Chaperone-mediated nuclear translocation of human telomerase and Post-translational regulation of telomere-associated protein, TRF1

Yu Young Jeong

The Graduate School

Yonsei University

Department of Integrated OMICS

for Biomedical Science

Chaperone-mediated nuclear translocation of human telomerase and Post-translational regulation of telomere-associated protein, TRF1

A Dissertation

Submitted to the Department of Integrated OMICS

for Biomedical Sciences

and the Graduate School of Yonsei University

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

Yu Young Jeong

August 2015

This certifies that the dissertation

of Yu Young Jeong is approved

_____

Thesis Supervisor: In Kwon Chung

_____

Jin Won Cho

_____

Kwang Chul Chung

_____

Nam-On Ku

_____

Hyun-Soo Cho

The Graduate School

Yonsei University

August 2015

Acknowledgement

박사과정을 마무리 하면서, 지금의 결실을 맺기까지 부족한 저를 지도해 주시고 이끌어주신 모든 분들께 감사의 인사를 드립니다.

먼저 지도교수님이신 정인권 교수님 감사합니다. 바쁘신 와중에도 연구 및 논문지도에 힘써주셔서 감사 드립니다. 또한 학위논문 심사를 위해 시간을 내어주시고 조언과 축하의 말을 건네주신 조진원 교수님, 정광철 교수님, 구남온 교수님, 조현수 교수님께도 감사 드립니다.

연구에 재미를 붙일 수 있게 도와주시고 지금까지도 초심을 잃지 않도록 잘 가르쳐 주신 김경남 교수님 감사 드립니다. 학부 때 학과공부에 대한 열정을 심어주시고 이끌어주신 모든 분자생물학과 교수님들–황성빈 교수님, 좌남수 교수님, 하정실 교수님, 김양희 교수님, 임하나 교수님께도 감사 드립니다.

석사 졸업 후에도 제 연구에 관심을 가져주시며 충고와 조언을 아낌없이 해 주시는 유지창 교수님께도 감사 드립니다. 또한 박사과정에 들어오면서부터 많은 조언과 관심으로 지금까지 지켜봐 주시고 응원해주신 손영종 교수님 감사 드립니다. 그리고 지금까지 제가 거쳐왔던 세 곳의 연구실에서 함께 공부하고 실험하며 힘이 되어준 모든 연구실 동료들, 선배님, 후배님들 감사합니다. 석사 때부터 지금까지 힘이 되어주시는 박철용 선생님, 수인언니, 선희언니, 특히 친구 류경리에게 감사의 마음을 전합니다. 오랜 시간 추억이 되어준 레지나 동기들–엄진수, 정주완, 염수복, 임세미, 박지희, 그리고 나의 어린시절 친구들–권나영, 이승은에게도 고마운 마음을 전합니다.

학업기간 동안 아낌없는 응원으로 늘 저에게 힘을 실어주시는 사랑하는 가족, 친지들에게도 깊은 감사를 드립니다. 부족한 딸이지만, 늘 믿어주시고 연구에 집중할 수 있도록 격려해 주시는 최고의 부모님, 사랑합니다. 감사합니다.

힘이 들 때면 늘 쉼터가 되어준 오빠네 가족에게도 감사의 말을 전합니다.

앞으로도 영원한 동료이자 조언자로써 함께할 허준영 박사님 감사합니다.

마지막으로 하느님께 감사 드립니다.

2015 년 6 월

정유영

Table of Contents

List of Figure

Chapter I. Hsp90 binding immunophilin FKBP52 modulates telomerase activity by promoting nuclear transport of hTERT

Chapter II. NEDD8 ultimate buster-1(NUB1) promotes the proteasome-mediated proteolysis of TRF1

Chapter III. NEDD8 modification promotes cytosolic localization of TRF1 and regulates the TRF1 stability

Abbreviations

1. hTERT. human telomerase reverse transcriptase
2. TERC, telomerase RNA component
3. hTRF1, human telomere repeat binding factor 1
4. hTRF2, human telomere repeat binding factor 2
5. POT1, protection of telomeres1
6. TIN2, TRF1-interacting nuclear factor 2
7. TPP1, TIN2-interacting protein 1
8. RAP1. TRF2-interacting telomere protein 1
9. SHR, steroid hormone receptor;
10. PPIase, peptidyl prolyl isomerase;
11. TPR, tetratricopeptide repeat
12. Hsp90, heat shock protein 90
13. FKBP, FK506 binding protein
14. TRAP, telomeric repeat amplification protocol
15. NPC, nuclear pore complex
16. NLS, nuclear localization signal
17. NUB1, NEDD8 ultimate buster-1
18. UBL, ubiquitin-like
19. UBA, ubiquitin associated
20. 19S RP, 19S regulatory particle
21. 20S CP, 20S core particle
22. FAT10, HLA-F adjacent transcript 10
23. HLA-F, major histocompatibility complex, class I, F
24. Fbx4, F-box protein 4
25. NEDD8, neural precursor cell expressed developmentally down-regulated 8
26. SUMO, Small Ubiquitin-like Modifier
27. TNKS1, TRF1-interacting ankyrin related ADP-ribose polymerase
28. PARP, poly(ADP-ribose) polymerase

29. CK2, casein kinase 2
30. RLIM, ring finger protein, LIM domain interacting
31. HR, homologous recombination;
32. ALT, alternative lengthening of telomeres
33. CRLs, cullin–RING ligases
34. WT, wild type
35. PD, PARP dead
36. GAPDH, glyceraldehydes 3-phosphate dehydrogenase
37. CHX, cycloheximide
38. FBS, fetal bovine serum
39. DMEM, Dulbecco's Modified Eagle Medium
40. NP-40, nonidet P-40
41. BSA, bovine serum albumin
42. PIC, protease inhibitor cocktail
43. PCR, polymerase chain reaction
44. DAPI, 4,6-diamidino-2-phenylindole
45. siRNA, small interfering RNA

Summary

Telomeres are specialized nucleoprotein complexes found at the end of linear eukaryotic chromosomes required for genome integrity (Smogorzewska and de Lange, 2004). Mammalian telomeric DNA is composed of tandem TTAGGG repeats bound by a series of associated proteins which are required for the protection and maintenance of telomeres from being recognized as double-strand DNA breaks (de Lange, 2002; Griffith et al., 1999). Most human normal somatic cells show a progressive loss of telomeric DNA during successive rounds of cell division due to a DNA end-replication problem (Cerone et al., 2005). Telomere homeostasis is regulated by telomerase and the six-subunit protein complex named shelterin, which consists of TRF1, TRF2, POT1, TIN2, TPP1, and Rap1 (Chong et al., 1995; Liu et al., 2004). The shelterin complex plays an essential role in maintaining the integrity of telomere length and structure.

Because the enzymatic activity of telomerase correlates with hTERT expression, hTERT is believed to be a key component of the telomerase holoenzyme. Several proteins have been identified to be involved in nuclear transport of hTERT. The molecular chaperones Hsp90 and p23 have been shown to interact with hTERT (Forsythe et al., 2001; Holt et al., 1999), and these interactions are required to maintain hTERT in a conformation enabling nuclear translocation. Although the nuclear localization of hTERT is an important step in telomerase assembly, the precise molecular mechanism underlying nuclear transport of hTERT remains unresolved. In the first chapter, we demonstrate that FKBP52-dependent linkage between hTERT-Hsp90 and dynein motor is required for efficient nuclear transport of hTERT. Our finding provides a new route for modulating telomerase activity by regulating its nuclear import efficiency in human cancer cells.

Among the shelterin subunits, TRF1 negatively regulates the telomere length by restricting telomerase access to the telomeres. The overexpression of TRF1 promotes telomere shortening, whereas the loss of TRF1 from the telomeres has been shown to induce abnormal telomere elongation (Smogorzewska et al., 2000; van Steensel and de Lange, 1997), suggesting that an abundance of TRF1 at the telomeres should be tightly controlled for maintaining telomere length. Therefore, the cellular level of TRF1 at the

telomeres has been shown to be tightly regulated. TRF1 is subject to extensive post-translational modification (Walker and Zhu, 2012) and it is evident that the abundance of TRF1 has been shown to be regulated through interactions with various proteins. Recent studies have revealed the existence of numerous modifying enzymes, such as tankyrase 1, Fbx4, RLIM, CK2, Pin1, and Plk1, which contribute to the regulation of stability, binding activity, and localization of TRF1 (Walker and Zhu, 2012).

In the second chapter, we describe a novel function of NUB1 for regulating the abundance of TRF1 in mammalian cells. We identified NEDD8 ultimate buster-1 (NUB1) as a negative regulator of TRF1 by directly interacting with TRF1. The overexpression of NUB1 significantly decreased the levels of TRF1 and also reduced the half-life of TRF1, whereas the depletion of NUB1 by the RNA interference stabilized the endogenous TRF1. These findings demonstrate that NUB1 plays a critical role in regulating the level of TRF1 at the telomeres. We also identified the novel post-translational modification of TRF1 (i.e., neddylation). We found for the first time that TRF1 is a target for NEDD8-conjugation *in vitro* and *in vivo* and the treatment of MLN4924, which is an NAE inhibitor, efficiently reduces the neddylation of TRF1. We also identified that NEDP1, which is NEDD8-specific protease, directly interacts with TRF1 *in vitro* and clearly abolished the neddylation of TRF1 *in vitro* and *in vivo*. The present findings suggest that novel post-translational modification of TRF1, called neddylation for controlling the stability and subcellular localization, provides a potential mechanism for telomere maintenance.

Chapter I

Hsp90 binding immunophilin FKBP52 modulates telomerase activity by promoting nuclear transport of hTERT

1. Abstract

Telomerase is a unique ribonucleoprotein enzyme that is required for continued cell proliferation. To generate catalytically active telomerase, hTERT should be transported to the nucleus and assembled with the telomerase RNA component. The molecular chaperones Hsp90 and p23 have been shown to maintain hTERT in a conformation enabling nuclear translocation. However, the regulatory role of chaperones in nuclear transport of hTERT remains unclear. In this work, we demonstrate that immunophilin FKBP52 links the hTERT-Hsp90 complex to dynein/dynactin motor, thereby promoting the cytoplasmic transport of hTERT to the nucleus along microtubule tracks. FKBP52 interacts with the hTERT-Hsp90 complex through the TPR domain binding to Hsp90 as well as with the dynamitin component of the dynein-associated dynactin complex through the PPIase domain. Depletion of FKBP52 inhibits nuclear transport of hTERT and results in a cytoplasmic accumulation. The resulting cytoplasmic hTERT is rapidly degraded through ubiquitin-dependent proteolysis, thereby abrogating telomerase activity. In addition, overexpression of dynamitin, which is known to dissociate dynein/dynactin motor from its cargoes, reduced telomerase activity. These results provide a molecular mechanism by which FKBP52 modulates telomerase activity by regulating nuclear transport of hTERT.

Keywords: Telomerase, hTERT, Nuclear transport, FKBP52, Hsp90, Dynein/dynactin motor

2. Introduction

Telomeres, the specialized nucleoprotein complexes found at the ends of linear chromosomes, are essential for chromosome integrity [1,2]. Properly capped telomeres protect chromosome termini from being recognized as DNA damage sites [3]. The maintenance of functional telomeres requires telomerase, a unique ribonucleoprotein enzyme that contains the telomerase reverse transcriptase (TERT), the telomerase RNA component (TERC) and several additional proteins for assembly and activity [4,5]. Telomerase adds telomere repeats to the chromosome ends by reverse transcription. In humans, telomerase activity is up-regulated in a majority of cancer cells but repressed in most normal somatic cells, indicating that activation of telomerase is required for tumor growth and survival [6].

Because the enzymatic activity of telomerase correlates with hTERT expression, hTERT is believed to be a key component of the telomerase holoenzyme [7,8]. To generate catalytically active telomerase, hTERT should enter the nucleus through the nuclear pore complex (NPC) [9]. Several proteins have been identified to be involved in nuclear transport of hTERT. The molecular chaperones Hsp90 and p23 have been shown to interact with hTERT, and these interactions are required to maintain hTERT in a conformation enabling nuclear translocation [10,11]. On the other hand, binding of CHIP and Hsp70 to hTERT causes dissociation of p23 from hTERT, resulting in a failure of nuclear import. The resulting cytoplasmic hTERT is ubiquitinated by CHIP and subsequently degraded by the proteasome [12]. Importantly, the association of hTERT with CHIP appears to be cell cycle-dependent. CHIP-mediated hTERT degradation is not activated in S phase during which telomerase acts on telomeres [12]. Although the nuclear localization of hTERT is an important step in telomerase assembly, the precise molecular mechanism underlying nuclear transport of hTERT remains unresolved.

Steroid hormone receptors (SHRs) and some other transcription factors have been shown to interact with Hsp90 in the cytoplasm [13]. The association of FKBP52 with cargo-Hsp90 heterocomplexes is required for their nuclear transport [14]. FKBP52 consists of a

N-terminal FK1 domain that contains peptidyl prolyl isomerase (PPIase) activity, a middle FK2 domain that is structurally similar to FK1 but lacks PPIase activity and a C-terminal tetratricopeptide repeat (TPR) domain that confers Hsp90 binding ability [15]. FKBP52 has also been shown to co-immunoprecipitate the dynein-dynactin complex, suggesting that the motor proteins are involved in nuclear transport of the Hsp90-binding cargo proteins [16-18]. Because Hsp90 binding to hTERT is essential for its nuclear import as well as assembly of the telomerase holoenzyme [10,11], we hypothesized that FKBP52 could play a potential role in nuclear transport of hTERT in a fashion similar to SHRs and other transcription factors.

