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The F-box protein β-TrCP promotes ubiquitination of TRF1 and regulates the ALT-associated PML bodies formation in U2OS cells

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ABSTRACT

The telomeric repeat binding protein 1 (TRF1) is a major factor of the mammalian telosome/shelterin and negatively regulates telomere length by inhibiting access of telomerase at telomere termini in telomerase-positive cells. In telomerase-negative cancer cells, TRF1 also plays a critical role in the mechanism called alternative lengthening of telomeres (ALT) and is essential for formation of the ALT-associated PML bodies (APBs). It was reported that TRF1 can be degraded by the ubiquitin–proteasome pathway, involving in two regulation factors, Fbx4 and RLIM. Here, we reported that β-TrCP1, a member of the F-box family protein with ubiquitin ligase activity, is a novel TRF1-associating protein. β-TrCP1 interacts with TRF1 in vivo and in vitro and promotes its ubiquitination. Overexpression of β-TrCP1 reduces endogenous TRF1 protein levels, while inhibition of β-TrCP1 by siRNA stabilizes TRF1. Moreover, we found that β-TrCP1 is essential for regulation of promyelocytic leukemia body recruitment of TRF1 in U2OS cells. These results reveal that β-TrCP1 is involved in the negative regulation of TRF1 and represents a new pathway for APB formation in telomerase-negative cells.

1. Introduction

Telomeres are the specialized protective nucleoprotein complexes which protect the end of eukaryotic chromosome from end-to-end fusion between chromosome termini [1,2]. Collapse of telomere length homeostasis is implicated in aging and cancer [1]. Telomere maintenance depends on precise associations between telomeric DNA and its binding proteins [3]. It is clear that six major protein subunits (TRF1, TRF2, RAP1, TIN2, POT1, and TPP1) form the specific telomeric complex called telosome/shelterin which participates in telomere regulation [4,5]. Telomeric repeat binding factor 1 (TRF1) was the first identified telomere-associated protein [6]. TRF1 binds double-stranded telomeric DNA and interacts with some important proteins that are essential for the maintence of telomeric DNA [7]. TRF1 negatively controls the telomere length and regulates the mitotic progression of dividing cells [8]. It has been reported that post-translational modification of TRF1 sush as ubiquitination by Fbx4 or RLIM and phosphorylation by Plk1 or CDK1 modulates telomere length [9–12]. In telomerase-negative cancer cells, TRF1 also plays an important role in maintaining the lengths of telomeres, named alternative lengthening of telomeres (ALT) mechanism [5]. TRF1 is required for the assembly of ALT-associated PML bodies (APBs) and sumoylation of TRF1 by MMS21/NSE2 promotes APBs formation in ALT cells, indicating the importantance of the post-translational modification of TRF1 in cellular regulation [13].

The ubiquitin-proteasome pathway is important for degradation of proteins, regulating critical cellular functions. Ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) sequentially label target proteins for ubiquitination and proteasomal degradation [14]. The interaction between specific E3s and substrates has been thought to determine the selectivity of substrate in the ubiquitin-proteasome system [15]. The F-box protein beta-transducin repeat-containing protein (β-TrCP) serves as the substrate recognition subunit in the SCF (Skp1-Cullin1-F-box protein) ubiquitin protein ligase complex, which mediates the ubiquitination and subsequent proteasomal degradation of target proteins [16]. β-TrCP consists of an N-terminal F-box domain, a C-terminal WD40-repeats domain and an a-helical domain [17]. The F-box domain is responsible for binding to Skp1, whereas the WD40-repeat domain mediates the interaction with the target proteins. There is a growing list of identified downstream ubiquitin substrates of β-TrCP, demonstrating a

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critical role for β-TrCP in regulating cell cycle progression, DNA damage and cellular apoptosis [18–21]. However, further studies are required to provide the experimental evidence to explore the precise function of this E3 ligase.

In this study, we report a novel ubiquitin-dependent TRF1 degradation mechanism. In a search for proteins capable of interacting with TRF1, We found that the F-box protein β-TrCP1 mediates TRF1 degradation. β-TrCP1 increases TRF1 turnover by targeting its degradation through the proteasome in a ubiquitin dependent manner. We also found β-TrCP1 is essential for regulation of promyelocytic leukemia (PML) body recruitment of TRF1 in U2OS cells, indicating that the regulation of TRF1 stability by β-TrCP1 maybe control the APBs assembly in U2OS cells. Overall, these results demonstrate that β-TrCP1 is involved in the negative regulation of TRF1 stability and represents a new pathway for APB formation in telomerase-negative cells.

