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SUMO-modification and elimination of the active DNA demethylation enzyme TDG in cultured human cells

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ABSTRACT

Thymine DNA glycosylase (TDG) is a base excision repair enzyme that interacts with the small ubiquitin-related modifier (SUMO)-targeted ubiquitin E3 ligase RNF4 and functions in the active DNA demethylation pathway. Here we showed that both SUMOylated and non-modified forms of endogenous TDG fluctuated during the cell cycle and in response to drugs that perturbed cell cycle progression, including hydroxyurea and nocodazole. Additionally, we detected a SUMOylation-independent association between TDG and RNF4 *in vitro* as well as *in vivo*, and observed that both forms of TDG were efficiently degraded in RNF4-depleted cells when arrested at S phase. Our findings provide insights into the *in vivo* dynamics of TDG SUMOylation and further clarify the TDG–RNF4 interaction.

1. Introduction

TDG is a member of the uracil DNA glycosylase superfamily and initiates base excision repair (BER) by releasing thymine or uracil from guanine/thymine (G/T) and guanine/uracil (G/U) mismatches arising from hydrolytic deamination of 5-methyl-cytosine (5mC) and cytosine bases that are paired with guanines [1–4]. Recently, another major role of TDG has emerged. TDG was shown to efficiently excise 5-carboxylcytosine (5caC) and 5-formylcytosine (5fC), key oxidation products of 5mC in genomic DNA, in an active DNA demethylation pathway of mammalian cells [5]. Together this suggests that the investigation of TDG structure and function is important not only to understand BER but also to elucidate epigenetic gene expression and reprogramming.

Like other eukaryotic cellular proteins, TDG is subjected to posttranslational modifications. Two of the relatively well-characterized posttranslational modifications are the covalent conjugation of small ubiquitin-related modifiers (SUMOs) and ubiquitin. SUMO modification (SUMOylation) promoted the release of TDG from the abasic (apurinic/apyrimidinic; AP) site created after base excision, coordinating transfer of TDG to AP endonuclease 1 [1–5]. The crystal structure of the central region of SUMO-1/3-conjugated TDG indicated that SUMOylation on lysine 330 induced the formation of the protruded helix in TDG, which allowed its dissociation from the product AP site [2,6,7]. Ubiquitin modification (ubiquitylation) plays a pivotal role in controlling TDG stability during cell cycle progression. Hardeland et al. reported that cells entering S-phase degraded TDG via the ubiquitin–proteasome system and then maintained TDG-free conditions until G2 [8]. However, the molecular details underlying the regulation of TDG ubiquitylation, including identification of the ubiquitin E3 ligase(s) that ubiquitylates TDG during S-phase, remain largely uncharacterized.

A wide variety of proteins that contain the really interesting new gene (RING) domain are demonstrated to function as ubiquitin E3 ligases. Among these ligases, RING finger protein 4 (RNF4 or SNURF) specifically promotes ubiquitylation of proteins that have been modified by SUMOs. These ubiquitin ligases are classified into a special category of E3 ligases, termed the SUMO targeted ubiquitin ligases (STUbLs) [9]. Hu et al. demonstrated that RNF4 reactivated methylation-silenced reporters and promoted global DNA demethylation, suggesting a potential role for RNF4 in controlling epigenetic gene expression through active DNA demethylation [10]. Further mechanistic studies showed that RNF4 interacted with BER enzymes, including TDG, and the interaction appeared to occur by enhancing the enzymatic activities that repaired DNA

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G:T mismatches generated from 5mC deamination. Although it is plausible that both TDG and RNF4 are involved in the BER-driven active DNA demethylation pathway, very little is known about the molecular regulation of the TDG–RNF4 interaction.

In this study, we first investigated SUMOylation of endogenous TDG during cell cycle progression and in response to hydroxyurea and nocodazole, which arrest cell cycle progression at S phase and mitosis, respectively. Second, we assessed TDG–RNF4 interaction *in vitro* and *in vivo*. Finally, we assessed whether RNF4 was required for TDG elimination during S phase. Our findings provide insights into posttranslational regulation of TDG function and stability during cell cycle progression.