In this work, we analyzed whether FKBP52 modulates nuclear-cytoplasmic shuttling of hTERT. We demonstrate that FKBP52 associates with the hTERT-Hsp90 complex through the TPR domain, which is responsible for Hsp90 binding. FKBP52 also links this complex to cytoplasmic dynein motor through the interaction with dynamitin via the PPIase domain. Depletion of FKBP52 inhibits nuclear transport of hTERT and results in a cytoplasmic accumulation, thereby abrogating telomerase activity. In addition, overexpression of dynamitin, which is known to dissociate dynein motor from its cargoes [18], impairs nuclear localization of hTERT. These results suggest that FKBP52-dependent linkage between hTERT-Hsp90 and dynein motor is required for efficient nuclear transport of hTERT.

3. Materials and Methods

3.1 Yeast two hybrid screening

Yeast two-hybrid screening was performed as described [19]. Briefly, the yeast strain EGY48 harboring pLexA-hTERT (amino acids 946–1132) and pSH18-34 was transformed by the lithium acetate method with a HeLa cDNA library fused to the activation domain vector pB42AD (Clontech).

3.2 Cell culture and plasmids

The human lung carcinoma cell line H1299 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100μg/ml streptomycin in 5% CO2 at 37℃. The expression vectors for Myc-FKBP52, Myc-FKBP51 and Myc-dynamitin were constructed by inserting the full-length cDNAs into pRK5-Myc (Stratagene). The GST-FKBP52 expression vector was constructed by inserting the full-length cDNA into pGEX-4T-2, and the GST fusion proteins were purified according to the manufacturer's instructions (Amersham Biosciences). The Flag-hTERT expression vector has been described previously [20].

3.3 GST pulldown, immunoprecipitation and immunoblotting

The expression vectors were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 24 h followed by lysis. GST pulldown, immunoprecipitation and immunoblotting were performed as described previously [19]. Immunoprecipitation and immunoblotting were performed using anti-Flag (Sigma, St. Louis, MO), anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), anti-hTERT (ROCKLAND), anti-Hsp56 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FKBP51 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Hsp90 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Dynein IC (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies as specified. All the immunoblots are representatives of at least three experiments that demonstrated the similar results.

3.4 RNA interference

The siRNA duplexes were transfected into H1299 cells using RNAiMax transfection reagent (Invitrogen, Carlsbad, CA). The siRNA target sequences specific for FKBP52 were 5'-AAGTTCCATGCTTGAGCAATA-3' for siFKBP52-1 and 5'-AACTGTAACAAGGCCCTAGA A-3' for siFKBP52-2. The siRNA target sequences specific for FKBP51 was 5'-ACCTAATGCTGAGCTTATA-3'. The control siRNA sequence (5'-AATTCTCCGAACGTGT CACGT-3') was used as a control (Qiagen) and did not correspond to any known gene in the data bases.

3.5 Immunofluorescence and confocal microscopy

H1299 cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. Cells were then blocked in PBS containing 5% bovine serum albumin. DNA was stained with 4,6-diamino-2-phenylindole (DAPI) (Vectashield; Vector Laboratories, Burlingame, CA). The subcellular distribution of GFP-hTERT was monitored by confocal microscopy using a confocal laser-scanning microscope (LSM-700 Image, Carl Zeiss, Jena, Germany). Images were analyzed using the LSM-700 software.

3.6 Telomerase assay

The telomeric repeat amplification protocol (TRAP) was used as previously described [21].

3.7 *In vivo* ubiquitination assay

H1299 cells were co-transfected with Flag-hTERT and HA-ubiquitin and treated with 10 μM MG132 to inhibit proteasome function. Lysates were subjected to immunoprecipitation with anti-Flag M2 affinity resins (Sigma), followed by immunoblotting with anti-HA antibody to illuminate ubiquitin-conjugated hTERT.

4. Results

4.1 FKBP52 affects the nuclear localization of hTERT and telomerase activity

Hsp90-binding immunophilin FKBP52 has been shown to form complexes with steroid hormone receptors and some other transcription factors such as p53 and NF-κB and act as a positive regulator in their nuclear localization [14,22,23]. To extend this idea to other nuclear factors that undergo nuclear-cytoplasmic shuttling, we analyzed whether FKBP52 modulates the nuclear translocation of hTERT. The expression of endogenous FKBP52 was depleted using two different small interfering RNA (siRNA) duplexes (Fig. 1A). H1299 cells expressing GFP-hTERT were subjected to indirect immunofluorescence staining to monitor the subcellular localization of hTERT. Representative fluorescence images of GFP-hTERT are shown in Fig. 1B. Whereas GFP-hTERT was predominantly localized to the nucleus in the control siRNA cells, depletion of FKBP52 led to a significant decrease in total GFP-hTERT fluorescence intensity (Fig. 1B). However, the MG132 treatment rescued FKBP52 depletion-dependent reduction in GFP-hTERT fluorescence and resulted in a cytoplasmic accumulation of GFP-hTERT (Fig. 1B and C). These results suggest that, when FKBP52 was depleted, hTERT fails to enter the nucleus and is subsequently degraded by the proteasome. We next determined the effect of FKBP52 depletion on telomerase activity. Depletion of FKBP52 resulted in a significant reduction in telomerase activity compared to the control siRNA cells (Fig. 1D). To examine whether reduction in telomerase activity is due to a FKBP52 depletion-related decrease in the transcription levels of hTERT and TERC, the effect of FKBP52 depletion on gene expression of hTERT and TERC was evaluated using reverse transcription-PCR analysis. No significant difference was observed in the steady-state levels of hTERT mRNA and TERC transcript between FKBP52 knockdown cells and the control cells (Fig. 1E).

Because FKBP51 has been shown to function as a negative regulator of steroid hormone receptor activity [15], we examined whether depletion of FKBP51 influences the nuclear localization of hTERT. The expression of endogenous FKBP51 was depleted using

siRNA duplex (Fig. 2A). Whereas depletion of FKBP52 inhibits the nuclear translocation of hTERT, the majority of GFP-hTERT signal was detected in the nucleus even when FKBP51 was depleted (Fig. 2B and C). Depletion of endogenous p23, which inhibits nuclear localization of hTERT, was used as a control [12]. We further characterized the effect of FKBP51 depletion on telomerase activity and found that telomerase activity was not affected by FKBP51 depletion (Fig. 2D). Taken together, these results demonstrate that FKBP52, but not FKBP51, is required for nuclear localization of hTERT and telomerase activity.

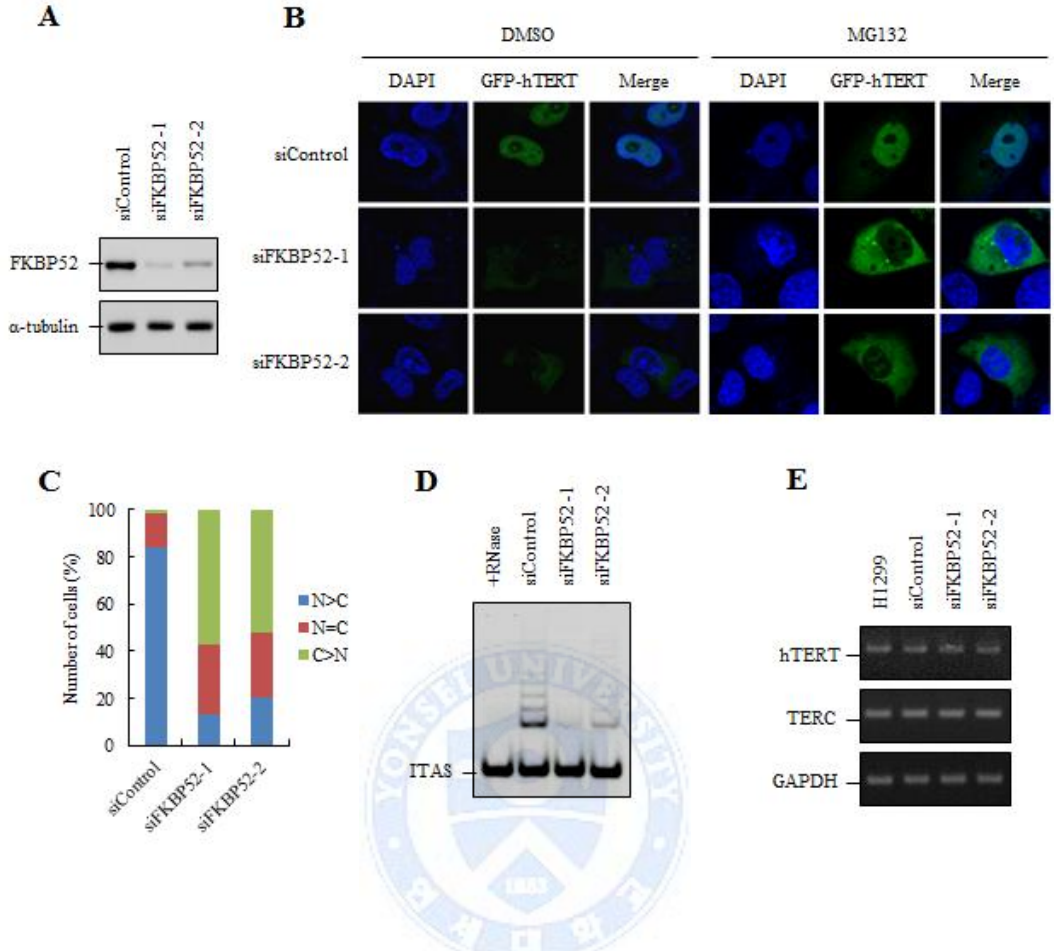

Figure 1. Depletion of FKBP52 inhibits nuclear transport of hTERT and reduces telomerase activity. A) H1299 cells were transfected with control siRNA (siControl) or FKBP52 siRNAs (siFKBP52-1 and siFKBP52-2). The protein level of endogenous FKBP52 was measured by immunoblotting as indicated. B) H1299 cells transfected with GFP-hTERT and FKBP52 siRNAs were treated with or without 10 μM MG132 for 2 hr and subjected to immunofluorescence staining to monitor the subcellular localization of GFP-hTERT. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue). C) After transfection, H1299 cells were treated with 10 μM MG132 for 2 hr and subjected to quantitative analysis of the location of GFP-hTERT within the cells. In each experiment, more than 100 GFP-hTERT-expressing cells were evaluated quantitatively for nuclear (N, blue bars), nuclear-cytoplasmic (N+C, red bars), and cytoplasmic (C, green bars) fluorescence. D) H1299 cells transfected with control siRNA or FKBP52 siRNAs were analyzed for telomerase activity by the TRAP assay. To test RNA-dependent extension, RNase A (0.25 mg/ml) was added to the extracts before the primer extension reaction. ITAS represents the internal telomerase assay standard. E) H1299 cells transfected with control siRNA or FKBP52 siRNAs were subjected to reverse transcription-PCR analysis for the expression of hTERT and TERC genes.

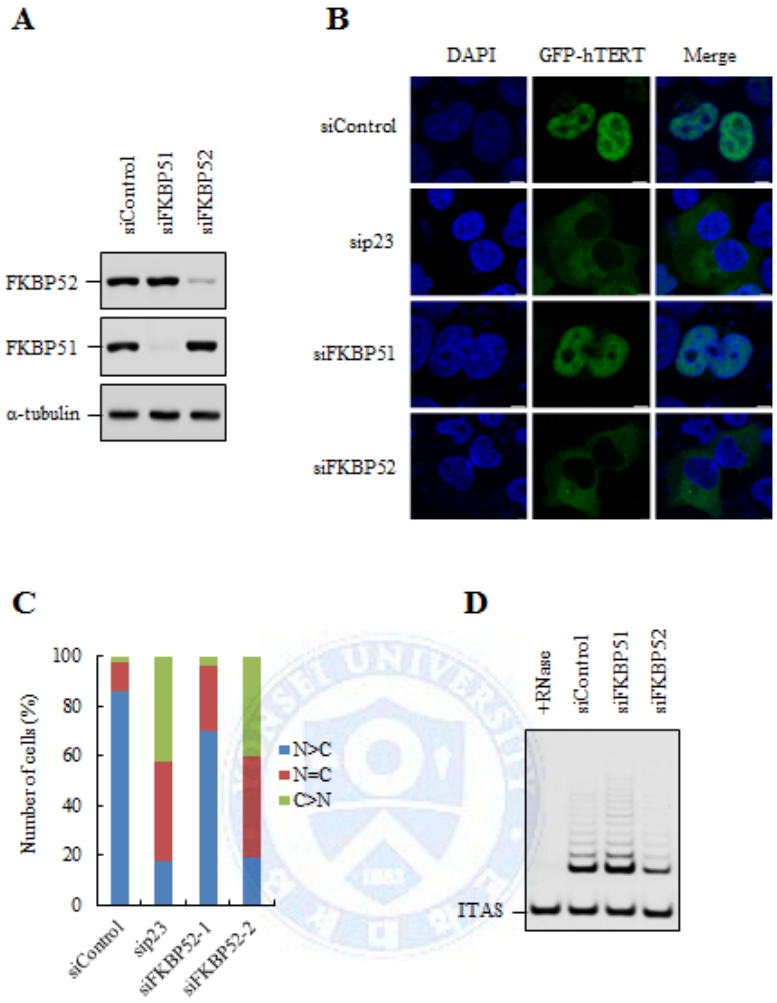

Figure. 2. Depletion of FKBP51 does not affect the nuclear localization of hTERT and telomerase activity. A) H1299 cells were transfected with FKBP51 siRNA or siFKBP52 siRNA. The protein levels of endogenous FKBP51 and FKBP52 were measured by immunoblotting as indicated. B) H1299 cells transfected with GFP-hTERT and p23 siRNA or FKBP51 siRNA or FKBP52 siRNA were subjected to immunofluorescence staining to monitor the subcellular localization of GFP-hTERT. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue). C) After transfection, H1299 cells were subjected to quantitative analysis of the location of GFP-hTERT within the cells. In each experiment, more than 100 GFP-hTERT-expressing cells were evaluated quantitatively for nuclear (N, blue bars), nuclear-cytoplasmic (N+C, red bars), and cytoplasmic (C, green bars) fluorescence. D) H1299 cells transfected with FKBP51 siRNA or FKBP52 siRNA were analyzed for telomerase activity by the TRAP assay. To test RNA-dependent extension, RNase A (0.25 mg/ml) was added to the extracts before the primer extension reaction. ITAS represents the internal telomerase assay standard.