2. Materials and methods

2.1. Reagents

Mouse monoclonal anti-GFP antibody was purchased from BD Biosciences Pharmingen (San Diego, CA). Anti-Flag antibody, anti-Flag-conjugated agarose beads, 4,6-diamidino-2-phenylindole (DAPI), mouse monoclonal antibody to His were purchased from Cell Signaling (Beverly, MA). Mouse monoclonal anti-myc antibody, mouse monoclonal anti-β-TrCP1 and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-TRF1 antibody was purchased from Abcam (Cambridge, MA). Rhodamine-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Glutathione Sepharose was purchased from Amersham Biosciences (Piscataway, NJ). Ni-NTA resin was purchased from Qiagen (Valencia, CA).

2.2. DNA construction

To generate green fluorescent protein (GFP)-tagged and myc-his-tagged of TRF1, PCR-amplified cDNAs were cloned into pEGFP-C1 (Clontech) and pcDNA3.1/myc-His B (Invitrogen) vectors respectively. Flag-tagged TRF1 cDNA and mutants were cloned by inserting the PCR product into the pcDNA3-FLAG vector (Invitrogen).

2.3. Cell culture and transfection

HeLa and 293T cells, from American Type Culture Collection (Rockville, MD), were cultured as subconfluent monolayers in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS (Hyclone, Logan, UT) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) with 10% CO_2. Plasmids were transfected using Lipofectamine 2000 reagent according to the manufacturer's instructions.

2.4. Small interfering RNA

siRNA targeting β-TrCP1 was purchased from Qiagen (Valencia, CA). Scramble sequence was used as a control. All the siRNAs were transfected into HeLa cells using Lipofectamine 2000.

2.5. Immunoprecipitation

Human 293T cells were co-transfected with GFP-TRF1 and Flag-β-TrCP1. 36 h after transfection, cells were harvested and subjected to immunoprecipitation with anti-Flag M2 affinity gel as described previously [22]. For immunoprecipitation of transiently transfected cells, HeLa cells were generally collected 36 h after transfection of Flag-β-TrCP1 and its deletions, cells were harvested and subjected to immunoprecipitation with anti-Flag M2 affinity gel.

2.6. In vivo ubiquitination assays

For in vivo ubiquitination assays, Myc-His-tagged TRF1 and HA-tagged ubiquitin expression plasmids were co-expressed in HEK293 cells. 2 d later, cells were treated with MG132 (10 μM) for 6 h before protein extraction in 6 M guanidinium buffer. Ubiquitylated proteins were pulled down by Ni-NTA resin and washed extensively. Binding proteins were then subjected to immunoblotting using an appropriate antibody.

2.7. Immunofluorescence microscopy

Cells were grown on acid-treated glass coverslips. After transfection, immunofluorescence was carried out as described previously [23]. Fluorescence intensity of target protein labeling was measured by using the confocal microscope LSM510 NLO (Carl Zeiss) scan head mounted transversely to an inverted microscope (Axiovert 200; Carl Zeiss) with a 100×1.3 NA PlanApo objective. The images from double labeling were collected using a dichroic filter set with Zeiss image processing software (LSM 5, Carl Zeiss) as described previously [24].

3. Results

3.1. β-TrCP1 is a novel interacting protein of TRF1

To further explore the biological function of TRF1, we generated a U2OS cell line which stably expresses Flag-TRF1. As shown in Fig. 1A, the expression of Flag-TRF1 can be well detected in the stably transfected cells but not in the control cells. We next purified TRF1-associating protein complexes by immunoprecipitation from U2OS cells stably expressing Flag-TRF1. As shown in Fig. 1B, in addition to Tankyrase and several degradation products of TRF1, a protein band that migrated around 60 KDa was present in the anti-Flag M2 immunoprecipitates. Mass spectrometry analysis revealed that this band belonged to β-TrCP1. To confirm the interaction between TRF1 and β-TrCP1, 293T cells were co-transfected with Flag-β-TrCP1 and GFP-TRF1 or GFP vector alone, and co-immunoprecipitation assay was performed. As shown in Fig. 1C, GFP-TRF1 but not GFP can be precipitated by Flag-β-TrCP1. To further confirm the interaction between TRF1 and β-TrCP1, lysates of U2OS cells were immunoprecipitated with control IgG and anti-β-TrCP1 antibody and then subjected to immunoblotting analysis. As shown in Fig. 1D, TRF1 can be precipitated by the anti-β-TrCP1 but not the control IgG. To map the region in β-TrCP1 that interacts with TRF1, we constructed β-TrCP1-deletion mutants missing either the F-box (ΔF-box-β-TrCP1) which is required for Skp1 binding or the WD40 domains (ΔWD40-β-TrCP1) that are required for substrate binding (Fig. 1E), and co-immunoprecipitation experiment was performed. As shown in Fig. 1F, TRF1 protein interacted with wild-type (Wt) β-TrCP1 and ΔF-box-β-TrCP1, but not the ΔWD40-β-TrCP1, indicating that the F-box is not required for TRF1 binding to β-TrCP1.