2. Materials and methods

2.1. Plasmids

pIREShyg mammalian expression vector containing human TDG was used for generating pCDNA-FLAG-tagged TDGWT and mutant constructs. Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Agilent Technologies) and oligonucleotides shown Supplementary Table 1. Expression plasmid for hexahistidine (His_6)- or glutathione-S-transferase tagged TDG (GST-TDG) was described previously [6,7,11]. A plasmid expressing His_6- or GST-tagged RNF4 was generated using PCR with pET28 vector (Novagen). Oligonucleotide sequences of primers to generate the TDG, RNF4 and their deletion mutant expression vectors are described in Supplementary Table 1 or available upon request. All plasmids were sequenced to confirm that the correct recombinant had been obtained. pE1E2S1 and pE1E2S3 plasmids for bacterial SUMOylation were described previously [11]. Myc-tagged SUMO-$1/3_{GG}$ (WT) and Myc-SUMO-$1/3_{\Delta G}$ constructs were described previously and [12,13]. Myc-SUMO-1_{Q94P} and Myc-SUMO-3_{Q90P} mutants were generated by PCR. Primer sequences are available upon request.

2.2. Antibodies

The antibodies used in this study were anti-SUMO-1, anti-SUMO-2/3 (CST), anti-His_6 (SantaCruz), anti-GST (SantaCruz), anti-β-actin (MBL), anti-Myc (MBL), anti-FLAG (MBL), anti-cyclin A (CST) and anti-histone $H3_{S10P.}$ (CST) antibodies. The mouse monoclonal anti-TDG antibody (mab 9.11-1.1) and the mouse monoclonal anti-RNF4 antibody (mab 11-3.1) were generated by Urano. Rabbit polyclonal anti-TDG antibody was produced by Sugasawa. Rabbit polyclonal antibody GTX110473 purchased from GeneTex (Irvine, CA, USA).

2.3. Recombinant protein expression and GST-pull-down assay

SUMO-1 conjugated GST-TDG and SUMO-3 conjugated GST-TDG were prepared in the bacterial SUMOylation system [11]. Standard conditions of GST-pulldown assay were as described previously [12,13].

2.4. Cell culture, drugs and DNA transfection

HeLa cells were cultured under the standard conditions [12,13]. MG132 (Peptide Institute) and nocodazole (Sigma–Aldrich) was dissolved in dimethyl sulfoxide (DMSO) and added to culture medium as indicated in the text. Hydroxyurea was obtained from Wako Pure Chemical Industries. Lambda-phosphatase was purchased from BioAcademia. To synchronize HeLa cells, double thymidine block was performed as previously described [12]. Cells were transfected with plasmid DNAs by X-tremeGENE HP transfection reagent (Roche), according to the manufacturer's protocol.

2.5. siRNA and transfection

The siRNAs against RNF4 (siGENOME SMARTpool siRNA reagent M-006557-03-0005) were obtained from Thermo Fisher Scientific. Transfections were performed in 3.5×10-mm tissue culture dishes with 40 nM siRNA duplex and 6 μl of Lipofectamine RNAiMAX (Invitrogen). Experiments were performed 60 h after the transfection.

2.6. Indirect-immunofluorescence analysis

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Then cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min on ice. After the permeabilization, cells were blocked in 0.2% bovine serum albumin, 0.1% Triton X-100 in PBS for 5 min 3 times and incubated with primary antibody for 1 h, followed by an appropriate secondary antibody. The coverslips were mounted on slide glasses using glycerol–DABCO (Wako Pure Chemical Industries) and samples were analyzed with a DP72 microscope (Olympus). DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI).

2.7. Immunoprecipitation and immunoblot analyses

Cultured cells were rinsed with ice-cold PBS and lysed with the buffer containing 20 mM Hepes–NaOH, pH 7.5, 1% Triton X-100, 150 mM NaCl, 20 mM N-ethylmaleimide (NEM), and the protease inhibitors cocktail (Sigma–Aldrich) on ice for 30 min. After centrifugation at 15,000 rpm for 15 min, supernatant were incubated with 2 μg anti-FLAG or anti-Myc, rotated for 2 h at 4 °C. Protein G beads (GE Healthcare) and rotated for 1 h at 4 °C. After rotation, the beads were washed with 1% Triton X-100 buffer 3 times. Immunoblot analysis was carried out as described previously [12,13].