4.2 FKBP52 associates with hTERT

To investigate the molecular mechanism in which FKBP52 affects the nuclear localization of hTERT, we examined the association between hTERT and FKBP52 by the GST pulldown assay. GST-FKBP52, but not the control GST, precipitated Flag-hTERT expressed in H1299 cells, indicating that hTERT associates with FKBP52 in vitro (Fig. 3A). To determine whether hTERT and FKBP52 associate in vivo, H1299 cells were co-transfected with Flag-hTERT and Myc-FKBP52 expression vectors and subjected to immunoprecipitation. Myc-FKBP52 was detected in anti-Flag immunoprecipitates when Flag-hTERT was expressed (Fig. 3B). Likewise, Flag-hTERT was recovered in anti-Myc immunopecipitates when Myc-FKBP52 was expressed, indicating that hTERT associates with FKBP52 in mammalian cells.

Because the molecular chaperones Hsp90 and p23 have been shown to associate with hTERT for the assembly of active telomerase [10,11], we performed the GST pulldown assay to determine whether the hTERT-FKBP52 complex contains molecular chaperones. As shown in Fig. 3C, the pulldown of GST-FKBP52, but not the GST control, yielded the specific co-precipitation of endogenous hTERT, Hsp90 and p23, suggesting the in vivo existence of the hTERT-Hsp90-p23-FKBP52 heterocomplex. We also found that the intermediate chain of cytoplasmic dynein was precipitated by GST-FKBP52 (Fig. 3C). This observation suggests that dynein motor proteins could be involved in nuclear transport of hTERT in a FKBP52-dependent manner (see below). To determine the domain in hTERT that is responsible for the interaction with FKBP52, we generated a series of deletion fragments of TERT (Fig. 3D) and assessed binding of FKBP52 using the yeast two-hybrid system. We found that FKBP52 interacts with the hTERT fragments containing amino acid residues 595-946 (Fig. 3E).

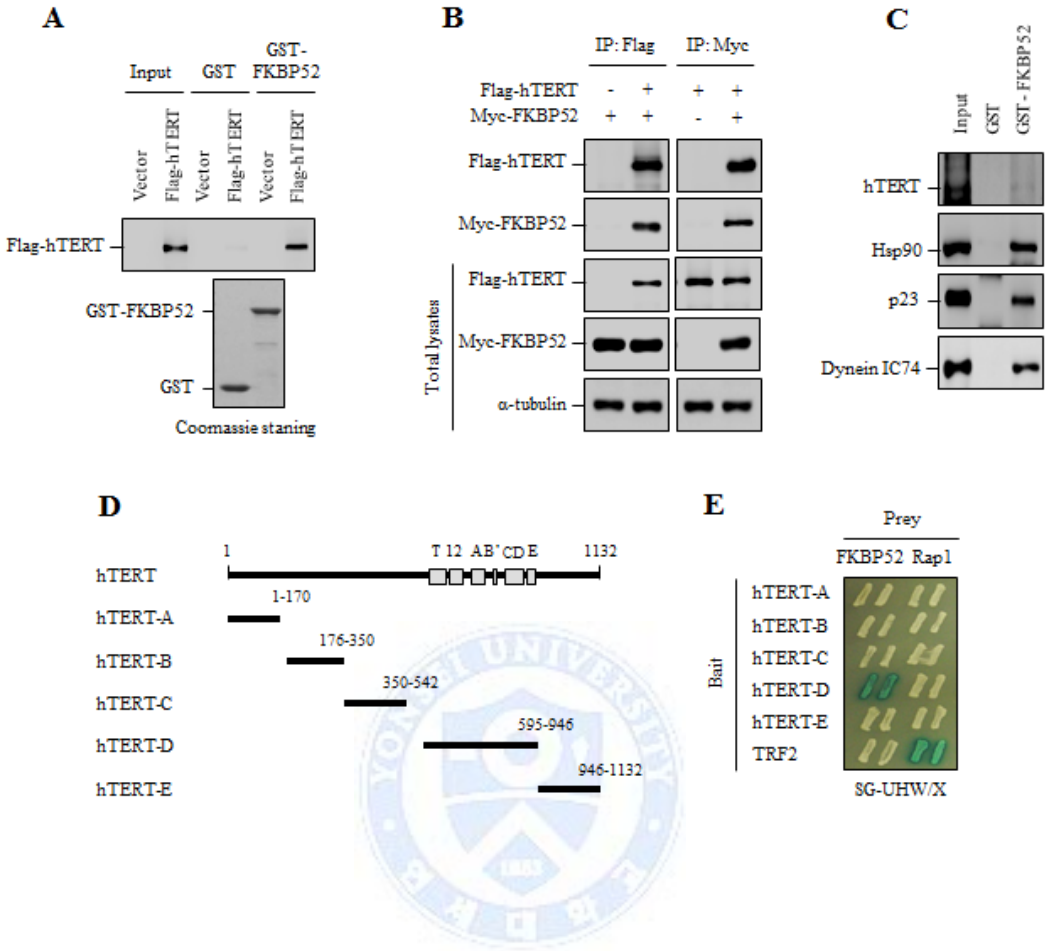

Figure. 3. FKBP52 interacts with hTERT. A) GST or GST-FKBP52 were immobilized on glutathione-Sepharose and incubated with ectopically expressed Flag-hTERT. Bound proteins were detected by immunoblotting with anti-Flag antibody. The purified GST fusion proteins were visualized by Coomassie staining. B) H1299 cells were co-transfected with Flag-hTERT and Myc-FKBP52 and subjected to immunoprecipitation with anti-Flag or anti-Myc antibodies, followed by immunoblotting to detect the precipitated proteins as indicated. C) GST or GST-FKBP52 were immobilized on glutathione-Sepharose and incubated with lysates from H1299 cells. Bound proteins were detected by immunoblotting with anti-hTERT, anti-Hsp90, anti0p23 and anti-dynein IC antibodies. D) Schematic representation of the hTERT domains involved in FKBP52 binding. E) Analysis of the physical interaction between hTERT and FKBP52 using the yeast two-hybrid assay. The blue signal on the SG-HWU/X plate indicates activation of the reporter gene. S, synthetic; G, galactose; H, histidine (-); W, tryptophan (-); U, uracil (-);X, X-Gal. Rap1 was used as the TRF2-binding control.

4.3. The interaction between hTERT and FKBP52 requires the presence of Hsp90

Although hTERT and FKBP52 are each able to interact with Hsp90, it is yet unclear whether FKBP52 alone is sufficient to interact with hTERT in the absence of Hsp90 binding. To address this issue, we generated FKBP52 deletion constructs lacking TPR domain or PPIase domain and determined the region in FKBP52 that is responsible for hTERT binding (Fig. 4A). When H1299 cells were co-transfected with Flag-hTERT and various Myc-FKBP52 fragments, Flag-hTERT was immunoprecipitated only by FKBP52 fragments containing the TPR domain, indicating that the TPR domain is responsible for hTERT binding (Fig. 4B). The TPR domain was also shown to be required for the interaction with Hsp90 (Fig. 4B). To verify this finding, we used FKBP52 mutant in which the lysine residue at 354 was replaced by arginine (K354A). The K354A mutation was shown to disrupt the interaction of FKBP52 with Hsp90 [24]. Co-immunoprecipitation experiments revealed that the K354A mutation severely impairs the interaction between hTERT and FKBP52 (Fig. 4C), suggesting that the interaction between hTERT and FKBP52 requires the presence of Hsp90. To further analyze the dependence of Hsp90 on hTERT transport to the nucleus, H1299 cells expressing GFP-hTERT were preincubated with geldanamycin (GA) to inhibit Hsp90 and subjected to indirect immunofluorescence staining. Under normal condition, total GFP-hTERT fluorescence intensity was reduced by the GA treatment compared to the untreated control cells (Fig. 4D). However, GA-dependent reduction in GFP-hTERT fluorescence was rescued by the presence of MG132, and the majority of GFP-hTERT signals was detected in the cytoplasm (Fig. 4D). These findings imply that Hsp90 is required for rapid and efficient nuclear transport of hTERT.

It was reported in previous works that, when nuclear import is inhibited, hTERT is accumulated in the cytoplasm, rapidly ubiquitinated and subsequently degraded by the proteasome [9,12]. Because FKBP52 is required for efficient nuclear transport of hTERT (Fig. 1B and C), we investigated the involvement of FKBP52 depletion in hTERT degradation. Depletion of FKBP52 led to a clear reduction in the level of Flag-hTERT (Fig. 4E). This reduction in Flag-hTERT was rescued by the MG132 treatment. To

determine whether hTERT is ubiquitinated before its proteasome-dependent degradation, H1299 cells were co-transfected with Flag-hTERT and HA-ubiquitin. To illuminate ubiquitin-modified hTERT, anti-Flag immunoprecipitates were evaluated by immunoblotting with anti HA-antibody. Whereas ubiquitinated hTERT was detected in the control siRNA cells in the presence of MG132, depletion of FKBP52 led to a marked increase in the level of ubiquitinated hTERT (Fig. 4F). Taken together, these results suggest that nuclear import of hTERT is inhibited by FKBP52 depletion and that the resulting cytoplasmic hTERT is ubiquitinated prior to proteasome-dependent degradation.

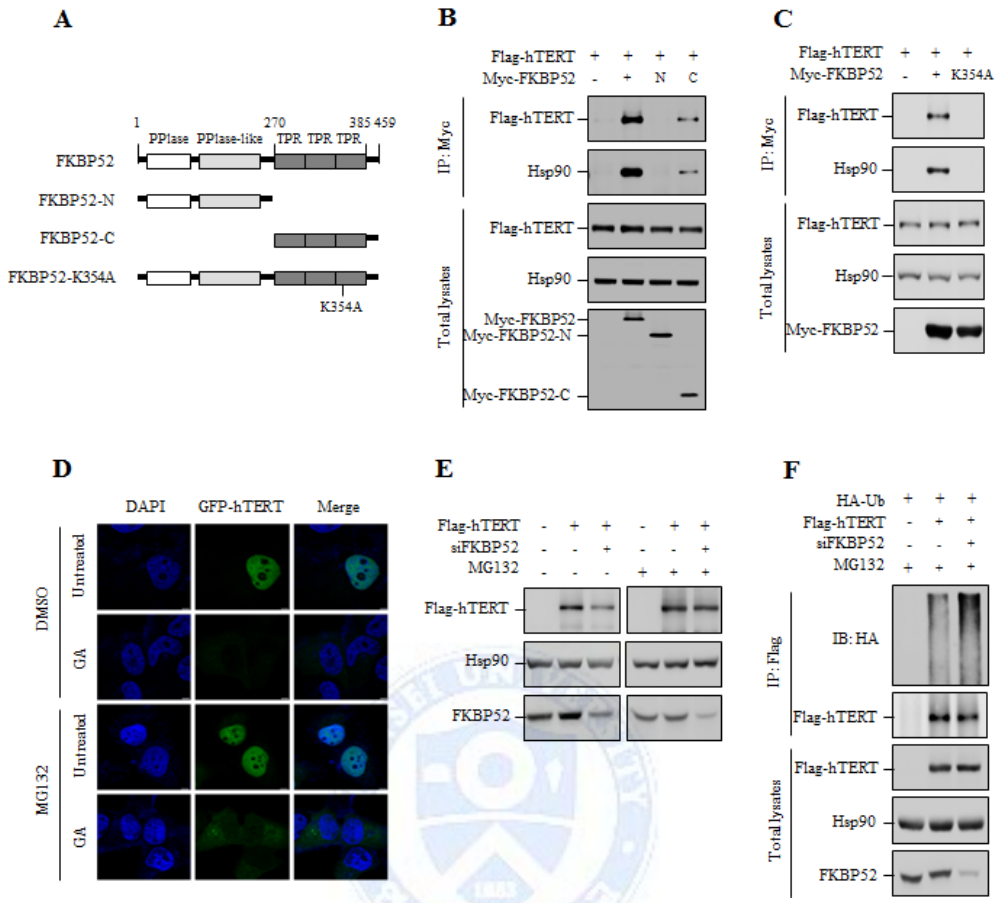

Figure 4. The interaction between hTERT and FKBP52 requires the presence of Hsp90. A) Schematic representation of the FKBP52 domains involved in hTERT binding. B) H1299 cells were co-transfected with Flag-hTERT and various truncated Myc-FKBP52 and subjected to immunoprecipitation with anti-Myc antibody, followed by immunoblotting to detect Flag-hTERT and Hsp90. C) H1299 cells were co-transfected with Flag-hTERT and either Myc-FKBP52 or Myc-FKBP52-K354A and subjected to immunoprecipitation with anti-Myc antibody, followed by immunoblotting to detect Flag-hTERT and Hsp90. D) H1299 cells transfected with GFP-hTERT were treated with or without 10 μM MG132 and 1 μM geldanamycine for 2 hr and subjected to immunofluorescence staining to monitor the subcellular localization of GFP-hTERT. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue). E) H1299 cells co-transfected with Flag-hTERT and FKBP52 siRNA were treated with or without 10 μM MG132 for 2 hr and subjected to immunoblotting to detect Flag-hTERT, Hsp90 and FKBP52. F) H1299 cells were co-transfected with HA-ubiquitin (HA-Ub), Flag-hTERT and FKBP52 siRNA and treated with or without 10 μM MG132 for 2 hr. Immunoprecipitation was carried out with anti-Flag antibody before probing with anti-HA antibody.

