZBTB1 is required for phospho-

KAP-1, associated with the NuRD complex, to form at these sites and enhances RAD18 loading through the local relaxation of chromatin (See schematic model Figure 6I).

Other proteins containing UBZ4 proteins function in DNA repair (Hofmann, 2009). Some TLS polymerases, including Pol η, and Pol κ contain UBZ3/4 domains, required for binding to monoubiquitinated PCNA (Bienko et al., 2005). RAD18 itself contains a UBZ4 domain, required for binding Lys63-linked polyubiquitin chains (Huang et al., 2009). Other DNA repair proteins that bind modified PCNA, such as SNM1A and WRNIP1, also contain UBZ4 domains (Bish and Myers, 2007; Yang et al., 2010). Furthermore, some nucleases, such as FAN1 and SLX4, are recruited to the monoubiquitin of FANCD2 and control the enzymatic processing of DNA interstrand cross-links (Kim and D'Andrea, 2012). Recent studies have identified another protein, DVC1, which contains a UBZ4 domain and regulates TLS. DVC1 functions at several levels in TLS. DVC1 is required for RAD18 binding and recruitment to UV damage sites where it promotes PCNA monoubiquitination (Centore et al., 2012; Ghosal et al., 2012). DVC1 also recruits the ubiquitin-selective chaperone p97 to stalled replication forks, thereby promoting the displacement of Pol η from PCNA (Davis et al., 2012; Mosbech et al., 2012).

A general role of chromatin remodeling complexes at UV damage sites

Recent reports implicate chromatin remodeling complexes in DNA repair at UV damage sites. The yeast INO80 chromatin remodeling complex promotes RAD18 recruitment at stalled replication forks, thus facilitating PCNA modification and TLS (Falbo et al., 2009). Similarly, deletion of the RSC remodeling complex in budding yeast decreases PCNA monoubiquitination (Niimi et al., 2012). Remodeling complexes also play an upstream role in the repair of CPDs, by promoting chromatin relaxation (Jiang et al., 2010). Whether ZBTB1 plays a broader role in the recruitment of these excision repair complexes remains to be determined.

The binding of ZBTB1, KAP-1, and the NuRD complex suggests that these proteins coordinate both transcriptional repression and DNA repair. In addition to repressing transcription, KAP-1, the NuRD complex, and HP1 (Heterochromatin protein 1) are rapidly localized to DNA lesions during the early stages of the DNA damage response (DDR) where they appear to play distinct roles in DNA repair (Baldeyron et al., 2011; Larsen et al., 2010; Smeenk et al., 2010; White et al., 2006). In unstressed conditions, ZBTB1 may maintain the transcriptional repression of heterochromatin, by complexing with other corepressors. The increased level of pKAP-1 in the ZBTB1-depleted cells, in the absence of exogenous DNA damage, may reflect greater signaling per lesion, due to the disruption of heterochromatin. On the other hand, upon UV damage, ZBTB1 appears to concentrate the pKAP-1-NuRD complexes to UV damage sites. KAP-1 phosphorylation promotes chromatin remodeling, thereby facilitating TLS. Therefore, the damage-dependent recruitment of ZBTB1 to DNA lesions is a distinct function of the protein, separable from its constitutive role in transcriptional repression. Of note, depletion of several NuRD complex subunits increases spontaneous DNA damage and reduces the efficiency of DNA repair by impairing the assembly of DNA repair factors, further highlighting the importance of chromatin remodeling in regulating DNA repair (Larsen et al., 2010).

The precise mechanism by which ZBTB1 promotes chromatin remodeling and downstream TLS events remains unknown. CPD hotspots often occur in heterochromatin, such as Alu repeat regions, which are associated with early nucleosome rearrangement (Zavala et al., 2013). Efficient TLS appears to require the pKAP-1-NuRD complex to relax the chromatin locally around the lesion. Perhaps ZBTB1 facilitates TLS by activating a local checkpoint, through the transient formation of pKAP-1 at DNA lesions. Previous studies have shown that the checkpoint kinase ATR is not required for PCNA monoubiquitination (Chang et al., 2006; Niimi et al., 2008). Likewise, we observed that, contrary to IR-induced DSB repair, inhibition of ATM and/or ATR did not block UV-inducible KAP-1 phosphorylation (Figure S6G), and KAP-1 shows distinct phosphorylation kinetics following UV and IR treatment (Figure S6H). Although the identity of the kinase responsible for UV-inducible KAP-1 phosphorylation remains unknown, these data indicate that KAP-1 phosphorylation is a complex process, dependent on distinct kinases activated by different DNA damage response pathways.

Although chromatin remodeling is required for efficient DNA repair of many types of DNA damage, ZBTB1 is specifically required for UV damage repair. It will be important to identify the specific ubiquitinated factor(s) recognized by ZBTB1 via its UBZ4 and BTB domains at UV-damage sites. Depletion of RNF8, a mediator of H2A ubiquitination at UV-damage sites during NER (Marteijn et al., 2009), did not affect ZBTB1 recruitment (data not shown). DVC1 knockdown also results in selective sensitivity to UV, and it requires an intact UBZ4 motif for its accumulation at the stalled replication forks, suggesting that the UBZ4 domain may confer specificity for the recognition of a subset of ubiquitinated proteins (Centore et al., 2012; Mosbech et al., 2012).