We next examined subcellular distribution of TRF1 and β-TrCP1 by immunofluorescence. As shown in Fig. 1G, some of endogenous β-TrCP1, but not all, appears to colocalize with GFP-TRF1 in a subset of U2OS cells. Therefore, the β-TrCP1 interacts and colocalizes with TRF1 in cells.

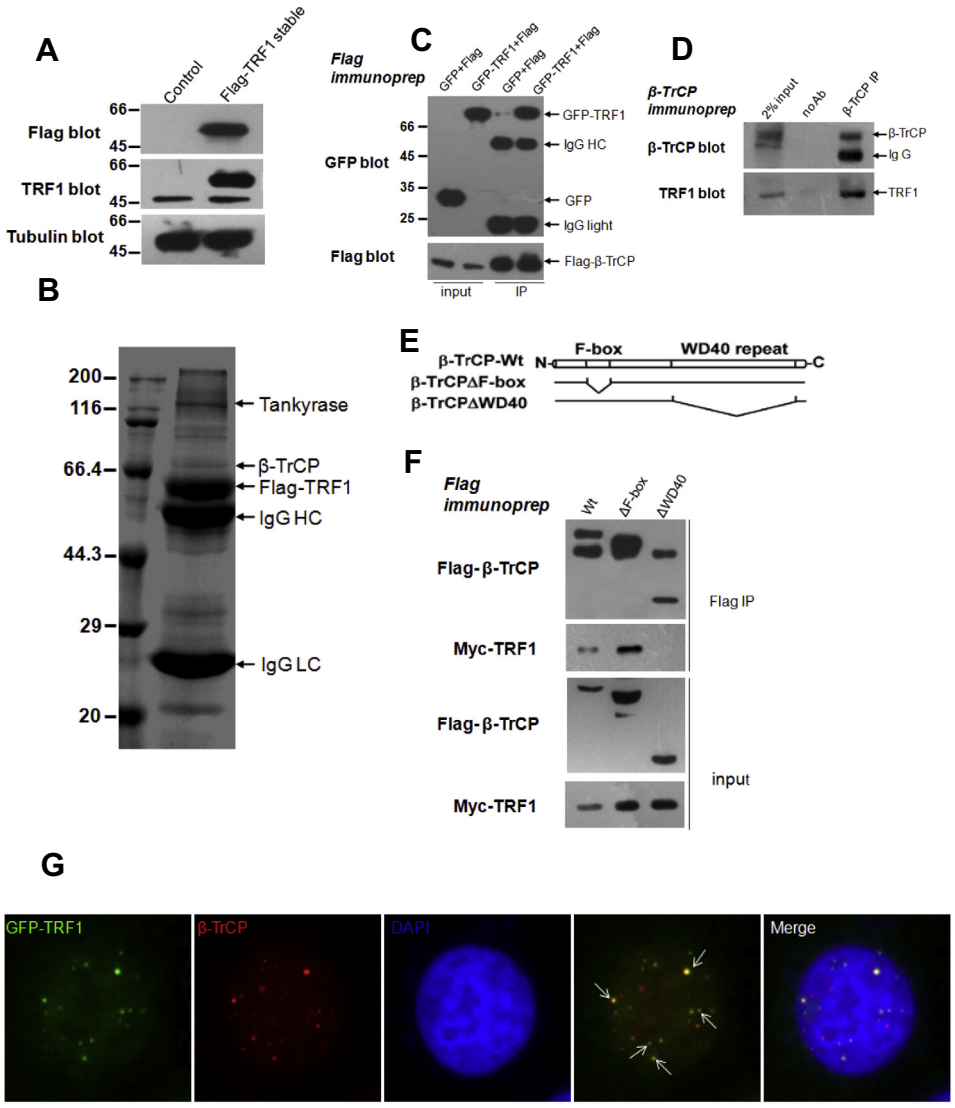

Fig. 1. β-TrCP1 is a novel TRF1-binding protein. (A) The Western blot shows Flag-TRF1 protein from stably expressing U2OS cells. (B) The Flag-TRF1 complexes were immunoprecipitated from the extracts of Flag-TRF1 stably expressing U2OS cells using an anti-Flag antibody-agarose beads. After binding, beads were extensively washed, and bound proteins were separated by SDS–PAGE gel. The indicated proteins were extracted from the gel, digested with trypsin, and the amino acid sequence of peptides was determined by mass spectrometry. (C) Co-immunoprecipitation of exogenously expressed TRF1 and β-TrCP1. 293T cells were co-transfected with GFP-TRF1 and Flag-β-TrCP1, lysed and immunoprecipitated with anti-Flag M2 affinity gel, using GFP as control, followed by immunoblotting analysis with GFP and Flag antibody respectively. (D) Endogenous interaction between TRF1 and β-TrCP1. U2OS cells were lysed and immunoprecipitated by anti-β-TrCP1 and control IgG. The immunoprecipitates were analyzed by immunoblotting with anti-β-TrCP1 antibody and anti-TRF1 antibody. (E) Schematic representation of wild-type and mutant β-TrCP1 proteins and their functional domains. (F) WD40 functional domain of β-TrCP1 is responsible for interaction between TRF1 and β-TrCP1. Flag-β-TrCP1 (Wt, ΔF-box or ΔWD40) were transfected into 293T cells together with Myc-TRF1 plasmids. Cell lysates were immunoprecipitated with anti-Flag M2 affinity gel followed by