3. Results and discussion

3.1. Detection of SUMOylated and non-modified forms of endogenous TDG

We used three TDG antibodies to analyze total cell lysates prepared from exponentially growing cultured HeLa and U-2 OS cells (Supplementary Fig. 1). Two prominent bands at around 60 kDa and 80 kDa were detected. The 80-kDa species was expected to be the mono-SUMOylated form of endogenous TDG, which has a molecular mass of 60 kDa. In HeLa cells transfected with siRNA against TDG, both 60- and 80-kDa bands were reduced (Fig. 1A), indicating that both bands were related to TDG. Since several group reported the covalent conjugation of TDG with SUMO *in vitro* and *in vivo* [1–5], we assumed that the 80-kDa band represented SUMOylated form of endogenous TDG. This was confirmed by exogenous expression of Myc-tagged SUMO-$1/3_{GG}$ in cultured HeLa cells. As shown in Fig. 1B, transfection of a vector expressing Myc-SUMO-$1/3_{GG}$ (WT), but not the SUMOylation-deficient mutant Myc-SUMO-$1/3_{\Delta G}$, gave rise to an increase of the 80-kDa species as well as a slight decrease of the 60-kDa species, supporting the idea that the 80-kDa band represented SUMOylated form of the 60-kDa of TDG. It should be mentioned that deSUMOylation, which antagonizes TDG SUMOylation, was actively occurring in asynchronously cultured cells, as transfection with the deSUMOylation-resistant SUMO mutant, Myc-SUMO-$1/3_{Q/P}$ [14], resulted in enhanced levels of the 80-kDa form (Fig. 1B). Indeed, inclusion of

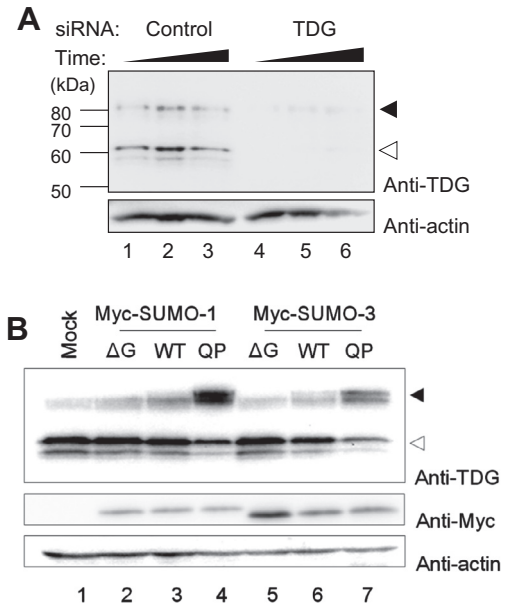

Fig. 1. Detection of non-modified and SUMOylated forms of endogenous TDG. (A) Exponentially growing HeLa cells were transfected with control siRNA (lanes 1–3) or siRNA against TDG (lanes 4–6), followed by incubation for 48 h (lanes 1and 4), 72 h (lanes 2 and 5) and 96 h (lanes 3 and 6). Cells were lysed directly in SDS sample buffer and proteins were subjected to 10% SDS–PAGE, followed by immunoblot analysis using anti-TDG (GTX; upper panel) or anti-actin (lower panel) antibody. The black arrowhead indicates the 80-kDa band of SUMOylated TDG. The white arrowhead shows the 60-kDa band of non-modified form of TDG. Molecular mass standards are shown on the left (kDa). (B) Exponentially growing HeLa cells were transfected with Myc-vetor alone (lane 1), Myc-SUMO-$1_{\Delta G}$ (lane 2), Myc-SUMO-1_{GG} (WT; lane 3), Myc-SUMO-$1_{Q/P}$ (lane 4), Myc-SUMO-$3_{\Delta G}$ (lane 5), Myc-SUMO-3_{GG} (WT; lane 6) or Myc-SUMO-$3_{Q/P}$ (lane 7), followed by incubation for 24 h. Cells were lysed directly in SDS sample buffer and proteins were separated by 10% SDS–PAGE, followed by immunoblot analysis using anti-TDG (upper panel), anti-Myc (middle panel) or anti-actin (bottom panel) antibody. The black arrowhead indicates the 80-kDa band of SUMOylated TDG. The white arrowhead shows 60-kDa band of non-modified form of TDG. The bands detected by anti-Myc antibody represent free forms of Myc-SUMOs.

NEM, an inhibitor of cysteine-based enzymes including deSUMOylation enzymes, during cell lysis often increased the levels of the 80-kDa species (Supplementary Fig. 1). Phosphatase-treatment of cell lysate did not show significant alteration of the band pattern in immunoblot analysis (Supplementary Fig. 2), implying that negligible fraction of cellular pool of TDG was phosphorylated.