Diversified functions of transcriptional regulators in DNA repair

Transcription and DNA repair processes are closely linked, often sharing the same protein complexes to exert dual functions (Fong et al., 2013). For instance, a stress-responsive NR4A nuclear receptor promotes DSB repair in addition to its role as a transcriptional coregulator (Malewicz et al., 2011). Some DNA repair factors also directly participate in gene transcription. Monoubiquitination of FANCD2, a central step in DNA interstrand cross-link repair, promotes transcription of *TAp63* and cellular senescence (Park et al., 2013). Also, XPC-containing NER complexes function as transcriptional coactivators of *Nanog*, to maintain embryonic stem cell pluripotency (Fong et al., 2011).

ZBTB family proteins generally function as transcriptional repressors, several of which are implicated in lymphocyte development and lymphomagenesis. *Zbtb1*$^{-/-}$ mice exhibit a defect in T cell development, resulting in a severe combined immunodeficiency (SCID) (Punwani et al., 2012; Siggs et al., 2012). This T cell defect is believed to result from the absence of Zbtb1-mediated transcriptional activity. Whether the T cell deficiency in these mice also results from heightened UV sensitivity or from a DNA repair defect remains untested.

ZBTB1 mutations in cancer

The Cancer Genome Atlas program (TCGA) allows comprehensive characterization of the human cancer genomes of many major tumor types, and helps identify clinically relevant somatic mutations that may have contributed to cancer pathogenesis (Chin et al., 2011). Increased replication stress and replication-associated DSB formation, due to deficient TLS activity, can lead to chromosomal aberrations, ultimately enhancing genome instability and influencing tumorigenesis. Interestingly, ZBTB1 mutations with a high functional impact score are concentrated on the UBZ4 domain. Elimination of the UBZ4 domain may result in a selective pressure, by decreasing ZBTB1-mediated PCNA monoubiquitination. Careful evaluation of the mutational status of other TLS genes in tumor samples, including polymerases and helicases, may provide insights to how the modulation of DNA damage tolerance mechanisms affects cancer progression.

EXPERIMENTAL PROCEDURES

Cell culture and plasmid construction

HeLa, HeLa S3, U2OS, and 293T cells were cultured in Dulbecco's Modified Eagles Medium supplemented with 10 % fetal bovine serum (FBS) following standard culture conditions and procedures. Human ZBTB1 cDNA (Clone ID: HsCD00079584) and DVC1 cDNA (Clone ID: HsCD00376679) were purchased form the Dana-Farber/Harvard Cancer Center DNA Resource Core. The full-length or deleted cDNA was PCR-amplified and subcloned to pcDNA3-Flag, pcDNA3-HA (Invitrogen), pEGFP-C1 (Clontech), pGEX-6P-1 (GE Healthcare Life Sciences), and pOZ-Flag-HA vectors to generate various constructs. Point mutations were introduced by QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and confirmed by DNA sequencing. For ZBTB1 rescue experiments, siRNA-resistant pMSCV-Flag-HA-ZBTB1 constructs were generated for retroviral transduction, and stable HeLa cells were selected in the presence of 1 μg/mL puromycin.

Transfection and siRNA

Plasmid transfection was performed using lipofectamine™ 2000 or LTX according to the manufacturer's protocols. siRNA duplexes were synthesized by Qiagen or Invitrogen and transfected using Lipofectamine RNAiMAX (Invitrogen). The siRNA sequences can be found in Supplemental Information.

Antibodies and chemicals

Information on antibodies and chemicals can be found in Supplemental Information.

Immunoblot analysis, fractionation and immunoprecipitation

Cells were lysed with 1 % NP40 lysis buffer (1 % NP40, 300 mM NaCl, 0.1 mM EDTA, 50 mM Tris [pH 7.5]) supplemented with protease inhibitor cocktail (Roche) and resolved by NuPAGE (Invitrogen) gels and transferred onto nitrocellulose membrane followed by antibody detection using enhanced chemiluminescence method (Western Lightening, Perkin Elmer). Some blots were developed with LAS-4000 Imaging system (GE Healthcare Life Sciences). For immunoprecipitation, cells were lysed with 150 mM NaCl buffer (1 % NP40,

150 mM NaCl, 0.1 mM EDTA, 50 mM Tris [pH 7.5]), and the lysates were incubated with anti-Flag agarose (Sigma) or anti-HA agarose (Sigma) followed by three times washing with 150 mM NaCl buffer. For cellular fractionation, cells were incubated with low salt permeabilization buffer (10 mM HEPES [pH 7.4], 10 mM KCl, 0.05% NP-40) on ice for 20 min. Following centrifugation, pellet was boiled in boiling lysis buffer (50 mM Tris [pH 6.8], 2 % SDS, 850 mM β-mercaptoethanol). Subcellular fractionation of HeLa cells using 100 mM NaCl concentration was performed as previously described (Kim et al., 2012).