immunodetection. (G) TRF1 is co-localized with β-TrCP1. U2OS cells expressing GFP-TRF1 (green) were treated with 10 μM MG132 for 4 h and analyzed by indirect immunofluorescence. Paraformaldehyde-fixed cells were stained with anti-β-TrCP1 antibodies (red). DNA was stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. β-TrCP1 promotes ubiquitination of TRF1

Our findings above demonstrated that TRF1 physically interacts with β-TrCP1. Given that the F-box protein β-TrCP1 can target specific substrates for proteasomal degradation, we speculated that β-TrCP1 may target TRF1 for ubiquitination and thereby promote its proteasome-mediated degradation. To examine whether β-TrCP1 ubiquitinates TRF1 in vivo, U2OS cells were co-transfected with myc-his-TRF1, HA-ubiquitin, and Flag-β-TrCP1, lysed under denaturing conditions, and then subjected to pull-down assay using Ni-NTA agarose beads. As shown in Fig. 2A, overexpression of β-TrCP1 caused an increase in TRF1 ubiquitination. To exclude the possibility that other cellular proteins bounded with β-TrCP1 from the transfected U2OS cell lysates may influence TRF1 ubiquitination, U2OS cells were transfected with different amount of Flag-β-TrCP1, and in vivo ubquitination assay was repeated. As shown in Fig. 2B, TRF1 was efficiently ubiquitinated by adding an increasing amount of Flag-β-TrCP1.

To further examine the in vivo role of β-TrCP1 in the ubiquitination of TRF1, U2OS cells were co-transfected with His-ubiquitin, myc-TRF1, and together Flag-β-TrCP1 WT or its ΔF-box mutant, and Ni-NTA pull-down assay was performed. As shown in Fig. 2C, TRF1 ubiquitylation was not enhanced by ΔF-box-β-TrCP1 overexpression. In summary, these data indicate that β-TrCP1 is an E3 ligase for TRF1 ubiquitination in U2OS cells.