3.2. TDG SUMOylation in cells undergoing S phase and in hydroxyurea-treated cells

When lysate prepared from synchronously cycling cultured HeLa cells was investigated by immunoblot analysis using anti-TDG antibody, we found that both SUMOylated and non-SUMOylated form of endogenous TDG fluctuated during cell cycle (Fig. 2A). Notably, lysate from cells at 2, 4 and 6 h after release from double-thymidine-block, which synchronizes cells at S phase, exhibited little TDG signal of both 60 and 80 kDa. This observed reduction of TDG during S phase was recapitulated in asynchronously cultured HeLa cells treated for 16 h with hydroxyurea, which activates the intra-S-phase checkpoint pathway to block S-phase progression (Fig. 2B, lane 3); Neither SUMOylated nor non-modified forms of TDG were detectable in cells treated with hydroxyurea, confirming reduction of both forms of TDG in cells undergoing S phase. Treatment of cells with MG132 proteasome inhibitor, led to recovery of both forms of TDG in hydroxyurea-treated cells (Fig. 2B, lane 6), implying that the reduction of TDG might be mediated by the ubiquitin–proteasome degradation system, consistent with previous observations by Hardeland et al. [8]. When asynchronous HeLa cells were immunostained with anti-TDG antibody, a significant fraction of cells exhibited an absence of TDG in the nucleus (Fig. 2C). These TDG-eliminated cells were able to incorporate 5-ethynyl-2′-deoxyuridine (EdU), a nucleotide analog (Supplementary Fig. 3), indicating that these cells were in S phase, thus supporting the idea of TDG degradation during S phase.

3.3. TDG SUMOylation after S phase and in nocodazole-exposed cells

In contrast to the reduction of TDG during S phase, both SUMOylated and non-modified forms of TDG were readily detectable in lysates prepared from synchronized cell culture at 8, 10, 12, 14 and 16 h after release from double thymidine block (Fig. 2A). Because cells at these points might represent G2 phase (8 and 10 h), mitosis (8 and 10 h) and G1 phase (12, 14 and 16 h), these data indicated that both SUMOylated and non-SUMOylated forms of TDG might be stable after S phase to subsequent mitosis and G1 phase. The immunostaining of mitotic cells further supported this notion, as most mitotic cells were heavily stained with anti-TDG antibody (Fig. 2C). Interestingly, exposure of asynchronous cells for 16 h to nocodazole, an agent interfering with microtubule polymerization and thus causing cells to arrest at mitosis, induced accumulation of both SUMOylated and non-modified forms of TDG (Fig. 2B, lane 4), confirming that TDG was stable during G2 phase/mitosis. It should be noted that the SUMOylated form of TDG appeared to be augmented compared with the non-modified form TDG in nocodazole-exposed cells, suggesting an unknown mechanism(s) that regulates augmentation of TDG SUMOylation during mitosis. Given that phosphatase-treatment of the nocodazole-exposed cell lysate did not show significant alteration of the band pattern in immunoblot analysis (Supplementary Fig. 2), augmentation of TDG SUMOylation likely does not involve TDG phosphorylation.

3.4. Effect of TDG mutation on SUMOylation

In addition to the investigation on TDG SUMOylation in response to cell cycle progression/arrest, we generated various TDG mutants and elucidated the regulation of TDG SUMOylation with respect to TDG structural and/or functional transfer. The TDG mutants expressed in HeLa cells were shown in Fig. 2C: D133A and E310Q are the SUMO-interacting motif (SIM) mutants [15], N140A is the active site mutant [16], M269H is the DNA-binding mutant [16], and K330R is the SUMOylation-deficient mutant [1,6,7]. When TDG_{D133A}, TDG_{E310Q} and TDG_{M269H} were transiently overexpressed in HeLa cells, the SUMOylated forms of these mutant proteins were barely detectable, whereas the non-modified form of each mutant was efficiently accumulated (Fig. 2D). These patterns were nearly identical to that of the SUMOylation-deficient TDG_{K330R} mutant, suggesting that the SIM and DNA-binding regions of TDG are critical for TDG SUMOylation.