4.4 FKBP52 links hTERT to the dynein/dynactin motor complex

It has been well documented that the cargo-Hsp90 heterocomplex associates with dynein motors, suggesting that the motor proteins are involved in the movement of cargoes to the nucleus on microtubule tracts [17,18]. FKBP52 interacts with cargo-bound Hsp90 through its TPR domain as well as with the dynamitin component of the dynein-associated dynactin complex through its PPIase domain [22]. We asked whether nuclear transport of hTERT is regulated by the dynein motor complex in a similar pattern as other cargoes. Since FKBP52 linkage to dynein is indirect through the dynamitin component, H1299 cells were co-transfetcted with Flag-hTERT and Myc-dynamitin and subjected to immunoprecipitation. Myc-dynamitin was detected in anti-Flag immunoprecipitates when Flag-hTERT was expressed (Fig. 5A). Myc-dynamitin associates with Flag-hTERT at a reduced level when FKBP52 was depleted (Fig. 5A), suggesting that the association of hTERT with dynamitin is mediated by FKBP52. These results demonstrate that FKBP52 acts as the molecular linker between hTERT and the dynein motor complex.

Overexpression of dynamitin has been shown to impair nuclear accumulation of the cargo proteins by inhibiting cytoplasmic dynein function [25]. To characterize the effect of dynamitin overexpression on nuclear transport of hTERT, H1299 cells were co-transfected with GFP-hTERT and Myc-dynamitin and subjected to indirect immunofluorescence staining to determine subcellular localization of GFP-hTERT. Whereas GFP-hTERT was predominantly localized to the nucleus in the control cells, overexpression of Myc-dynamitin resulted in a clear cytoplasmic accumulation of GFP-hTERT (Fig. 5B and C). We also measured telomerase activity by the TRAP assay. Overexpression of Myc-dymanitin decreased telomerase activity in a dose-dependent manner (Fig. 5D). These results suggest that dymanitin overexpression-dependent reduction in telomerase activity could be due to the decreased nuclear transport of hTERT.

If the hTERT-Hsp90-p23-FKBP52-dynein motor complex is transported to the nucleus along microtubules, we would expect that treatment of cells with microtubule disrupting drug colchicine inhibits nuclear accumulation of hTERT. To address this possibility, H1299 cells were treated with colchicine and examined the subcellular localization of

hTERT by indirect immunofluorescence staining. Pretreatment with colchicine resulted in the suppression of the hTERT transport to the nucleus (Fig. 5E and F), demonstrating that nuclear transport of hTERT requires cytoplasmic dynein and a functional microtubule network.

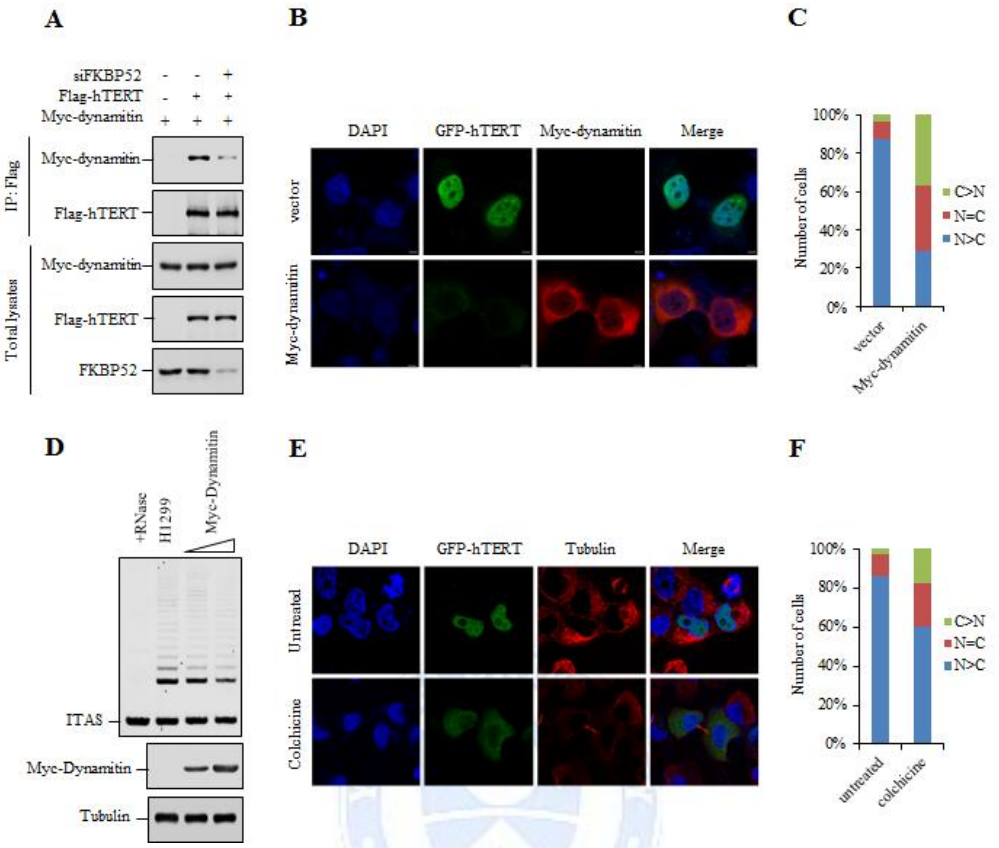

Figure. 5. FKBP52 links hTERT to the dynein/dynactin motor complex. A) H1299 cells were co-transfected with Flag-hTERT, Myc-dynamitin and FKBP52 siRNA and subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting to detect Myc-dynamitin and Flag-hTERT. B) H1299 cells transfected with GFP-hTERT and Myc-dynamitin were subjected to immunofluorescence staining to monitor the subcellular localization of GFP-hTERT. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue). C) After transfection, H1299 cells were subjected to quantitative analysis of the location of GFP-hTERT within the cells. In each experiment, more than 100 GFP-hTERT-expressing cells were evaluated quantitatively for nuclear (N, blue bars), nuclear-cytoplasmic (N+C, red bars), and cytoplasmic (C, green bars) fluorescence. D) H1299 cells transfected with increasing amount of Myc-dynamitin were analyzed for telomerase activity by the TRAP assay. To test RNA-dependent extension, RNase A (0.25 mg/ml) was added to the extracts before the primer extension reaction. ITAS represents the internal telomerase assay standard. E) H1299 cells transfected with GFP-hTERT were treated with 1uM of colchicine for 1 hr and subjected to immunofluorescence staining to monitor the subcellular localization of GFP-hTERT. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue). F) After transfection, H1299 cells were subjected to quantitative analysis of the location of GFP-hTERT within the cells. In each experiment, more than 100 GFP-hTERT-expressing cells were evaluated quantitatively for nuclear (N, blue bars), nuclear-cytoplasmic (N+C, red bars), and cytoplasmic (C, green bars) fluorescence.

5. Discussion

For telomere elongation by telomerase, newly synthesized hTERT should be transported to the nucleus and assembled with the telomerase RNA component [9]. Because the size of hTERT (~124 kDa) precludes a nuclear transport mechanism by passive diffusion, it should undergo active nuclear import pathway to set proper telomerase activity [26,27]. Here, we provide compelling evidence for a role of FKBP52 in the nuclear translocation of hTERT. We demonstrate that FKBP52 acts as a molecular linker between hTERT-Hsp90 and the cytoplasmic dynein motor complex, favoring the cytoplasmic transport of hTERT to the nucleus along microtubule tracks. Indeed, depletion of FKBP52 inhibits nuclear import of hTERT, resulting in a cytoplasmic accumulation of hTERT. Cytoplasmic hTERT is degraded through ubiquitin-dependent proteolysis, thereby abrogating telomerase activity. Degradation of cytoplasmic hTERT is efficiently inhibited by the MG132 treatment, suggesting that proteasomal degradation of hTERT occurs after inhibition of nuclear transport. These data provide a molecular mechanism by which FKBP52 modulates telomerase activity.

In this work, we show that FKBP52 interacts with the hTERT-Hsp90 heterocomplex by means of the TPR domain binding to Hsp90 as well as with the dynamitin component of the dynein-associated dynactin complex by means of the PPIase domain. These findings suggest that the interactions of FKBP52 with Hsp90 and dynamitin are not mutually exclusive. Based on our data presented in this work, we propose a model to explain how FKBP52 contributes to hTERT trafficking to the nucleus. In this model, FKBP52 acts as a scaffold to bring hTERT-bound Hsp90 (via its TPR domain) to the cytoplasmic dynein motor complex (via its PPIase domain) (Fig. 6). This suggests that FKBP52 performs a fundamental function in the nuclear translocation of hTERT. Indeed, prevention of this linkage by a TPR domain point mutation of FKBP52 that is unable to interact with Hsp90 inhibits the interaction with hTERT. We also found that overexpression of dynamitin blocks dynein function by dissociating the motor from hTERT and impairs nuclear accumulation of hTERT, resulting in reduced telomerase activity.

It has been recently reported that hTERT contains a bipartite nuclear localization signal (NLS) that is responsible for nuclear import [9]. Several nuclear import receptors such as importin α and importin 7 have been shown to interact with hTERT through the bipartite NLS and mediate nuclear import of hTERT [9,28]. Redundancy in nuclear import pathways is observed for many other cargo substrates like ribosomal proteins and histones [29,30]. Likewise, it is likely that hTERT can be imported to the nucleus by multiple nuclear import pathways. On the other hand, the molecular chaperone network is essential for maintaining a balance between folding and degradation of hTERT [12]. When the Hsp90 and p23 molecular chaperones are assembled on hTERT, the hTERT-Hsp90-p23 complex is linked to cytoplasmic dynein through FKBP52 and is transported towards the nucleus via association with microtubules. The critical question that remains to be answered is how the molecular chaperones modulate protein folding of hTERT for its nuclear transport. Possible explanation is that the association of hTERT with Hsp90 and p23 may cause a conformational change that exposes the bipartite NLS on the protein surface. Upon binding to the molecular chaperones, hTERT is recognized by members of the importin superfamily and translocated to the nucleus via the NPC. In contrast, the binding of CHIP and Hsp70 to the hTERT-Hsp90-p23 complex causes dissociation of p23 from hTERT and may induce a conformational switch that disrupts a linkage between FKBP52 and the hTERT-Hsp90 complex, resulting in a failure of nuclear transport of hTERT. It may also induce a hTERT conformation that is unable to interact with importins. Indeed, Hsp70 associates with hTERT but readily dissociates when telomerase is folded into its active form [12]. Thus, it will be interesting to explore structural and functional roles of multichaperone complexes assembled on hTERT in several intermediate assembly steps during the nuclear translocation process of hTERT.

Although nuclear protein import is dependent on NLS within cargo proteins recognized by importins, microtubules have also been shown to facilitate dynein-mediated nuclear transport of NLS-containing cargo proteins, including p53 [31], retinoblastoma protein [32], parathyroid hormone-related protein [33], NF-κB [34] and dystrophin Dp71d [35]. In a similar manner to other systems, cells treated with microtubule-perturbing drug

colchicine showed reduced movement of hTERT towards the nucleus, implying the microtubule-dependent nuclear transport of hTERT. It has been previously shown that, following microtubule-dependent trafficking towards the nucleus, a cargo can be released from microtubules and recognized by importins prior to nuclear import [36]. As proposed in other NLS-containing cargo proteins, our results suggest that importins may compete with microtubule for hTERT binding. The bipartite NLS of hTERT could be recognized by importins near the nuclear periphery, dissociating hTERT from dynein and subsequently mediating nuclear import through the NPC.