Fluorescence microscopy

For GFP fluorescence, cells were grown on coverslip, UVC irradiated, fixed with 3 % paraformaldehyde for 10 min at room temperature, washed three times with PBS and mounted with DAPI-containing mounting medium (Vector Lab). For local cell irradiation, cells were irradiated with 100 J/m^2 UVC using 5 μm Nucleopore Track-Etched membrane filter (Whatman). Cells were fixed and permeabilized with CSK extraction buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 0.2 % Triton X-100). After treatment of 2 N HCl for 30 min at room temperature to denature DNA, cells were incubated with 5 % bovine serum albumin (BSA) in PBS. DNA lesions were detected by anti-cyclobutane pyridimine dimers (CPDs) antibody (Cosmo Bio) and visualized with Alexa Fluor® 568 goat anti-mouse IgG (Molecular Probes) in 2 % BSA. For detection of PNCA foci, cells were pre-extracted and sequentially fixed with 3 % paraformaldehyde for 10 min at 4 °C and with ice-cold methanol for 10 min at −20 °C. For double staining of XPC and CPDs, cells were first incubated with anti-XPC (Genetex) antibody and Alexa Fluor® 488 goat anti-rabbit IgG, fixed again, DNA was denatured, and incubated with anti-CPD antibody. Images were captured using a Zeiss AX10 fluorescence microscope and AxioVision software.

Mutation analysis of the UBZ4 domain from TCGA data

21 TCGA datasets were screened for mutations in genes containing a UBZ4 domain i.e. WRNIP, Pol κ, RAD18, SLX4, FAN1, SNM1A, FAAP20, DVC1, and ZBTB1 (http://www.cbioportal.org/public-portal/). Only nonsense and frameshift mutations leading to a deletion, and mutations with high functional impact score were selected for the analysis. Mutations were then considered to specifically target a UBZ4 motif when they were located in or within 50 bp before a UBZ4 domain.

Statistical analysis

P values for statistical analyses were obtained using Student's *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- The UBZ4 domain of ZBTB1 is frequently mutated in cancer
- ZBTB1 is required for RAD18-dependent PCNA monoubiquitination
- Recr0uitment of ZBTB1 to UV damage sites is required for PCNA modification
- ZBTB1 regulates KAP-1-dependent chromatin remodeling following UV damage

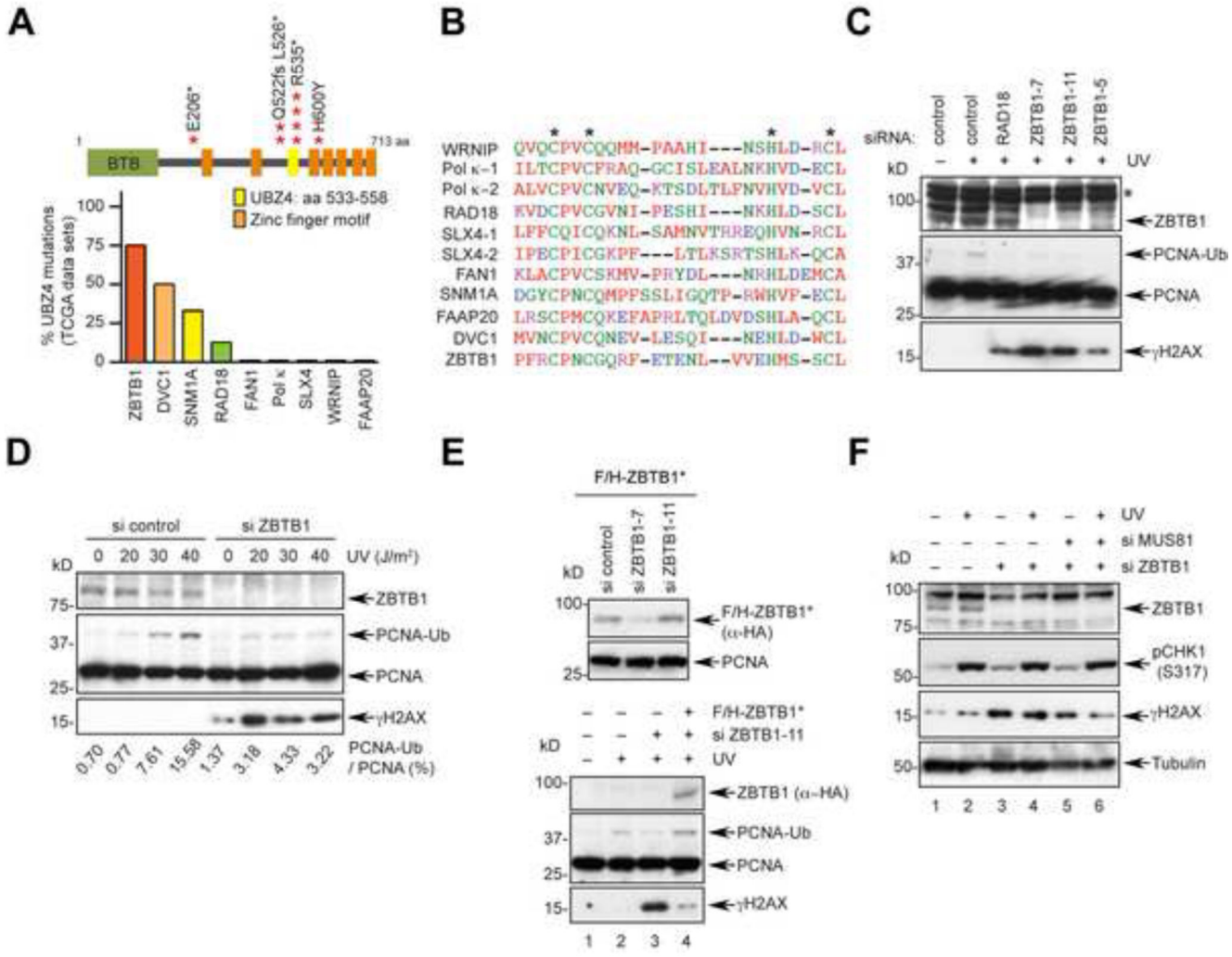

Figure 1. ZBTB1 is a UBZ4 domain-containing protein required for PCNA monoubiquitination following UV damage