3.5. RNF4 interaction with TDG in a SUMOylation-independent manner

To assess functional role of TDG SUMOylation, we investigated the interaction of TDG with RNF4. Because RNF4 is a member of the STUbLs, which possess high affinity interacting domains to SUMO, and was demonstrated to be associated with TDG for BER-driven active DNA demethylation (Fig. 3A), it seemed conceivable that TDG SUMOylation might contribute to the association with RNF4 [9,10]. To test this, we first expressed Myc-RNF4 together with either FLAG-TDG_{WT}, which produced both

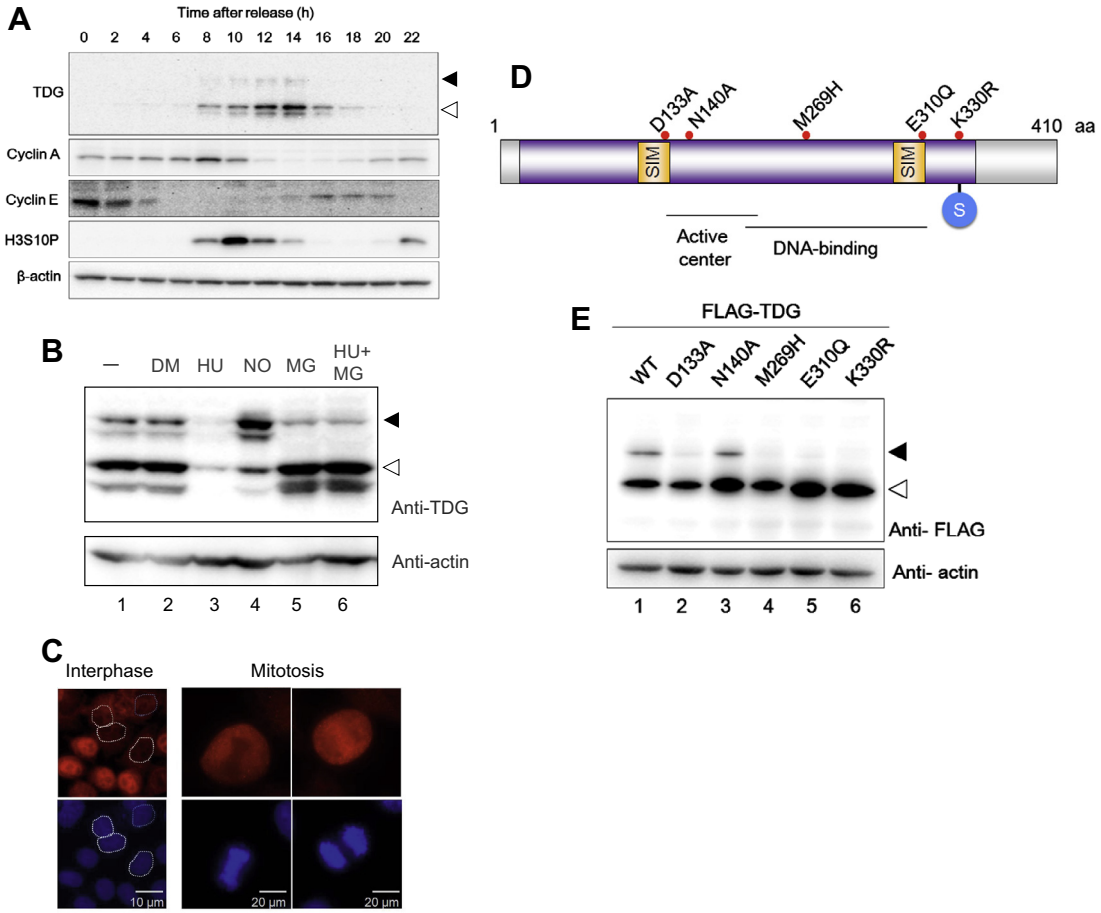

Fig. 2. TDG SUMOylation in cells arrested at different points in the cell cycle and the effect of TDG mutations on SUMOylation. (A) Immunoblot analyses of total cell lysate from synchronized cells at indicated times after release of double-thymidine block (h). Blots were probed with anti-TDG, anti-cyclin A (marker for S/G2 phase), anti-cyclin E (marker for S phase), anti-phosphorylated histone H3 (marker for mitosis), or anti-β actin antibody as indicated. The black arrowhead indicates the 80-kDa band of SUMOylated TDG. The white arrowhead shows 60-kDa band of non-modified form of TDG. (B) HeLa cells were cultured under the standard conditions (lane 1), in the presence of DMSO (lane 2), 5 mM hydroxyurea (lane 3), 1.0 μg/ml nocodazole (lane 4), 20 μM MG132 (lane 5), 5 mM hydroxyurea plus 20 μM MG132 (lane 6). After 16 h incubation, cells were lysed directly in SDS sample buffer and proteins were subjected to 10% SDS–PAGE, followed by immunoblot analysis using anti-TDG (GTX; upper panel) or anti-actin antibody (lower panel). The black and white arrowheads indicate the 80-kDa band of SUMOylated TDG and 60-kDa band of non-modified form of TDG, respectively. (C) Subcellular localization of endogenous TDG was detected by indirect immunofluorescence analysis using anti-TDG antibody (upper). Interphase nucleus and mitotic chromosomes were visualized by DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI) staining (bottom). Bars and numbers indicate scale of the pictures. (D) Schematic representation of human TDG structure and the positions of mutated amino acid residues (aa). (E) The expression plasmid vectors of TDG_{WT} and TDG mutants as indicated in the figure were transfected into HeLa cells. After 16 h incubation, cells were lysed directly in SDS sample buffer and proteins were separated by 10% SDS–PAGE, followed by immunoblot analysis using anti-TDG (upper panel) or anti-actin (lower panel) antibody. The black and white arrowheads indicate the 80-kDa band of SUMOylated TDG and 60-kDa band of non-modified form of TDG, respectively.