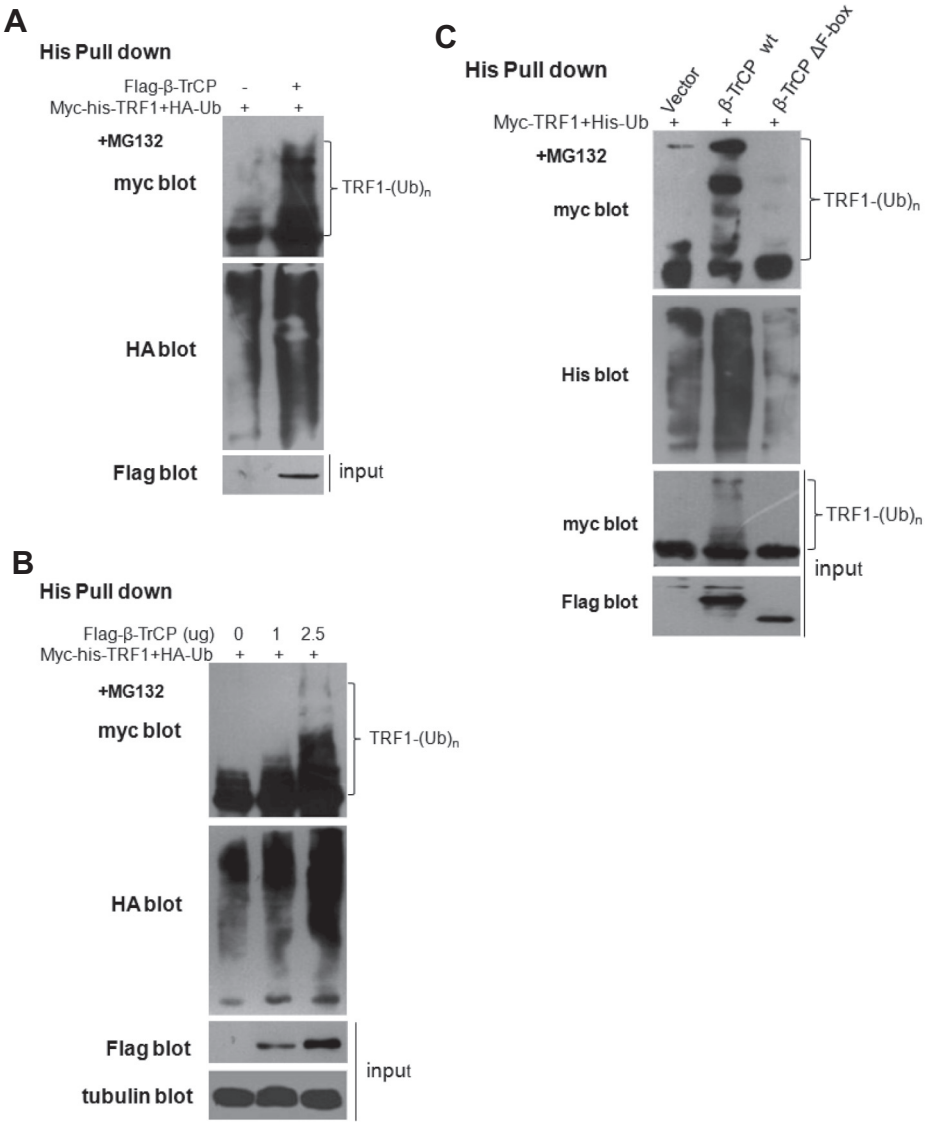

Fig. 2. β-TrCP1-mediated ubiquitination of TRF1 in vivo. (A and B) 293T cells were transfected with Myc-his-TRF1, Flag-β-TrCP1 and HA-Ub expression plasmids. 24 h after transfection, cells were treated with or without 25 μM proteasome inhibitor MG132 for another 6 h. The cells were lysed under denaturing conditions and then immunoprecipitated with Ni-NTA resin. The ubiquitination of TRF1 was detected with anti-myc and anti-HA antibodies. (C) 293T cells were transfected with vector, Myc-TRF1, Flag-β-TrCP1 (Wt or ΔF-box) and His-Ub expression plasmids. 24 h after transfection, cells were treated with or without 25 μM proteasome inhibitor MG132 for another 6 h. The cells were lysed under denaturing conditions and then immunoprecipitated with Ni-NTA resin. The ubiquitination of TRF1 was detected with anti-myc and anti-His antibodies. Myc-TRF1 and Flag-β-TrCP1 (Wt or ΔF-box) were detected in whole-cell lysates by immunoblotting.

3.3. β-TrCP1 regulates the stability of TRF1

Because β-TrCP1 promotes ubiquitination of TRF1, we next examined its ability to regulate the protein level of TRF1. U2OS cells were co-transfected with GFP-TRF1 and increasing amounts of Flag-β-TrCP1. As shown in Fig. 3A, the steady state of TRF1 was reduced by β-TrCP1 overexpression, indicating that β-TrCP1 can enhance the degradation of Tara. Next, U2OS cells were transfected with or without Flag-β-TrCP1, and endogenous TRF1 was detected using anti-TRF1 antibody. As shown in Fig. 3B, Overexpression of β-TrCP1 resulted in a clear reduction in the protein level of TRF1, and such turnover of TRF1 was blocked by the MG132 treatment, indicating that TRF1 degradation is mediated by the proteasome. We next tested the effect of depletion of β-TrCP1 on TRF1. As shown in Fig. 3C, β-TrCP1 siRNA efficiently suppressed the expression of endogenous β-TrCP1 protein, and such siRNA depletion caused an increase of TRF1 protein level as expected.