(A) Mutations of ZBTB1 in cancer are concentrated on the UBZ4 domain. Mutations found in primary tumor samples from TCGA datasets are indicated. The percentage of all UBZ4-specific mutations found in cancers for each UBZ4-containing protein is shown as bargraph. (B) Peptide sequence alignment of various UBZ4 domains. (C) HeLa cells transfected with siRNA oligos were treated with 30 J/m^2 UVC for 3 hr, and cell lysates were analyzed by immunoblot. Asterisk denotes nonspecific bands. (D) siRNA-transfected HeLa cells were treated with the indicated doses of UVC for 3 hr. The percentage of ubiquitinated PCNA was quantified by ImageJ. (E) (left) Confirmation of siRNA-resistant ZBTB1. HeLa cells stably expressing Flag-HA-tagged ZBTB1 resistant to siRNA-11 (F/H-ZBTB1*) were treated with either siRNA-7 or siRNA-11 and analyzed by immunoblot. (right) Immunoblot of F/H-ZBTB1*-expressing HeLa cells treated with siRNA and UVC for 3 hr. (F) MUS81 suppresses γH2AX hyper-activation caused by ZBTB1 knockdown. See also Figure S1 and Table S1.

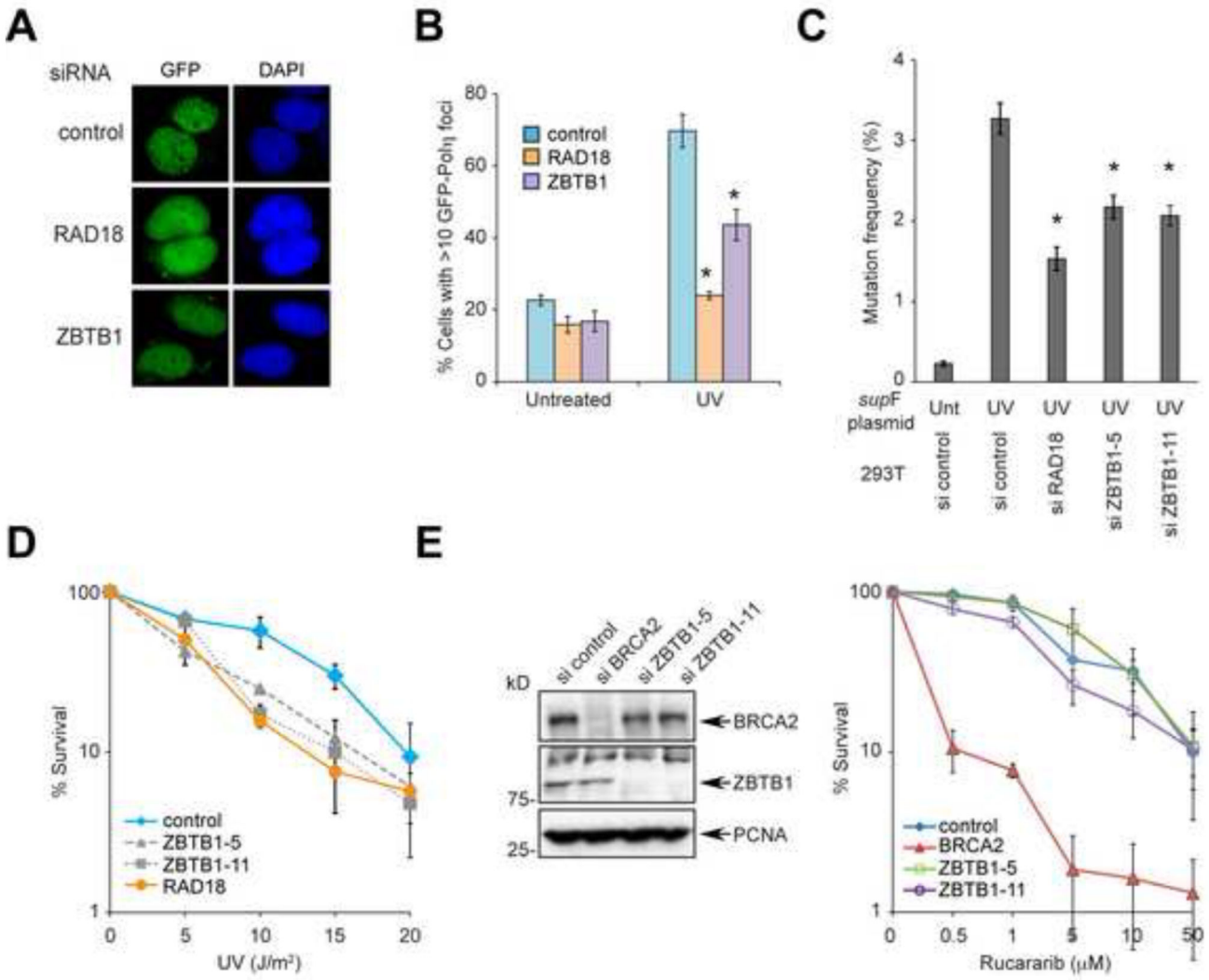