SUMOylated and non-modified forms of TDG, or the FLAG-TDG_{K330R}, SUMOylation-deficient mutant, and evaluated reciprocal immunoprecipitation of TDG and RNF4. As shown in Fig. 3B, Myc-RNF4 equally co-immunoprecipitated with either wild-type or mutant FLAG-TDG, suggesting that RNF4 interacts with TDG in a TDG SUMOylation-independent fashion *in vivo*. When FLAG-TDG_{WT} signal in the fraction immunoprecipitated with anti-Myc antibody was overexposed, SUMOyated form of FLAG-TDG_{WT} was barely detectable (Fig. 3C), further emphasizing that RNF4 bound to TDG regardless of SUMOylation of TDG.

Our results illustrating that SUMOylation was nonessential for TDG binding to RNF4 was also revealed in *in vitro* binding experiments using recombinant proteins. When SUMO-1/3-conjugated GST-TDG or non-modified GST-TDG was mixed with His_6-RNF4, the recombinant RNF4 bound to both SUMOylated and non-modified forms of GST-TDG (Fig. 3D). GST-fusion protein fused to N-terminal half of RNF4 preferentially bound recombinant TDG compared with that GST fused to C-terminal half of RNF4, which contains a RING finger domain (Fig. 3A and E). These results suggested direct interaction between TDG and RNF4 in the absence of SUMO, supporting the idea that TDG SUMOylation is not necessary for the association with RNF4.

3.6. RNF4-independent reduction of TDG in hydroxyurea-treated cells

Because RNF4 is an ubiquitin E3 ligase, it is possible that TDG–RNF4 interaction might be involved in TDG degradation during S phase. To assess the role of RNF4 in regulating the stability of TDG, we compared the amount of SUMOylated and non-modified forms of TDG in RNF4-depleted and non-depleted cells. After transfection of siRNAs, cells were maintained under standard culture conditions for 24 h, followed by incubation in the culture medium containing hydroxyurea or nocodazole for 16 h. We found that even though ~90% of RNF4 was depleted (Fig. 4A), SUMOylated TDG accumulated efficiently in nocodazole-arrested mitotic cells and both SUMOylated and non-modified TDG were eliminated in hydroxyurea-treated S phase arrested cells (Fig. 4B). These data indicated that RNF4-depletion had a negligible impact on SUMOylation in mitotic cells as well as reduction of TDG during S phase.