In this study, we identify Hsp90-binding immunophilin FKBP52 as a molecular linker between hTERT-Hsp90 and the dynein motor complex and characterize a mechanism underlying dynein/microtubule-dependent nuclear transport of hTERT. FKBP52 links directly the hTERT-Hsp90 complex to dynamitin, which in turn connects this complex to the dynein motor prior to microtubule-dependent movement towards the nucleus. This nuclear transport model of hTERT provides a new route for modulating telomerase activity by regulating its nuclear import efficiency in human cancer cells.

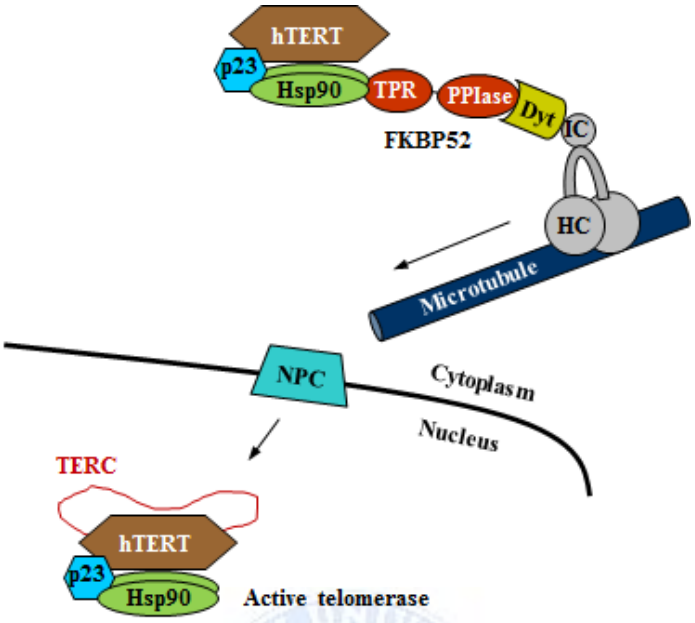

Figure. 6. Model for FKBP52-mediated nuclear transport of hTERT. FKBP52 links the hTERT-hsp90 complex to dynein for cytoplasmic transport of hTERT to the nucleus along microtubule tracks.

6. References

[1] E.H. Blackburn, Switching and signaling at the telomere, Cell 106 (2001) 661-673.

[2] A. Smogorzewska, T. de Lange, Regulation of telomerase by telomeric proteins, Annu. Rev. Biochem. 73 (2004) 177-208.

[3] W. Palm, T. de Lange, How shelterin protects mammalian telomeres, Annu. Rev. Genet. 42 (2008) 301-334.

[4] C. Autexier, N.F. Lue, The structure and function of telomerase reverse transcriptase, Annu. Rev. Biochem. 75 (2006) 493-517.

[5] A. Bianchi, D. Shore, How telomerase reaches its end: mechanism of telomerase regulation by the telomeric complex, Mol. Cell 31 (2008) 153-165.

[6] N.W. Kim, M.A. Piatyszek, K.R. Prowse, C.B. Harley, M.D. West, P.L. Ho, G.M. Coviello, W.E. Wright, S.L. Weinrich, J.W. Shay, Specific association of human telomerase activity with immortal cells and cancer, Science 266 (1994) 2011-2015.

[7] T.M. Nakamura, T.R. Cech, Reversing time: origin of telomerase, Cell 92 (1998) 587-590.

[8] J. Wang, J. Wang, L.Y. Xie, S. Allan, D. Beach, G.J. Hannon, Myc activates telomerase, Genes Dev. 12 (1998) 1769-1774.

[9] J. Chung, P. Khadka, I.K. Chung, Nuclear import of hTERT requires a bipartite nuclear localization signal and Akt-mediated phosphorylation, J. Cell Sci. 125 (2012) 2684-2697.

[10] S.E. Holt, D.L. Aisner, J. Baur, V.M. Tesmer, M. Dy, M. Ouellette, J.B. Trager, G.B. Morin, D.O. Toft, J.W. Shay, W.E. Wright, M.A. White, Functional requirement of p23 and Hsp90 in telomerase complexes, Genes Dev. 13 (1999) 817-826.

[11] H.L. Forsythe, J.L. Jarvis, J.W. Turner, L.W. Elmore, S.E. Holt, Stable association of hsp90 and p23, but Not hsp70, with active human telomerase, J. Biol. Chem. 276 (2001) 15571-15574.

[12] J.H. Lee, P. Khadka, S.H. Baek, I.K. Chung, CHIP promotes human telomerase reverse transcriptase degradation and negatively regulates telomerase activity, J. Biol.

Chem. 285 (2010) 42033-42045.

[13] D.F. Smith, D.O. Toft, Minireview: the intersection of steroid receptors with molecular chaperones: observations and questions, Mol. Endocrinol. 22 (2008) 2229-2240.

[14] J.C. Sivils, C.L. Storer, Mario D Galigniana, M.B. Cox, Regulation of steroid hormone receptor function by the 52-kDa FK506-binding protein (FKBP52), Curr. Opin. Pharmacol. 11 (2011) 314-319.

[15] C.L. Storer, C.A. Dickey, M.D. Galigniana, T. Rein, M.B. Cox, FKBP51 and FKBP52 in signaling and disease, Trends Endocrinol. Metab. 22 (2011) 481-490.

[16] M.D. Galigniana, C. Radanyi, J.M. Renoir, P.R. Housley, W.B. Pratt, Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus, J. Biol. Chem., 276 (2001) 14884-14889.

[17] G.M. Wochnik, J. Rüegg, G.A. Abel, U. Schmidt, F. Holsboer, T. Rein, FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells, J. Biol. Chem. 280 (2005) 4609-4616.

[18] M.D. Galigniana, A.G. Erlejman, M. Monte, C. Gomez-Sanchez, G. Piwien-Pilipuk, The hsp90-FKBP52 complex links the mineralocorticoid receptor to motor proteins and persists bound to the receptor in early nuclear events, Mol. Cell. Biol. 30 (2010) 1285-1298.

[19] G.E. Lee, E.Y. Yu, C.H. Cho, J. Lee, M.T. Muller, I.K. Chung, DNA-protein kinase catalytic subunit-interacting protein KIP binds telomerase by interacting with human telomerase reverse transcriptase, J. Biol. Chem. 279 (2004) 34750-34755.

[20] J.H. Kim, S.M. Park, M.R. Kang, S.Y. Oh, T.H. Lee, M.T. Muller, I.K. Chung, Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT, Genes Dev. 19 (2005) 776-781.

[21] J.H. Kim, J.H. Kim, G.E. Lee, J.E. Lee, I.K. Chung, Potent inhibition of human telomerase by nitrostyrene derivatives, Mol. Pharmacol. 63 (2003)1117-1124.

[22] M.D. Galigniana, J.M. Harrell, H.M. O'Hagen, M. Ljungman, W.B. Pratt, Hsp90-binding immunophilins link p53 to dynein during p53 transport to the nucleus, J. Biol. Chem. 279 (2004) 22483-22489.

[23] A.G. Erlejman, S.A. De Leo, G.I. Mazaira, A.M. Molinari, M.F. Camisay, V. Fontana, M.B. Cox, G. Piwien-Pilipuk, M.D. Galigniana, NF-κB transcriptional activity is modulated by FK506-binding proteins FKBP51 and FKBP52: a role for peptidyl-prolyl isomerase activity. J. Biol. Chem. 289 (2014) 26263-26276.

[24] A.J. Ramsey, L.C. Russell, S.R. Whitt, M. Chinkers, Overlapping sites of tetratricopeptide repeat protein binding and chaperone activity in heat shock protein 90, J. Biol. Chem. 275 (2000) 17857-17862.

[25] K.A. Melkonian, K.C. Maier, J.E. Godfrey, M. Rodgers, T.A. Schroer, Mechanism of dynamitin-mediated disruption of dynactin, J. Biol. Chem. 282 (2007) 19355-19364.

[26] I.K. Poon, D.A. Jans, Regulation of nuclear transport: central role in development and transformation? Traffic 6 (2005) 173-186.

[27] M. Stewart, Molecular mechanism of the nuclear protein import cycle, Nat. Rev. Mol. Cell. Biol. 8 (2007) 195-208.

[28] C. Frohnert, S. Hutten, S. Wälde, A. Nath, R.H. Kehlenbach, Importin 7 and Nup358 promote nuclear import of the protein component of human telomerase, PLoS One 9 (2014) e88887.

[29] S. Jakel, D. Gorlich, Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells, EMBO J. 17 (1998) 4491-4502.

[30] P. Muhlhausser, E.C. Muller, A. Otto, U. Kutay, Multiple pathways contribute to nuclear import of core histones, EMBO Rep. 2 (2001) 690-696.

[31] P. Giannakakou, D.L. Sackett, Y. Ward, K.R. Webster, M.V. Blagosklonny, T. Fojo, p53 is associated with cellular microtubules and is transported to the nucleus by dynein, Nat. Cell Biol. 2 (2000) 709-717.

[32] D.M. Roth, G.W. Moseley, D. Glover, C.W. Pouton, D.A. Jans, A microtubule-facilitated nuclear import pathway for cancer regulatory proteins. Traffic 8 (2007) 673-

686.

[33] D.M. Roth, G.W. Moseley, C.W. Pouton, D.A. Jans, Mechanism of microtubule-facilitated "fast track" nuclear import, J. Biol. Chem. 286 (2011) 14335-14351.

[34] I. Mikenberg, D. Widera, A. Kaus, B. Kaltschmidt, C. Kaltschmidt, Transcription factor NF-kappaB is transported to the nucleus via cytoplasmic dynein/dynactin motor complex in hippocampal neurons, PLoS One 2 (2007) e589.

[35] R. Suárez-Sánchez, A. Aguilar, K.M. Wagstaff, G. Velez, P.M. Azuara-Medina, P. Gomez, A. Vásquez-Limeta, O. Hernández-Hernández, K.G. Lieu, D.A. Jans, B. Cisneros, Nucleocytoplasmic shuttling of the Duchenne muscular dystrophy gene product dystrophin Dp71d is dependent on the importin α/β and CRM1 nuclear transporters and microtubule motor dynein, Biochim. Biophys. Acta 1843 (2014) 985-1001

[36] K.M. Wagstaff, D.A. Jans, Importins and beyond: non-conventional nuclear transport mechanisms, Traffic 10 (2009)1188-1198.

Chapter II

NEDD8 ultimate buster-1 (NUB1) promotes the proteasome-mediated proteolysis of TRF1

1. Abstract

The human telomeric protein TRF1 is one of the telomere-associated proteins, which is coated at the chromosome ends to protect telomere. In particular, TRF1 negatively regulates telomere extension by inhibiting telomerase access to the ends of the telomeres, suggesting that the TRF1 at the telomeres is tightly regulated for controlling telomere length. Here, we identified NEDD8 Ultimate Burster1 (NUB1), a negative regulator of the NEDD8 conjugation system, by recruiting free NEDD8 and its conjugates to the proteasome for degradation as a novel TRF1-interacting protein. NUB1 interacts with TRF1 *in vitro* and *in vivo* through its N-terminal region in an NEDD8-independent manner. We found that overexpression of NUB1 decreased levels of TRF1 and also reduced the half-life of TRF1, whereas depletion of endogenous NUB1 expression stabilized levels of endogenous TRF1. A previous report found that the ubiquitin-like domain at the N-terminal region of NUB1 interacted with the 26S proteasome subunit (S5a/PSMD4). We also found that the interaction between TRF1 and PSMD4 was enhanced by NUB1 overexpression, but decreased by NUB1 knockdown, suggesting that NUB1 might play an adaptor role between TRF1 and proteasome for degradation. These findings demonstrate that NUB1 plays a critical role in controlling the proteasomal degradation of TRF1 through physical interaction for telomere homeostasis.

Keywords : Telomere, TRF1, NUB1, UBL-UBA protein family, S5a/PSMD4, proteasomal degradation

2. Introduction

Telomeres are specialized nucleoprotein complexes found at the end of linear eukaryotic chromosomes required for genome integrity [1]. Mammalian telomeric DNA is composed of tandem TTAGGG repeats bound by a series of associated proteins which are required for the protection and maintenance of telomeres from being recognized as double-strand DNA breaks [2,3]. Most human normal somatic cells show a progressive loss of telomeric DNA during successive rounds of cell division due to a DNA end-replication problem [4]. However, in most tumor cells, steady-state telomere length is tightly regulated by telomerase which is a specialized reverse transcriptase that adds DNA to the chromosome termini to compensate for telomere loss during DNA replication. These observations suggest that abnormal telomere shortening or elongation is implicated in aging and cancer.

Telomere homeostasis is regulated by telomerase and the six-subunit protein complex named shelterin, which consists of TRF1, TRF2, POT1, TIN2, TPP1, and Rap1 [5,6]. The shelterin complex plays an essential role in maintaining the integrity of telomere length and structure. Among the shelterin components, both TRF1 and TRF2 bind to duplex telomeric DNA as dimers though the C-terminal DNA-binding motif that is closely related to the Myb domain and an internal conserved TRF homology domain. TRF2 is best known for its role in telomere protection, and it stabilizes a terminal loop structure called the t-loop [7]. In comparison, TRF1 negatively regulates telomerase-dependent telomere extension by inhibiting telomerase access to the ends of telomeres. The overexpression of TRF1 accelerates telomere shortening whereas the loss of TRF1 from telomeres has been shown to induce telomerase-dependent telomere elongation, implying that the cellular amount of TRF1 on telomeres should be tightly regulated for maintaining telomere length [8,9].