Figure 2. ZBTB1 regulates TLS and cellular survival following UV damage

(A) U2OS cells were sequentially transfected with indicated siRNAs and GFP-Pol η, treated with 15 J/m^2 UVC for 14 hr, fixed, and examined by fluorescence microscopy. (B) Quantification of cells displaying more than ten GFP-Pol η foci. Data shown are mean ± standard deviation (SD) from three independent experiments. * $p < 0.05$ compared with siRNA control. (C) The mutation frequency in damaged (1,000 J/m^2 UVC) *sup*F plasmid recovered from siRNA-treated 293T cells was determined. Data shown are mean ± SD from three independent experiments. * $p < 0.05$. (D) Clonogenic survival of siRNA-transfected HeLa cells treated with increasing doses of UVC and plated for 12 days. (E) (left) Knockdown efficiency of HeLa cells used for viability assay. (right) Luminescence viability assay of siRNA-treated HeLa cells following Rucaparib treatment. BRCA2 siRNA serves as positive control. See also Figure S2.

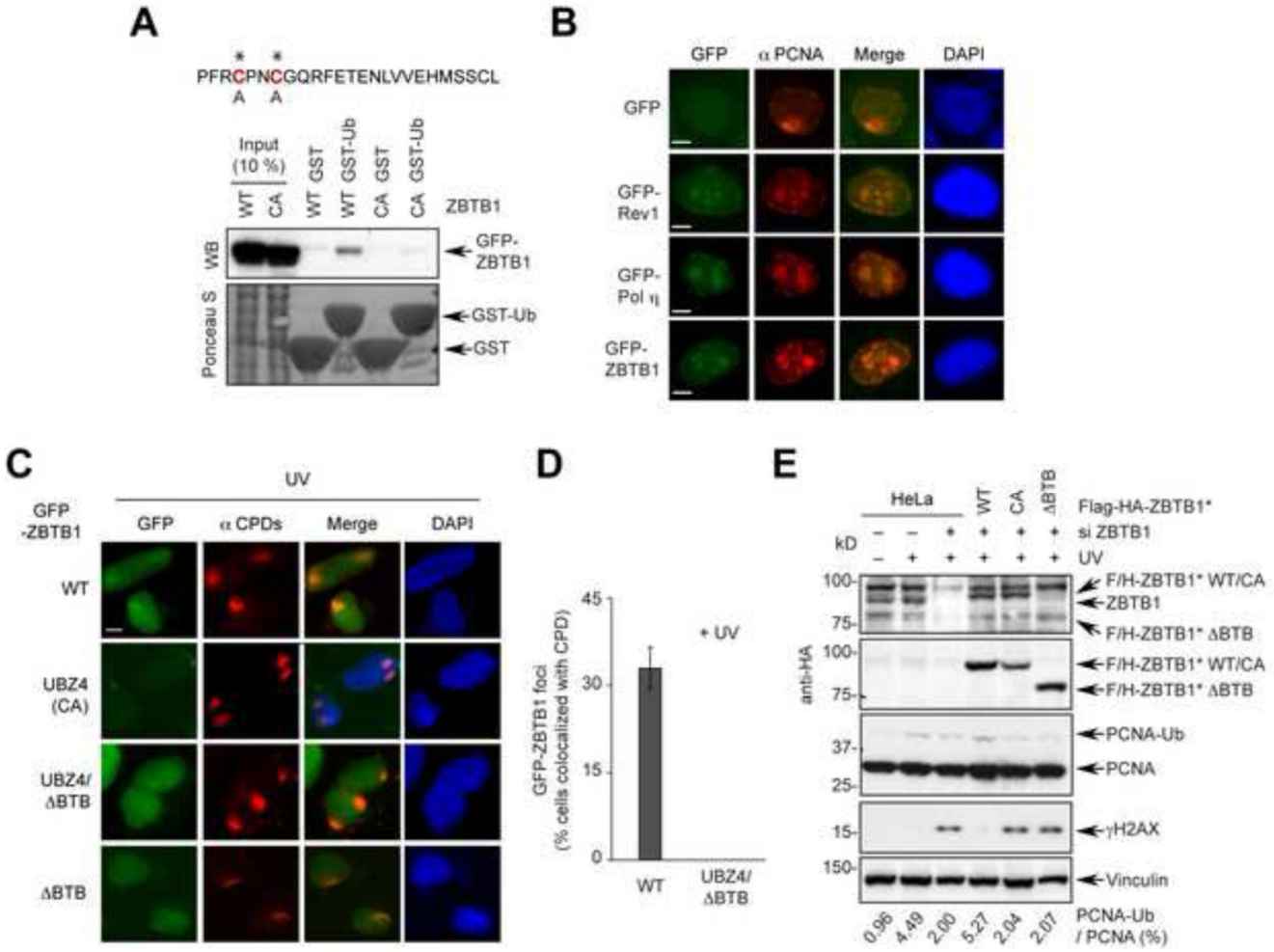

Figure 3. The UBZ4 domain of ZBTB1 is required for targeting to UV damage sites and for PCNA monoubiquitination