To further investigate whether the negative regulation of TRF1 protein level by β-TrCP1 is through modulating TRF1 degradation, U2OS cells were transfected with or without β-TrCP1, incubated with cycloheximide (CHX) to block new protein synthesis, and then analyzed by immunoblotting with anti-TRF1 antibody. As shown in Fig. 3D and 3E, overexpression of β-TrCP1 caused a clear reduction in the half-life of TRF1, which was stabilized by the proteasome inhibitor MG132 treatment. Taken together, these data indicate that β-TrCP1 can negatively regulate the steady-state level of TRF1 protein in U2OS cells.

3.4. β-TrCP1 regulates APB formation in ALT cells

Given that telomere elongation is associated with the formation of APB in U2OS cells, our previous report also showed that the interaction between PML3 and TRF1 is essential for APBs assembly in U2OS cells [25]. We therefore reason that β-TrCP1 may regulate the APB formation in ALT cells. To this end, GFP-TRF1 was co-transfected

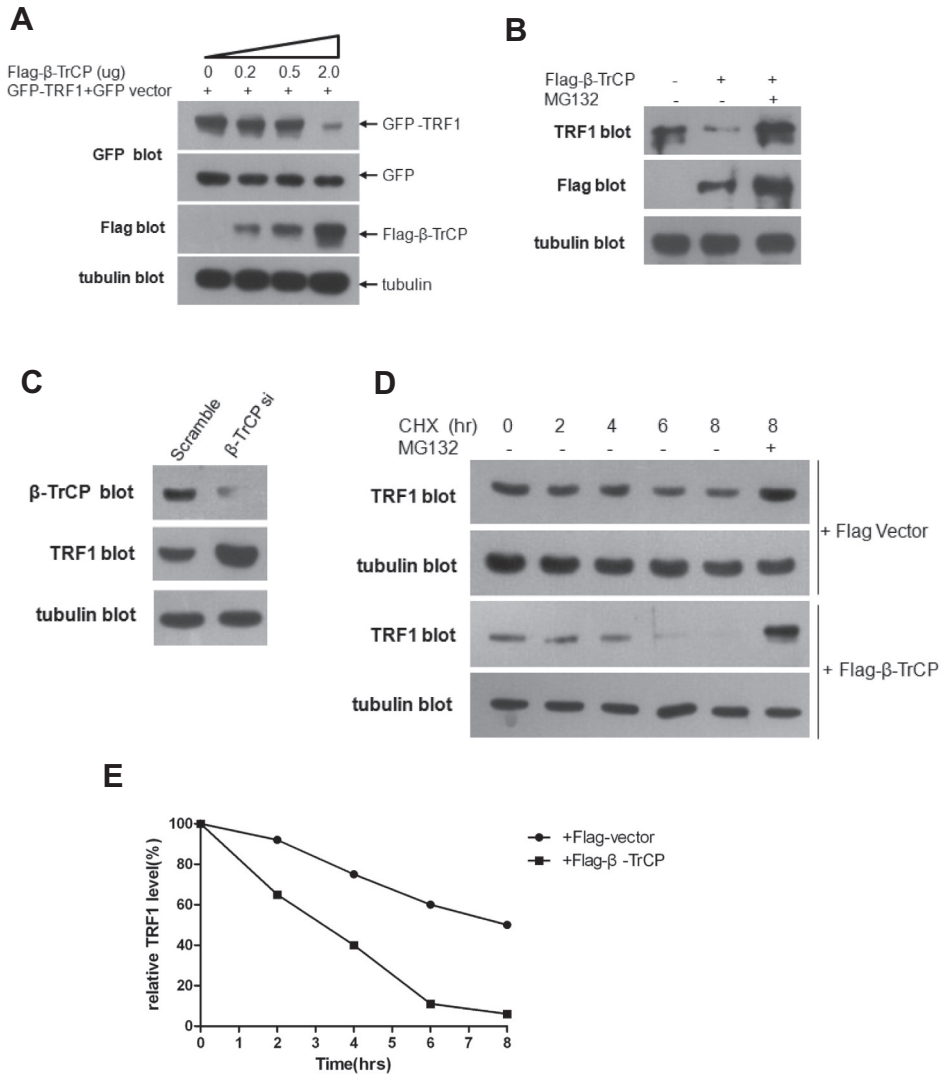

Fig. 3. β-TrCP1 dependent degradation of TRF1. (A) Overexpression of wild-type β-TrCP1 decreases TRF1 protein level in a dose-dependent manner. U2OS cells were co-transfected with GFP-TRF1 and an increasing amount of Flag-β-TrCP1 wild-type plasmids. 24 h after transfection, cells were lysed and subjected to immunoblotting assay with anti-Flag or anti-GFP antibody. Transfection efficiency was monitored by expressing a constant amount of GFP expression plasmid in each sample. Tubulin was used as a loading control. (B) U2OS cells were transfected with Flag-β-TrCP1 plasmid and treated with or without 10 μM MG132 for 8 h. Untreated cells were used as a control. 24 h after transfection, cells were lysed and subjected to immunoblotting assay with anti-Flag or anti-TRF1 antibody. Tubulin was used as a loading control. (C) Inhibition of β-TrCP1 increases TRF1 protein level. U2OS cells were transfected with oligonucleotides (control or siRNA for β-TrCP1). 48 h after transfection, cells were harvested and followed by immunoblotting analysis with anti-β-TrCP1 or anti-TRF1 antibody. Tubulin was used as the loading control. (D) Overexpression of β-TrCP1 reduces the half-life of TRF1. U2OS cells were transfected with p3XFLAG-myc-CMV-24 vector or Flag-β-TrCP1 expression plasmid. 