TRF1 is subject to extensive post-translational modification [10] and it is evident that the abundance of TRF1 has been shown to be regulated through interactions with various proteins. We have previously identified casein kinase 2 (CK2) as a TRF1-interacting protein, and CK2-mediated phosphorylation is required for the efficient telomere binding

of TRF1, suggesting the role of CK2 in determining the level of TRF1 at the telomeres [11]. Polo-like kinase 1 also interacts and phosphorylates TRF1 and is involved in both TRF1 overexpression-induced apoptosis and the telomere-binding ability of TRF1 [12]. Moreover, Pin1 has been shown to be an essential regulator of TRF1 stability [13] and guanine nucleotide-binding protein-like 3 (GNL3L) has also been reported to bind and stabilize TRF1 protein by inhibiting its ubiquitination and binding to Fbx4 [14]. On the other hand, tankyrase 1, which is a member of the poly(ADP-ribose) polymerase (PARP) family, has been shown to interact with the acidic domain in the N-terminus of TRF1. The ADP-ribosylation of TRF1 by tankyrase1 dissociates TRF1 from telomeres, thus enabling telomerase access to the telomeres and resulting in telomere elongation [15,16]. The dissociated telomere-unbound TRF1 by the activation of tankyrase 1 is rapidly ubiquitinated by E3 ubiquitin ligases, such as Fbx4 [17] and RLIM [18], and subsequently degraded by the proteasome.

In this study, we identified NEDD8 ultimate buster-1 (NUB1) as a novel TRF1-interacting protein using yeast two-hybrid screening. NUB1 is an interferon-inducible protein and possesses a UBL (ubiquitin-like) domain at the N-terminal region and two ubiquitin-associated domains at the C-terminus [19,20]. Previously, NUB1 has been shown to directly interact with NEDD8 (neural precursor cell expressed developmentally down-regulated 8), which is an ubiquitin-like modifier, and PSMD4, which is a 26S proteasome subunit [20,21,22]. Thus far, the crucial function of NUB1 is the recruitment of the NEDD8 and NEDD8-conjugated protein to the 26S proteasome for degradation [19,20,23]. Here, we showed that NUB1 promotes TRF1 degradation by enhancing the interaction between TRF1 and 26S proteasome. These findings suggest that NUB1 is a negative regulator of TRF1 protein level and represents a new pathway for modulating TRF1 function at the telomeres.

3. Materials and Methods

3.1 Yeast Two hybrid Screening

Yeast two-hybrid screening was performed as described [24]. Briefly, the full-length TRF1 cDNA was fused to the LexA DNA binding domain and transformed by the lithium acetate method into the EGY48 yeast strain. Expression of the LexA-TRF1 fusion protein was verified by Western blotting using anti-LexA antibody. The stable strain was transformed again with a HeLa cDNA library fused to the activation domain vector pB42AD (Clontech).

3.2 Cell culture and plasmids construction

The human embryonic kidney cell line HEK293 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100units/ml penicillin, and 100μg/ml streptomycin in 5% CO2 at 37°C. The expression vectors for Flag-TRF1, Myc-NUB1, V5-FBX4, were constructed by inserting the full-length cDNAs into pRK5-Flag, pRK5-Myc and pRK5-V5(Stratagene), respectively. The expression vectors for GST-TRF1 and GST-NUB1 were constructed by cloning the full-length and truncated fragments from the TRF1 or NUB1 cDNA into pGEX-4T-2 (GE Healthcare).. The GST fusion proteins were expressed and purified by using a glutathione-Sepharose 4B according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

3.3 GST Pull down, Immunoprecipitation, immunoblotting and antibodies

The expression vectors were transiently transfected into HEK293 cells using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen) for 24 h followed by lysis. For GST pull down assay, lysates were precleared with glutathione-Sepharose 4B (Amersham Biosciences) and incubated with glutathione-Sepharose beads containing GST fusion proteins for 2 h at 4℃. For immunoprecipitation, lysates were preincubated with protein A-Sepharose (Amersham Biosciences) and incubated with anti-Flag (Sigma) or anti-Myc (Santa Cruz Biotechnology) antibodies precoupled with protein

A-Sepharose beads for 2 h at 4℃. Bound proteins were analyzed by SDS-PAGE, followed by immunoblotting. Immunoblotting were performed using anti-Flag (Sigma), anti-Myc (Santa Cruz Biotechnology), anti-NUB1 (Santa Cruz Biotechnology), anti-NEDD8 (Cell signaling), anti-tubulin (Santa Cruz Biotechnology) and anti-hTRF1#370 (a gift from Titia de Lange, Rockefeller University) antibodies as specified. All the immunoblots are representatives of at least three experiments that demonstrated the similar results.

3.4 RNA interference

The siRNA duplexes were transfected into HEK293 cells using Lipofectamine2000 transfection reagent (Invitrogen). The siRNA target sequences specific for NUB1 were 5'-ACCGTACTTAGACATAGCTAA-3' for siNUB1-1 and 5'-TAGAGGCTTATGAGTATCTTA -3' for siNUB1-2. The siRNA target sequences specific for NEDD8 were 5'-CATAATGAG GCATCATATA-3'. The scrambled sequence (5'-AATCGCATAGCGTATGCCGTT-3') was used as a negative control and did not correspond to any known gene in the data bases.

3.5 *In vivo* ubiquitination assay

HEK293 cells were co-transfected with Flag-TRF1 and HA-ubiquitin and treated with 10 μM MG132 to inhibit proteasome function. Lysates were subjected to immunoprecipitation with anti-Flag M2 affinity resins (Sigma), followed by immunoblotting with anti-HA antibody to illuminate ubiquitin-conjugated TRF1.

4. Results

4.1 Identification of NUB1 as a TRF1-interacting partner

To identify TRF1-interacting factors, we screened a HeLa cell cDNA library using the yeast two-hybrid system. Using the full-length TRF1 as bait, 26 positive clones were obtained and sequenced. One of the isolated clones contained the cDNA encoding NUB1 (NEDD8 ultimate buster-1) (Fig. 1A), which was identified as a negative regulator of the NEDD8 conjugation system by recruiting NEDD8 and its conjugates to the proteasome for degradation [19-21, 25]. RAP1, which was known to interact with TRF2[26], and PINX1, which was known to interact with TRF1[27], were used as positive controls. To confirm the direct interaction between TRF1 and NUB1, we performed GST pull-down experiments. GST-NUB1, but not the control GST, precipitated Flag-TRF1 expressed in HEK293 cells, indicating that TRF1 interacts with NUB1 *in vitro* (Fig. 1B). Likewise, GST-TRF1, but not the control GST, precipitated Myc-NUB1 expressed in HEK293 cells. To determine whether TRF1 and NUB1 associate *in vivo*, HEK293 cells were co-transfected with Flag-TRF1 and Myc-NUB1 expression vectors and subjected to immunoprecipitation. Flag-TRF1 was detected in anti-Myc immunoprecipitates when Myc-NUB1 was expressed (Fig. 1C). Likewise, Myc-NUB1 was recovered in anti-Flag immunopecipitates when Flag-TRF1 was expressed. To further substantiate the direct interaction between TRF1 and NUB1, we performed GST-pull down experiments with purified proteins. As expected, His-TRF1 was precipitated with GST-NUB1, but not the control GST (Fig. 1D). It has been well documented that NUB1 can interact with NEDD8 directly and reduce the free NEDD8 and NEDD8-conjugates through proteasomal degradation. To examine whether the interaction between TRF1 and NUB1 *in vivo* was NEDD8-dependent, HEK293 cells were co-transfected with Flag-TRF1, Myc-NUB1 and NEDD8 siRNA and subjected to immunoprecipitation. The interaction between Myc-NUB1 and Flag-TRF1 was not affected much when NEDD8 was depleted (Fig. 1E). These results suggest that NUB1 can interact with TRF1 in an NEDD8-independent manner.

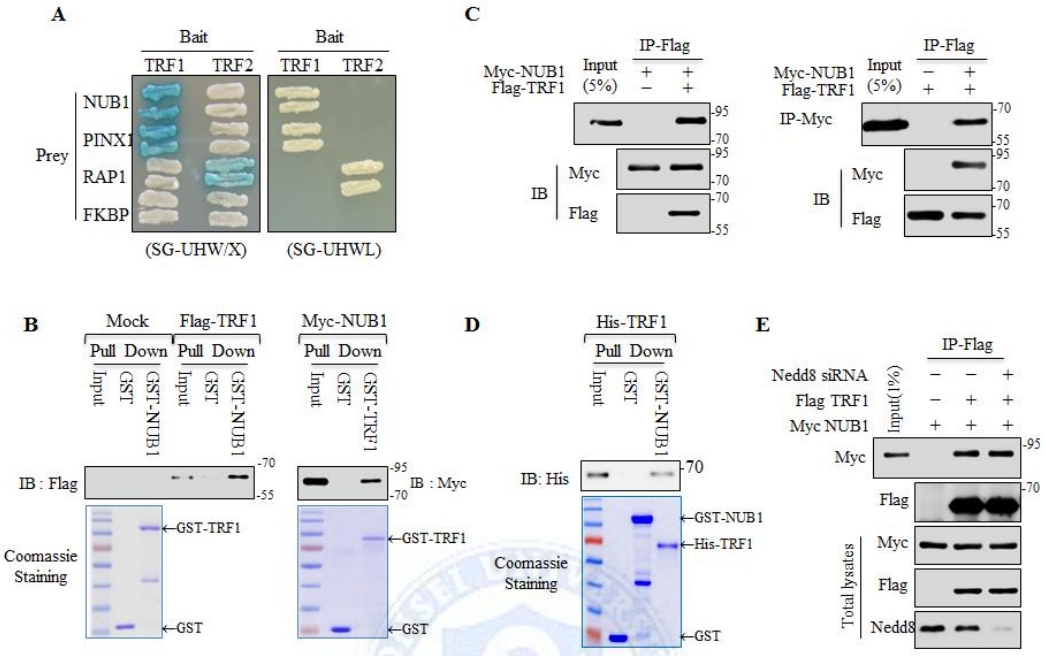

Figure 1. NUB1 interacts with hTERT *in vitro* and *in vivo*. (A) Analysis of the physical interaction between TRF1 and NUB1 using the yeast two-hybrid assay. PinX1, RAP1, and unrelated FKBP52 were used as the TRF1-binding, TRF2-binding, and negative control, respectively. The growth on the SG-HWUL plate and the blue signal on the SG-HWU/X plate indicate activation of the reporter genes, LacZ and LEU2, respectively. S, synthetic; G, galactose; H, histidine (-); W, tryptophan (-); U, uracil, (-); L, leucine (-); X, 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) (B) GST, GST-NUB1 and GST-TRF1 were immobilized on glutathione-Sepharose and incubated with ectopically expressed Flag-TRF1 or Myc-NUB1. Bound proteins were detected by immunoblotting with anti-Flag or anti-Myc antibodies. (C) HEK293 cells were co-transfected with Flag-TRF1 and Myc-NUB1 and subjected to immuno-precipitation with anti-Myc or anti-Flag antibodies, followed by immunoblotting with anti-Flag or anti-Myc antibodies. (D) GST, GST-NUB1 were immobilized on glutathione-Sepharose and incubated with purified His-TRF1. Bound proteins were detected by immunoblotting with anti-His antibody. (E) HEK293 cells were co-transfected with Flag-TRF1, Myc-NUB1 and NEDD8 siRNA and subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting to detect Myc-NUB1.

4.2 Identification of the domains in TRF1 and NUB1 required for their interactions

NUB1 consists of an ubiquitin-like (UBL) domain as a proteasome-interacting motif, an ubiquitin-associated (UBA) domain as a ubiquitin-related motif, and a pest domain as an NEDD8-interacting domain. To map the region in NUB1 responsible for TRF1 binding, we generated NUB1 deletion constructs lacking UBL or UBA domains (Fig. 2A). HEK293 cells were co-transfected with Flag-TRF1 and Myc-NUB1 or its truncate constructs and subjected to immunoprecipitation with anti-Flag antibody. Full-length of NUB1 and its N-terminal fragment (1-346) were bound to Flag-TRF1 (Fig. 2B). We also confirmed their interaction using *in vitro* binding assay with GST-fused full-length NUB1 and NUB1 truncates. Likewise, Flag-TRF1 was precipitated with GST-NUB1 and GST-NUB1-N(1-346), but not GST-NUB1-C(346-601) and control GST (Fig. 2C). These results indicate that TRF1 interacts with the N-terminal region containing the UBL domain in NUB1. Next, several Flag-tagged TRF1 fragments were tested for their interaction with NUB1 *in vivo* (Fig. 2D). HEK293 cells were co-transfected with Myc-NUB1 and various Flag-TRF1 fragments and subjected to immunoprecipitation. Full-length TRF1, TRF1-D2(1-263), and TRF1-D5(1-377) containing acidic domains were bound to Myc-NUB1, suggesting that the acidic domain of TRF1 is important for NUB1 binding (Fig. 2E). Domain study showed that N-terminal region of NUB1 and the acidic domain of TRF1 were responsible for their interaction.