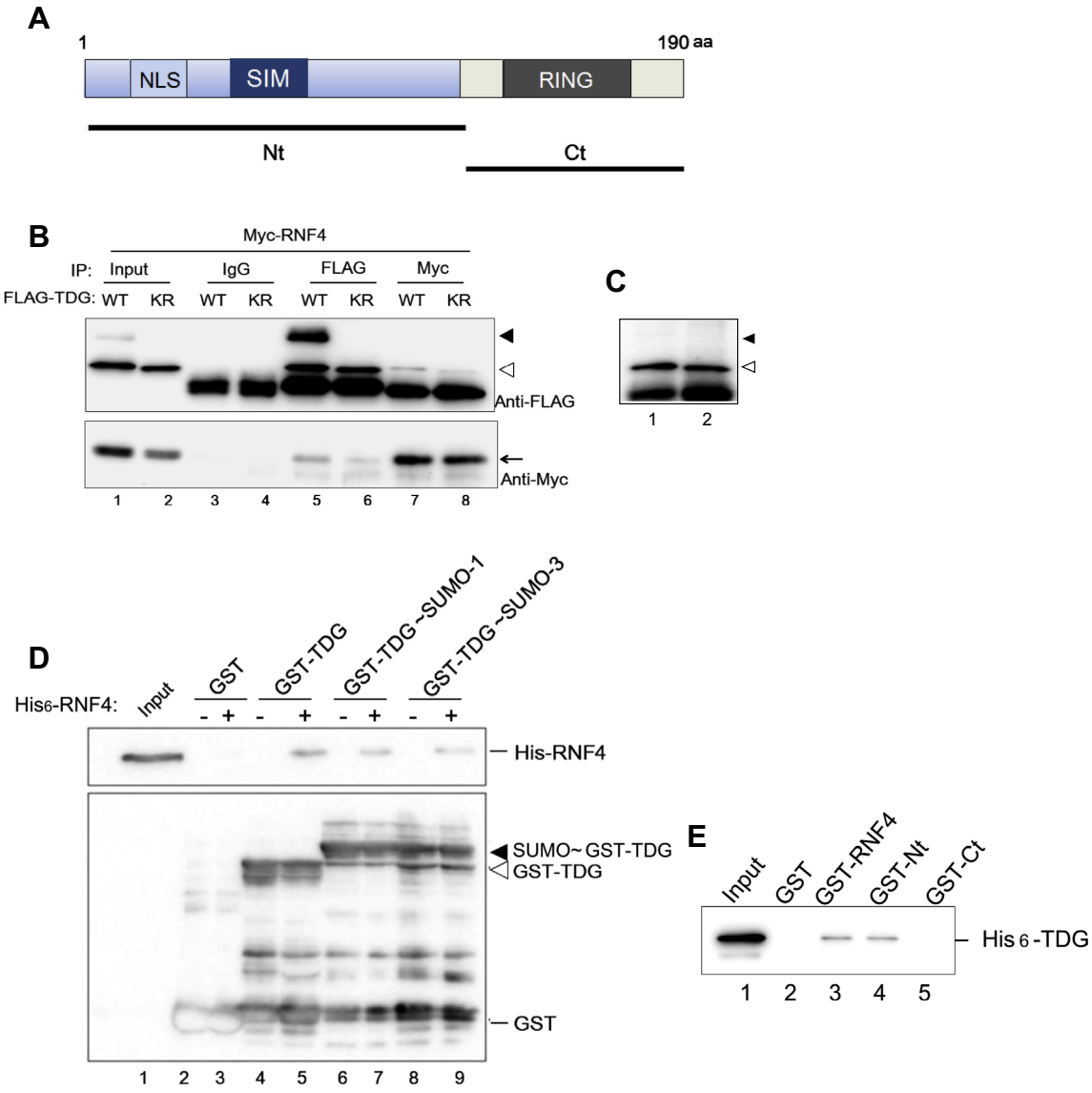

Fig. 3. RNF4 interaction with TDG independent of SUMOylation. (A) Schematic representation of RNF4 structure and domains used for GST-pulldown assay. Numbers represent amino acid residues (aa). NLS: nuclear localization signal. SIM: SUMO-interacting motif. RING: RING-finger domain. (B) Immunoprecipitation assay. Exponentially growing HeLa cells were transfected with Myc-RNF4 together with either FLAG-TDG_{WT} (lanes 1, 3, 5 and 7) or FLAG-TDG_{K330R} mutant (lanes 2, 4, 6 and 8), followed by incubation for 24 h. Cells were harvested and subjected to immunoprecipitation using either anti-FLAG (lanes 5 and 6) or anti-Myc (lanes 7 and 8) antibody. 10 μl of each immunoprecipitated sample was subjected to 10% SDS–PAGE, followed by immunoblot analysis using anti-FLAG (upper panel) or anti-Myc (lower panel) antibody. IgG was used for as a control (lanes 3 and 4). The black and white arrowheads indicate the position of SUMOylated TDG and non-modified TDG, respectively. (C) Immunoblot analysis shown in lanes 7 and 8 in B was repeated under the same conditions as in B, except for 25 μl of the sample volume, to improve intensity of FLAG-TDG signal. The black and white arrowheads indicate the position of SUMOylated TDG and non-modified TDG, respectively. (D) Aliquots of lysates containing bacterially expressed His_6-RNF4 (lanes 3, 5, 7 and 9) or the binding buffer (lanes 2, 4, 6 and 8) were incubated with either GST alone (lanes 2 and 3), GST-TDG (lanes 4 and 5), SUMO-1-modified GST-TDG (lanes 6 and 7), or SUMO-3-modified GST-TDG (lanes 8 and 9), followed by precipitation with glutathione-Sepharose beads. Following extensive washing of the precipitated beads, the associated proteins were separated by SDS–PAGE and subjected to immunoblotting analysis with anti-His_6 antibody (upper panel) or anti-GST antibody (lower panel). Black arrow indicates the position of the SUMOylated form of GST-TDG. White arrowhead represents the position of the non-modified GST-TDG. Lane 1 shows lysate containing His_6-RNF4 (input). (E) Aliquots of lysates containing His_6-TDG (lane 1) were incubated with either GST alone (lane 2), GST-RNF4 (lane 3), GST-Nt (lane 4) or GST-Ct (lane 5), followed by precipitation with glutathione-Sepharose beads. Following extensive washing of the precipitated beads, the associated proteins were separated by SDS–PAGE and subjected to immunoblotting analysis with anti-His_6 antibody.