24 h after transfection, cells were treated with cycloheximide (50 mg/ml) and harvested at indicated time points. The expression levels of TRF1 were determined by immunoblotting analysis with anti-TRF1 antibody. Tubulin was used as the loading control. (E) Graphical representation of relative TRF1 levels normalized against the tubulin loading control. The graph represents an average of three independent experiments.

into U2OS cells with Flag-β-TrCP1 or Flag-vector as control. As shown in Fig. 4A, analyses of 120 randomly sampled cells from three independent experiments demonstrated that β-TrCP1 coexpression increases the percentage of APB/PML body from 37.6% to 60.2% ($P < 0.05$). To confirm whether endogenous β-TrCP1 also shows the same activity, we next measured β-TrCP1 knockdown effect on APB formation. As shown in Fig. 4B, the results showed that knockdown β-TrCP1 by siRNA decreases the percentage of APB/PML body from 36.8% to 15.9% ($P < 0.05$). To exclude the possibility that the β-TrCP1-dependent regulation of APB is caused by changes in the protein expression of TRF1, we used TRF2 and PML colocalization to assess the β-TrCP1 effect on APB formation. As shown in Fig. 4C, β-TrCP1 knockdown by siRNA decreases the percentage of APB/PML body from 37.4% to 16.5%, indicating that β-TrCP1 is essential for APBs assembly in U2OS cells.

4. Discussion

Telomeres are essential for genome stability in eukaryotes. Dysfunctions in telomere assembly are associated with chromosomal instability that is implicated in cancer and ageing. TRF1 was known as an interactor which negatively regulates the telomere length by inhibiting the interaction between telomeres and telomerase. Interestingly, TRF1 and its binding proteins such as tankyrase and PinX1 locate to the centrosome at the onset of mitosis [26,27]. In addition, our previous study found that the interaction of PML3 with TRF1 is essential for APBs assembly in U2OS cells [25]. These suggest that the temporal and spatial dynamics of TRF1 is highly regulated, however, the underlying molecular mechanisms are less understood. In this study, we report a novel function of β-TrCP1 in regulating the abundance of TRF1 in U2OS