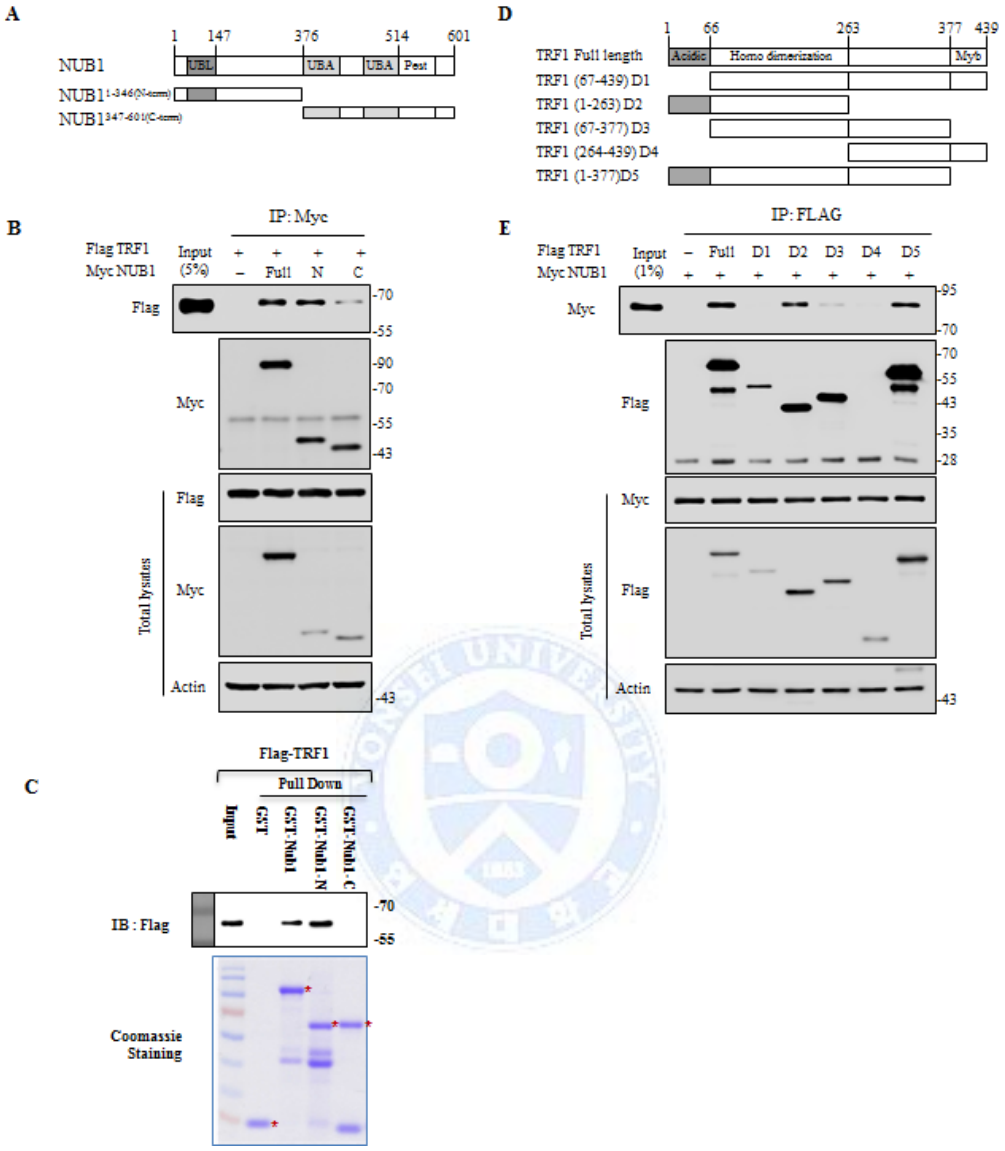

Figure 2. Identification of the domains in NUB1 and TRF1 required for their interaction. (A) Schematic representation of NUB1 and its deletion variants used in this study. (B) HEK293 cells were co-transfected with Flag-TRF1 and various Myc-NUB1 and subjected to immunoprecipitation with anti-Myc antibody, followed by immunoblotting with anti-Flag antibody. (C) GST, GST-NUB1 variants were immobilized on glutathione-Sepharose and incubated with ectopically expressed Flag-TRF1. Bound proteins were detected by immunoblotting with anti-Flag antibody. (D) Schematic representation of TRF1 and its deletion variants (E) HEK293 cells were co-transfected with various Flag-tagged TRF1 and Myc-NUB1 and subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-Myc antibody.

4.3 NUB1 promotes TRF1 recruitment to proteasome for degradation through S5a/PSMD4

Ubiquitin-mediated proteasomal degradation is one of the major mechanisms involved in the maintenance of the proper levels of cellular proteins. The 26S proteasome holoenzyme consists of two major subunits, the 19S regulatory particle (RP) and the 20S core particle (CP) [28]. Specific subunits of the proteasome 19S RP have been identified as ubiquitin receptors that are capable of recognizing both free ubiquitin and ubiquitin-conjugated proteins, and the most studied proteasome ubiquitin receptor is S5a/PSMD4.

It has been well documented that the ubiquitin-like–ubiquitin-associated (UBL–UBA) family of proteins regulates its interacting protein by promoting or inhibiting proteasomal degradation [29]. Both components interact with S5a/PSMD4 via its UBL domain and ubiquitin and ubiquitinated proteins via its UBA domain. A previous report stated that NUB1 directly interacts with S5a/PSMD4, which is a subunit of the 26S proteasome through its UBL domain, and it was shown that the C terminus of NUB1L binds to S5a/PSMD4 [21]. Another study reported the association of NUB1L with S5a/PSMD4 via the N-terminal UBL domain [30, 31]. Because we found NUB1 directly interacts with TRF1, we hypothesized that NUB1 mediates the recruitment of TRF1 to the proteasome through its UBL domain. First, to confirm the interaction between NUB1 and S5a/PSMD4 in vivo, HEK293 cells were co-transfected with GFP-PSMD4 and various Myc-NUB1 truncates and subjected to immunoprecipitation with the anti-Myc antibody, followed by immunoblotting using the anti-GFP antibody. As expected, NUB1 was associated with PSMD4 through its N-terminal region containing the UBL domain in accordance with the latter report (Fig. 3A). To examine whether NUB1 contributes to the interaction between TRF1 and the 26S proteasome, HEK293 cells were co-transfected with Flag-TRF1, Myc-NUB1, and GFP-PSMD4 expression vectors and treated with 10uM of MG132 for 2hrs. Lysates were subjected to immunoprecipitation with anti-Flag or anti-GFP antibodies, followed by immunoblotting using anti-GFP or anti-Flag antibodies, respectively. The interaction between Flag-TRF1 and GFP-PSMD4 was increased by the overexpression of NUB1 (Fig. 3B, 3C). On the other hand, NUB1

depletion led to a clear reduction of the association between Flag-TRF1 and GFP-PSMD4, suggesting that NUB1 acts as a physical linker between TRF1 and S5a/PSMD4 through a UBL domain at its N-terminal region for recruiting TRF1 to proteasome (Fig. 3D).

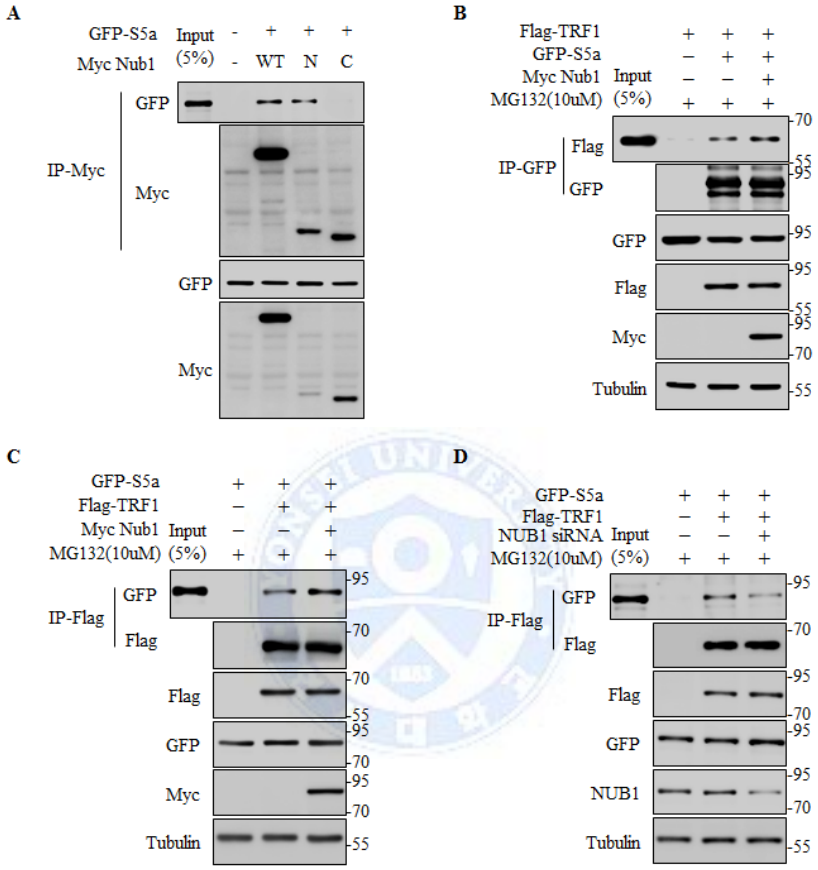

Figure 3. NUB1 act as a physical linker between the TRF1 and the S5a/PSMD4 (A) HEK293 cells were transfected with GFP-PSMD4 and Myc tagged NUB1 variants and subjected to immunoprecipitation with anti-Myc antibody, followed by immunoblotting with anti-GFP antibody. (B) HEK293 cells were transfected with GFP-PSMD4, Flag-TRF1 and Myc-NUB1 and treated with 10μM MG132 for 4 hours, and subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-GFP or anti-Flag antibodies. (C) HEK293 cells were transfected with GFP-PSMD4, Flag-TRF1 and Myc-NUB1 and treated with 10μM MG132 for 4 hours, and subjected to immunoprecipitation with anti-GFP antibody, followed by immunoblotting with anti-Flag antibodies (D) HEK293 cells were co-transfected with Flag-TRF1, Myc-NUB1 at 48h post-transfection with NUB1 siRNA(50nM) and treated with 10μM MG132 for 4 hours, and subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-GFP or anti-Flag antibodies.

4.4 NUB1 down-regulates TRF1 levels and stability

In earlier studies, the characterized function of NUB1 was recruiting NEDD8 and NEDD8 conjugates to proteasome for degradation [19, 20, 23]. However, our results showed that the interaction between TRF1 and NUB1 occurred in an NEDD8-indepenent manner. To examine whether NUB1 contributes to the levels of TRF1 in vivo, HEK293 cells were co-transfected with Flag-TRF1 and increasing doses of Myc-NUB1 and analyzed using immunoblotting with the anti-Flag antibody. The expression levels of Flag-TRF1 were decreased by NUB1 in a dose-dependent manner (Fig. 4A). Although the NEDD8-dependent mechanism of TRF1 has not yet been reported, to eliminate this possibility, we used MLN4924, which is the selective inhibitor of the NEDD8-Activating Enzyme (NAE) [32]. HEK293 cells were co-transfected with HA-TRF1 and increasing doses of Myc-NUB1 and treated with or without 1uM of MLN4924 for 20hrs and analyzed using immunoblotting with the anti-HA antibody. Interestingly, the expression levels of HA-TRF1 were decreased by NUB1 in a dose-dependent manner even when MLN4924 was treated (Fig. 4B). These data suggest that NUB1 reduces the level of TRF1 in an NEDD8-independent manner. Furthermore, we also examined the stability of TRF1 by NUB1 overexpression. HEK293 cells were co-transfected with Flag-TRF1 and Myc-NUB1 and treated with cycloheximide (100ug/ml) as indicated time to block new protein synthesis and analyzed using immunoblotting with the anti-Flag antibody. As shown in Fig. 4C and graphically in Fig. 4D, the overexpression of Myc-NUB1 reduced the half-life of TRF1 compared with the empty vector-expressing cells. Myc-Fbx4, which is a TRF1-specific ubiquitin E3 ligase, was used as a control [17, 33]. In all cases, the turnover of TRF1 was blocked by the MG132 treatment, indicating that the degradation of TRF1 was mediated by the proteasome. To further verify the involvement of NUB1 in regulating the levels of endogenous TRF1, we transiently expressed increasing doses of Myc-NUB1 in HEK293 cells and examined the levels of endogenous TRF1. The levels of endogenous TRF1 substantially decreased in Myc-NUB1-expressing cells compared with the empty vector-expressing cells (Fig. 4E). Myc-Fbx4 expression vector was used as a positive control. Additionally, to examine the effect of NUB1 on endogenous TRF1, the expression of endogenous NUB1 was depleted using two different small interfering RNA (siRNA) duplexes. The levels of endogenous TRF1 increased in the NUB1-depleted cells compared with the control cells (Fig. 4F). However, no significant difference was observed in the steady-state levels of TRF1 mRNA between NUB1 knockdown or over-expressing cells and the control cells (Fig. 4G). Taken together, these results suggest that

NUB1 modulates the cellular abundance and half-life of endogenous TRF1 by acting as a negative regulator of the TRF1 protein.