(A) Purified GST or GST-Ub was incubated with 293T lysates expressing GFP-tagged ZBTB1 wild-type or UBZ4 mutant (C536A & C539A), and analyzed by immunoblot and Ponceau S staining. (B) Anti-PCNA immunostaining in U2OS cells, transfected with GFP-tagged Rev1, Pol η, or ZBTB1 and treated with 100 J/m^2 UVC for 2 hr through a 5 μm micropore filter. GFP-tagged Rev1 and Pol η were served as positive controls. Scale bar: 5 μm. (C) Anti-CPD immunostaining of U2OS cells transfected with GFP-ZBTB1 wild-type or mutant treated with UVC through micropore filter. ΔBTB: aa1-142 deletion. (D) Quantification of cells in (C), showing the percentage of cells with GFP-ZBTB1 colocalizing with CPDs among cells displaying positive anti-CPD signal. (E) Immunoblot analysis of siRNA-treated HeLa cells stably expressing siRNA-resistant ZBTB1 variants. Note that cells displaying high expression of the unstable UBZ4 mutant were chosen for achieving similar expression among variants. See also Figure S3.

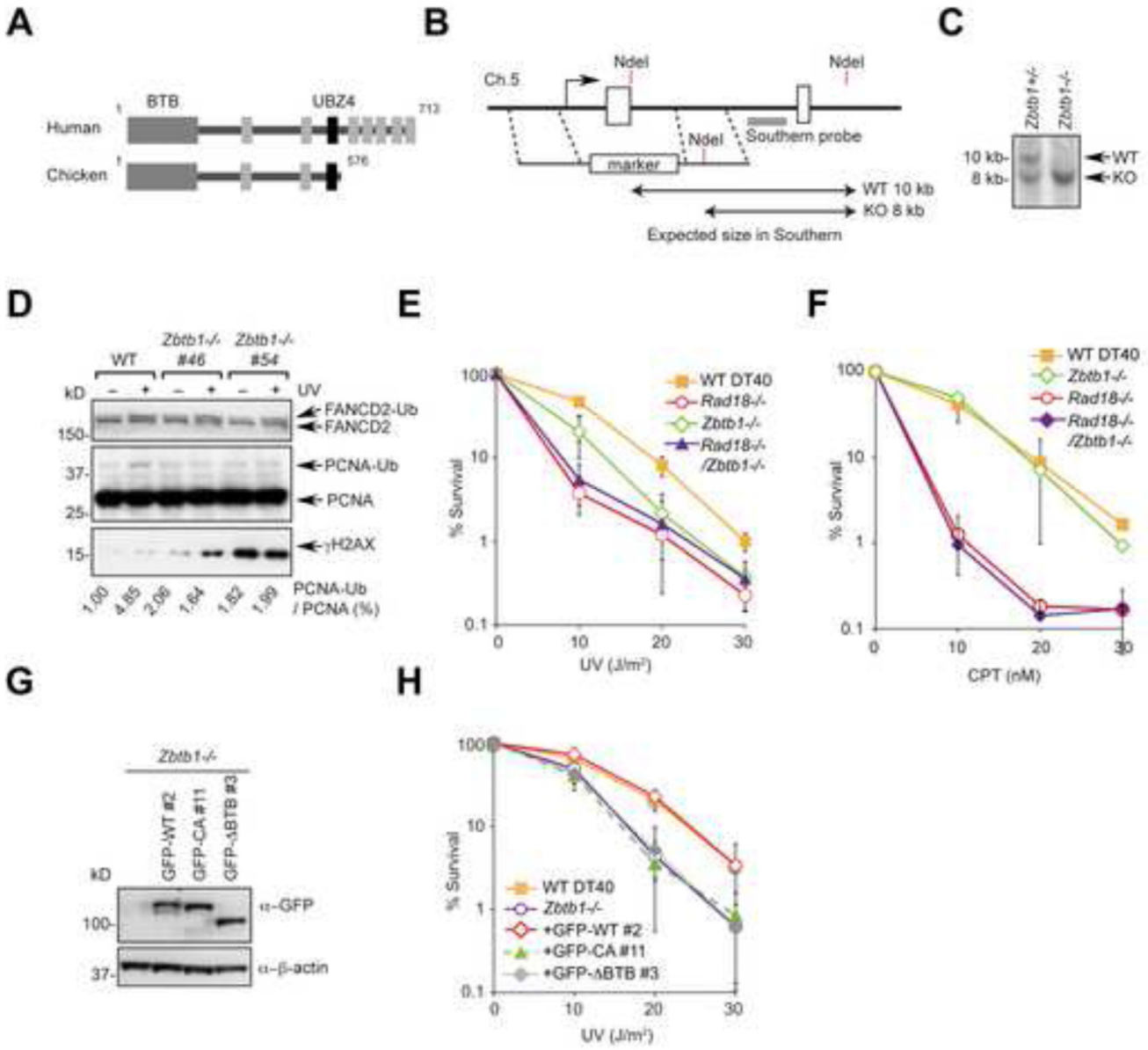

Figure 4. Generation and characterization of *Zbtb1* knockout in chicken DT40 cells

(A) Structural comparison of human and chicken ZBTB1. (B) The *Zbtb1* gene targeting scheme (C) Southern blot analysis of wild-type and knockout DT40 clones (D) Two independent *Zbtb1*$^{-/-}$ clones were treated with 30 J/m^2 UVC for 2 hr and PCNA ubiquitination was analyzed by immunoblot. (E) *Zbtb1* knockout cells are hypersensitive to UV damage, showing epistasis to *Rad18*. Data shown are mean ± SD from three independent experiments. (F) *Zbtb1* knockout cells are not sensitive to CPT. (G) Immunoblot showing the expression of human ZBTB1 variants in the *Zbtb1*$^{-/-}$ DT40 cells. (H) Cellular UV sensitivity of ZBTB1-reconstituted stable clones generated in (G). See also Figure S4.