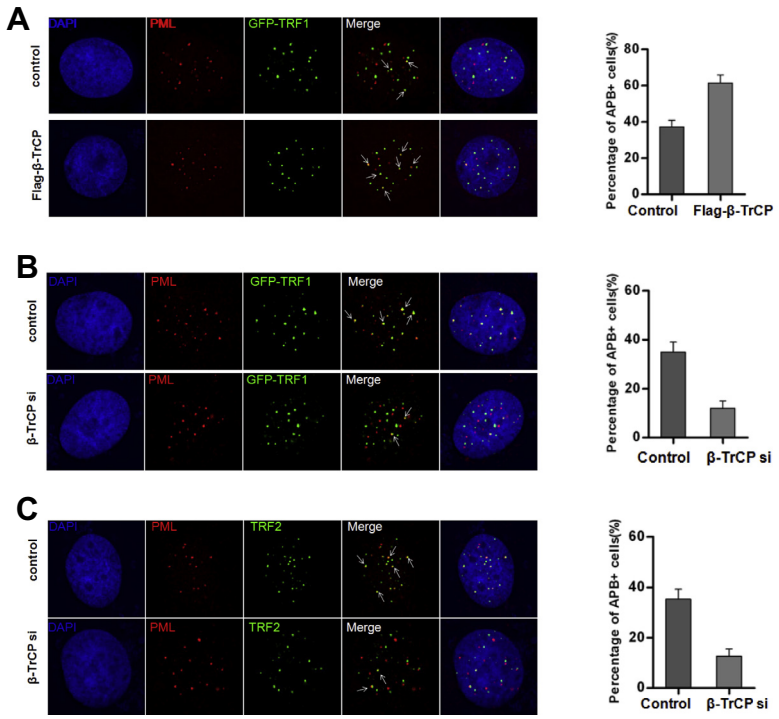

Fig. 4. Negative regulation of APB formation by β-TrCP1 in U2OS cells. (A) Overexpression of β-TrCP1 promotes APBs formation in U2OS cells. Flag-vector (as control) or Flag-β-TrCP1 was co-transfected with GFP-TRF1 into aliquots of U2OS cells respectively. 24 h after transfection, cells were extracted, fixed, and stained with PML antibody. (B and C) Inhibition of β-TrCP1 disrupts APBs formation in U2OS cells. Scramble or β-TrCP1 siRNA were transfected into aliquots of U2OS cells with GFP-TRF1 (B) or without GFP-TRF1 (C). 48 h after transfection, cells were extracted, fixed, and stained with PML antibody (B) or TRF2 antibody and PML antibody (C).

cells. β-TrCP1 interacts with TRF1 in vitro and in vivo and promotes its ubiquitination, thus acting as a negative regulator of TRF1. Whereas overexpression of β-TrCP1 reduces the half-life of endogenous TRF1 protein, depletion of β-TrCP1 results in an increase of endogenous TRF1 expression. Moreover, we found that β-TrCP1 is essential for regulation of PML body recruitment of TRF1 in U2OS cells. These results support the hypothesis that β-TrCP1 plays a critical role in regulating the level of TRF1 and participates in APBs assembly in U2OS cells.

Previous study reported that telomere-unbound TRF1 is degraded by ubiquitin-mediated proteolysis through proteasome pathway [28]. It has been showed that Fbx4 interacts with and targets TRF1 for ubiquitin-mediated degradation by acting as a substrate-specific adaptor of a Cul1-based ubiquitin ligase complex [11,29]. Recently, RLIM was shown to interact with TRF1 and can promote ubiquitination and degradation of TRF1 [9]. These results indicated that ubiquitination of TRF1 may play a critical role in modulating telomere length homeostasis. However, Recent studies showed that TRF1 is required for the assembly of APBs [25,30]. Moreover, it is reported that TRF1 is sumoylated by the MMS21/NSE2 SUMO ligase and such sumoylation of TRF1 is important for APBs formation, indicating that TRF1 is essential for the formation of APBs [31], but the detailed mechanism of APBs formation is unknown. In our present study, we showed another E3 ubiquitin ligase, β-TrCP1, which regulates ubiquitination and proteasome-mediated degradation of TRF1. Interestingly, overexpression of β-TrCP1 increases the percentage of APB/PML body, while inhibition of β-TrCP1 decreases the percentage of APB/PML body. All these findings validate that β-TrCP1 affects PML bodies composition and functions.

Our previous finding that during the formation of APBs, PML3 recruits telomeres to APBs through interacting with TRF1, upon the recruitment of telomeres to PML bodies. In addition, Mdm2 binds PML3 and promotes PML3 nuclear exclusion [32]. While β-TrCP1 controls Mdm2 protein abundance by ubiquitination pathways during cell cycle progression and in response to DNA damage [33,34]. Thus, we speculate that β-TrCP1 may involve in ALT mechanism by disrupting the interaction of PML3–Mdm2 and increasing association of PML3–TRF1 in APB/PML body, but decreasing the protein abundance of telomere-unbound TRF1. However, our hypotheses need to be further verified.

Taken together, our studies demonstrate a critical role of β-TrCP1 in TRF1 regulation and APBs assembly in U2OS cells, and represent a new pathway for APB formation in telomerase-negative cells.

Acknowledgments

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