In this study, we investigated TDG SUMOylation in cultured human cells and revealed that SUMOylation of endogenously expressed TDG was altered during cell cycle progression and in response to drugs that perturb cell cycle progression. We also generated several mutants of TDG with aberrant SUMOylation in cultured human cells. These are significant findings in enriching the examples of SUMOylation dynamics in vital cellular systems, which might be beneficial for elucidating the SUMO signaling network [17–19]. Additionally, we provided information regarding TDG–RNF4 interaction, which will be useful to study active DNA demethylation process regulated by TDG and RNF4 [10,20]. Indeed, the observation of TDG reduction in RNF4-depleted cells suggests the possibility of another ubiquitin E3 ligase(s), beside RNF4, that controls TDG degradation during S phase. Identification of such an E3 ligase(s) would be one of the important research avenues for future studies.

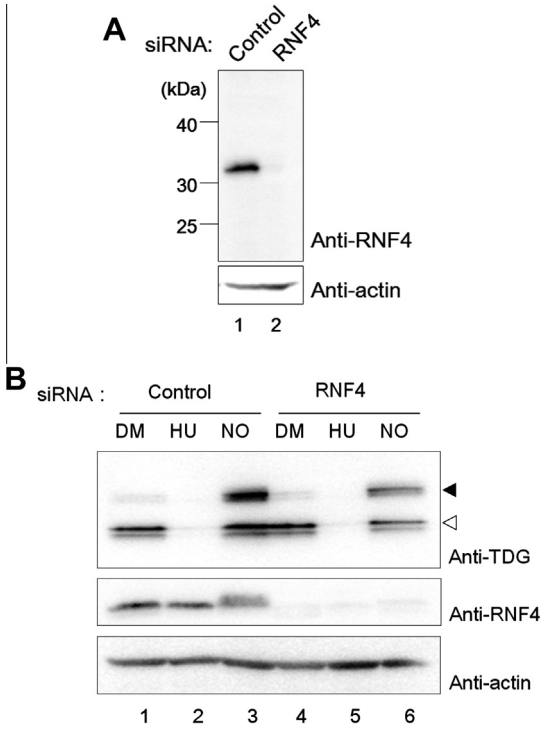

Fig. 4. RNF4-independent degradation of TDG. (A) Exponentially growing HeLa cells were transfected with control siRNA (lane 1) or siRNA against RNF4 (lane 2). After 24 h incubation, cells were lysed directly in SDS sample buffer and proteins were subjected to 10% SDS–PAGE, followed by immunoblot analysis using anti-RNF4 (upper panel) or anti-actin (lower panel) antibody. Molecular mass standards are shown on the left (kDa). (B) Exponentially growing HeLa cells were transfected with control siRNA (lanes 1–3) or siRNA against RNF4 (lane 4–6), followed by 24 h incubation. Then cells were cultured for 16 h under the standard conditions (lanes 1 and 4), in the presence of 5 mM hydroxyurea (lanes 2 and 5) or 1.0 μg/ml nocodazole (lanes 3 and 6). Cells were lysed directly in SDS sample buffer and proteins were subjected to 10% SDS–PAGE, followed by immunoblot analysis using anti-TDG (GTX; upper panel), anti-RNF4 (middle panel) or anti-actin (bottom panel) antibody. The black and white arrowheads indicate the position of SUMOylated TDG and non-modified TDG, respectively.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.004.

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