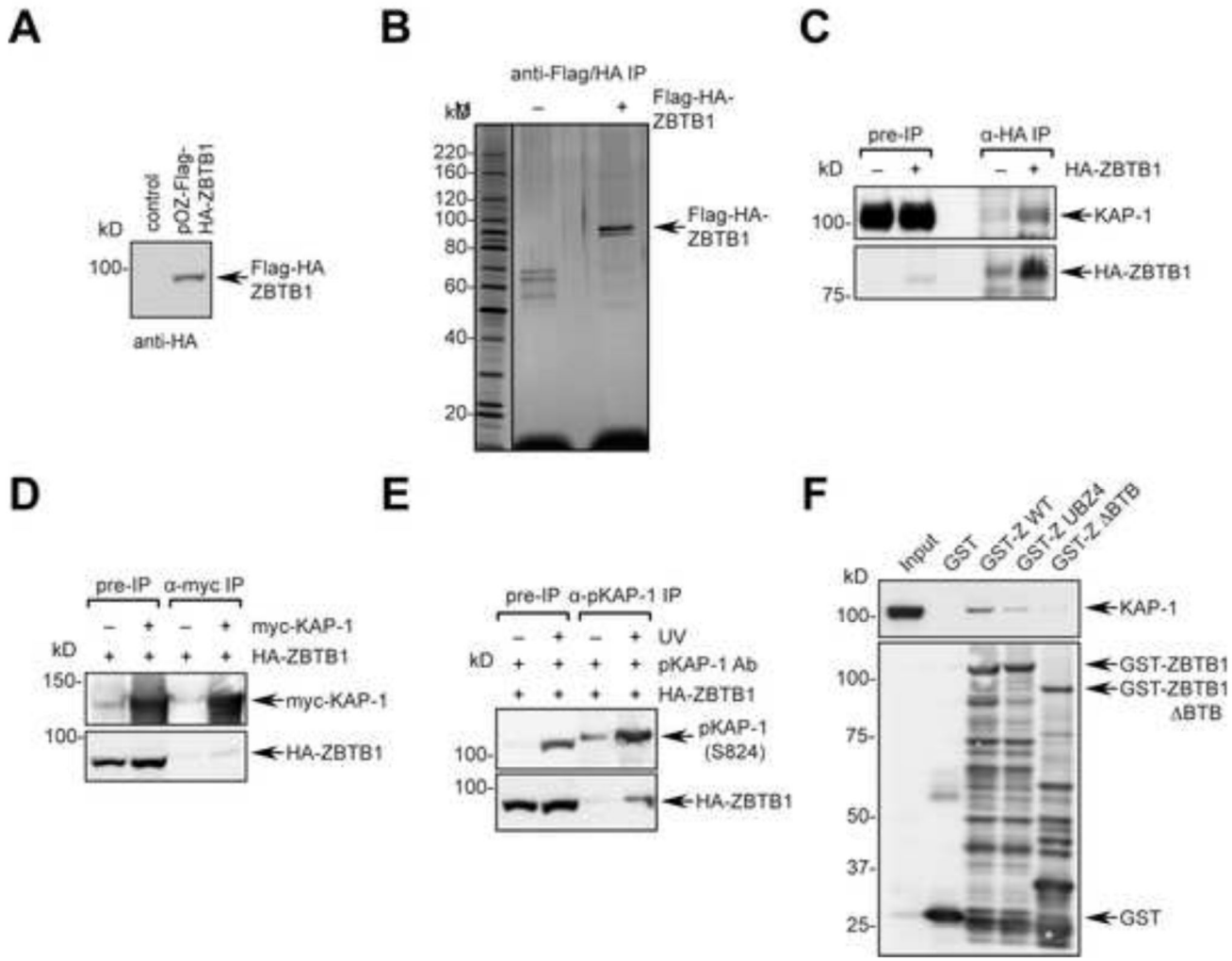

Figure 5. Identification of ZBTB1 binding proteins

(A) Immunoblot showing stable expression of Flag-HA-ZBTB1 in HeLa S3 cells. (B) Silver staining of a ZBTB1-containing complex isolated by Flag-HA tandem affinity purification from the nuclear extracts of HeLa S3 control or expressing Flag-HA-ZBTB1. (C) Cell lysates from 293T cells expressing HA-ZBTB1 were immunoprecipitated by anti-HA conjugated beads, and endogenous KAP-1 was analyzed by immunoblots. (D) 293T cells transfected with myc-KAP-1 and HA-ZBTB1 were subjected to anti-myc coimmunoprecipitation (E) 293T cells transfected with HA-ZBTB1 were left untreated or treated with 30 J/m^2 UV for 2 hr, and cell lysates were immunoprecipitated with anti-phospho KAP-1 (S842) antibody. (F) Purified GST or GSTZBTB1 wild-type, UBZ4, or ΔBTB mutant was incubated with 293T cell lysates, and associated KAP-1 was analyzed by anti-KAP-1 immunoblot. See also Figure S5 and Table S2.

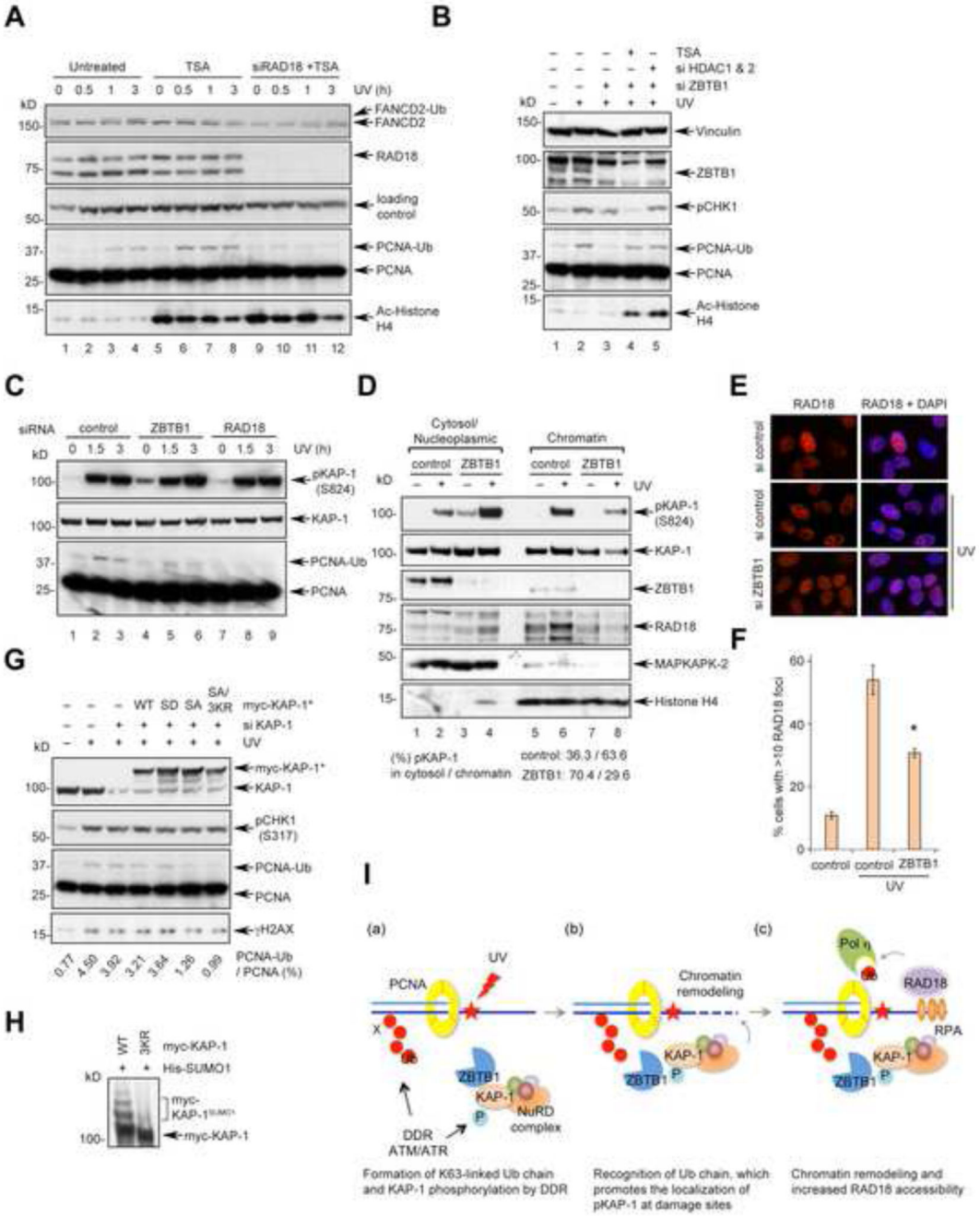

Figure 6. ZBTB1 controls KAP-1-dependent chromatin remodeling to promote PCNA monoubiquitination

(A) HeLa cells left untreated or treated with 400 nM Trichostatin A (TSA) for 14 hr were UVC-treated. Increased acetylated histone H4 serves as a marker for TSA treatment. (B) siRNA-transfected HeLa cells were treated with 30 J/m^2 UVC for 3 hr and analyzed by immunoblot. Where indicated, cells were pretreated with 400 nM TSA. (C) Immunoblot of siRNA-transfected HeLa cell lysates treated with 30 J/m^2 UVC for indicated times. (D) siRNA-transfected HeLa cells were treated with 30 J/m^2 UVC for 3 hr. Cells were lysed in

low salt permeabilization buffer to separate cytosol/nucleoplasmic (S) and chromatin fractions (P). (E) siRNA-transfected U2OS cells were treated with 40 J/m^2 UVC for 3 hr, and RAD18 foci were visualized by anti-RAD18 immunostaining. Representative images were shown. (F) Quantification of cells displaying more than 10 RAD18 foci in (E). Data shown are mean ± SD from three independent experiments. * $p < 0.05$ (G) Immunoblot of HeLa cells sequentially transfected with KAP-1 siRNA and siRNA-resistant myc-KAP-1* wild-type, S824D (SD), S824A (SA), or SA + 3KR (K554R, K779R and K804R) (H) myc-KAP-1 wild-type or 3KR mutant was cotransfected with His-tagged SUMO1 in 293T cells, and cell lysates were analyzed by anti-myc immunoblot. (I) Model depicting the ZBTB1-dependent PCNA monoubiquitination in TLS. See also Figure S6.