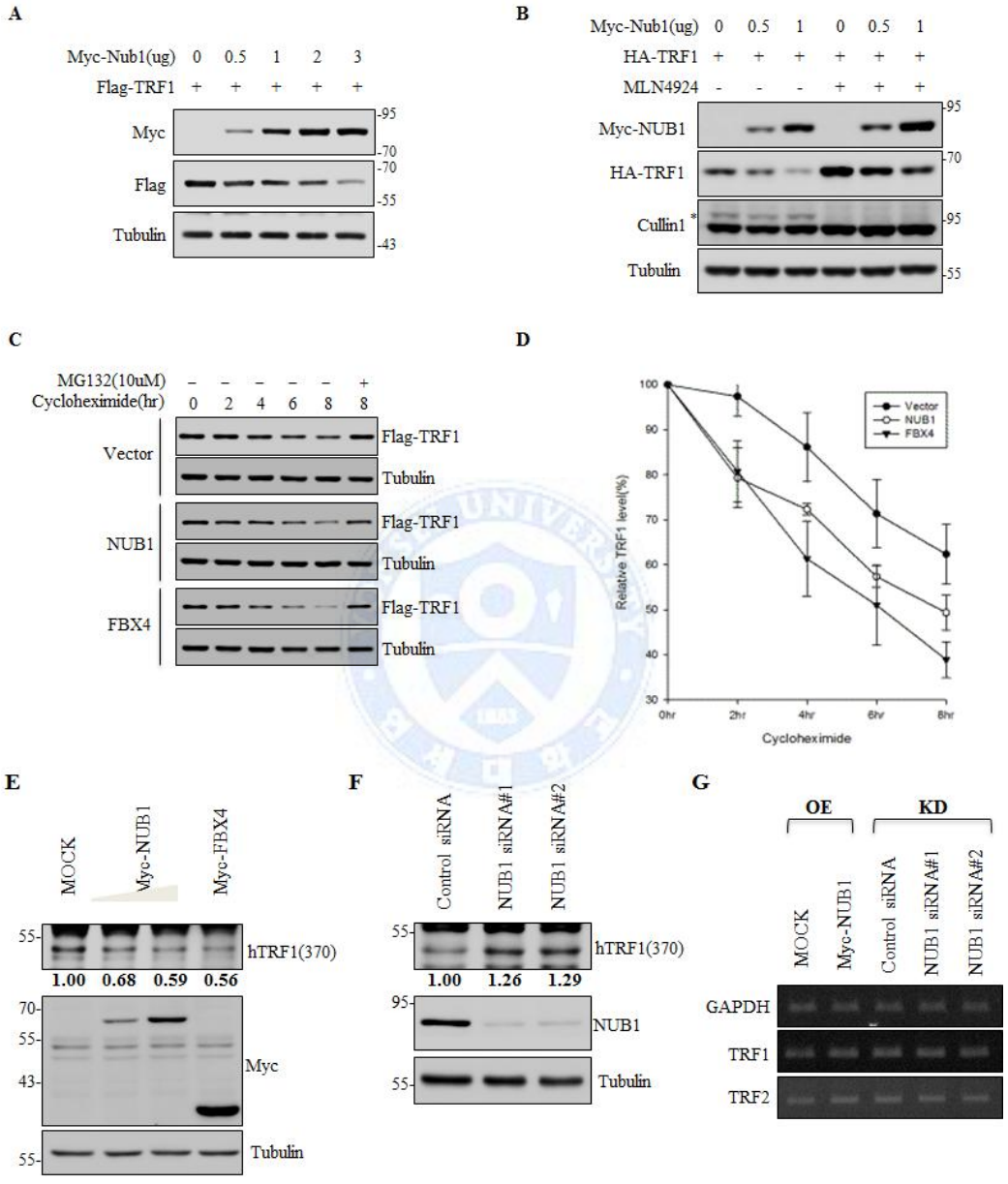

Figure 4. NUB1 down-regulates the TRF1 level and Stability. (A) HEK293 cells were co-transfected with Flag-TRF1 and increasing amounts of Myc-NUB1 as specified. The levels of ectopically expressed Flag-TRF1 and Myc-NUB1 were determined by immunoblotting with anti-Flag and anti-Myc antibodies, respectively. An antibody against tubulin was used as a loading control. (B) HEK293 cells were co-transfected with Flag-

TRF1 at 48h post-transfection with increasing amounts of NUB1 siRNA. Lysates were analyzed on immunoblots with anti-Flag or anti-tubulin antibodies. (C) HEK293 cells were transfected with Flag-TRF1, Myc-NUB1 or Myc-Fbx4 and treated with 100μg/ml cycloheximide together with, or without, 10μM of MG132 for the indicated times. The levels were analyzed on immunoblots with anti-Flag and anti-Myc antibodies. An antibody against tubulin was used as a loading control. (D) Graphical representation of the relative TRF1 levels normalized against the tubulin loading control. The TRF1 expression levels were quantified with the average and standard deviation from three independent experiments. (E) HEK293 cells were transfected with Myc-NUB1 or Myc-Fbx4 as indicated. The levels endogenous TRF1, Myc-NUB1 and, Myc-Fbx4 were determined by immunoblotting with anti-TRF1(#370) and anti-Myc antibodies, respectively. (F) HEK293 cells transfected with two different NUB1 siRNA duplexes and lysates were analyzed by immunoblotting using anti-TRF1(#370) and anti-NUB1 antibodies, respectively. (G) HEK293 cells transfected with Myc-NUB1 or NUB1 siRNAs were subjected to reverse transcription-PCR analysis for the mRNA levels of endogenous TRF1 and TRF2 genes.

4.5 Degradation of TRF1 by the 26S proteasome relies on NUB1 in a ubiquitin-independent manner

NUB1 is a unique member of the UBL–UBA family that interacts with NEDD8 instead of ubiquitin. Previous reports demonstrated that FAT10, which is the only ubiquitin-like modifier, can target proteins for degradation by the proteasome in a ubiquitin-independent manner [34, 35], and the degradation of FAT10 and FAT10 conjugates is accelerated by NUB1L [30, 31, 35]. We also identified that TRF1 is recruited to the 26S proteasome by NUB1. Next, we examined whether the ubiquitination of TRF1 is affected by the presence of NUB1. HEK293 cells were co-transfected with Flag-TRF1, Myc-NUB1, V5-Fbx4, and HA-Ubiquitin, treated with 10uM of MG132 for 4hrs, and subjected to immunoprecipitation with the anti-Flag antibody, followed by immunoblotting using the anti-HA antibody to illuminate ubiquitin-modified TRF1. Interestingly, we found that the ubiquitination of TRF1 was reduced by the overexpression of NUB1 even when Fbx4 was co-expressed (Fig. 5A). Furthermore, we confirmed the in vivo ubiquitination of TRF1 under denaturing conditions. HEK293 cells were co-transfected with HA-TRF1, Myc-NUB1, Myc-Fbx4, and His-Ubiquitin expression vectors and treated with 10uM of MG132 for 4hrs. Lysates were precipitated under denaturing conditions using Ni-NTA pull-down and the ubiquinated-TRF1 was analyzed through immunoblotting using the HA antibody. Consistently, the ubiquitination of TRF1 was decreased by NUB1, suggesting that NUB1 down-regulates TRF1 in a ubiquitin-independent manner (Fig. 5B).

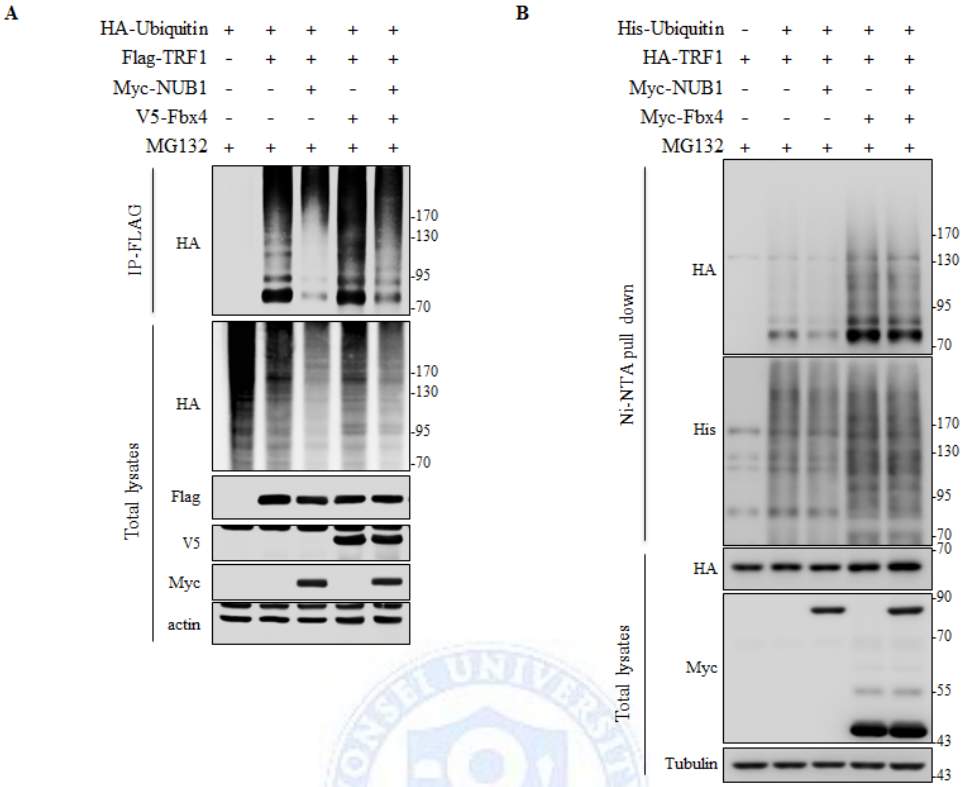

Figure 5. Degradation of TRF1 by the 26S proteasome relies on NUB1 in a ubiquitnation-independent manner. (A) HEK293 cells were co-transfected with Flag-TRF1, HA-Ubiquitin, Myc-NUB1 and V5-Fbx4 and treated with 10μM of MG132. Lysates were subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting using anti-HA antibody to detect Ubiquitin-conjugated TRF1. (B) HEK293 cells were transfected with His tagged Ubiquitin, HA-TRF1, Myc-NUB1 and Myc-Fbx4 as indicated, and performed Ni-NTA pull-down under denature condition followed by immunoblotting using anti-HA antibody.

5. Discussion

TRF1 negatively regulates the telomere length by restricting telomerase access to the telomeres. The overexpression of TRF1 promotes telomere shortening, whereas the loss of TRF1 from the telomeres has been shown to induce abnormal telomere elongation [8], suggesting that an abundance of TRF1 at the telomeres should be tightly controlled for maintaining telomere length. Therefore, the cellular levels of TRF1 at the telomeres has been shown to be tightly regulated by interactions with various proteins, such as tankyrase 1, Fbx4, RLIM, CK2, Pin1, and Plk1 [10]. Here, we describe a novel function of NUB1 for regulating the abundance of TRF1 in mammalian cells. We identified NUB1 as a negative regulator of TRF1 by directly interacting with TRF1. The overexpression of NUB1 significantly decreased the levels of TRF1 and also reduced the half-life of TRF1, whereas the depletion of NUB1 by the RNA interference stabilized the endogenous TRF1. These findings demonstrate that NUB1 plays a critical role in regulating the level of TRF1 at the telomeres.

NUB1 is composed of several domains, including the ubiquitin-like domain, two ubiquitin-associated domains, a bipartite nuclear localization signal, and a PEST sequence, and it is a unique member of the UBL–UBA family that interacts with NEDD8 instead of ubiquitin [19, 20]. To explain the function of NUB1 on TRF1, there are several possibilities. The first possibility is that TRF1 is the target for NEDD8 conjugation, resulting in NEDD8-conjugated TRF1 being recruited by NUB1 to the 26S proteasome for degradation. Although TRF1 is subject to extensive post-translational modification [10], the NEDD8 conjugation of TRF1 has not yet been determined, and we found that the interaction between TRF1 and NUB1 was not affected by the depletion of endogenous NEDD8 (Figure 1E), implying that the interaction between NUB1 and TRF1 occurs in an NEDD8-independent manner. Unexpectedly, we also found that the MLN4924 treatment dramatically increased the level of TRF1, suggesting that further study is required to assess the biological function of the NEDD8-dependent mechanism of TRF1. The second possibility is that NUB1 is an adaptor between S5a of the 19 S proteasome and TRF1. A

previous report has shown that NUB1 interacts with NEDD8 and NEDD8-conjugates through its PEST domain in the C-terminus and also interacts with S5a through its UBL domain in the N-terminus [19-21, 31]. Based on our findings on the physical association between NUB1 and TRF1, we focus on the second possibility for determining the NEDD-independent functions of NUB1 on TRF1 in this study.

A recent study suggested that the UBL–UBA protein family, which has an N-terminal ubiquitin-like (UbL) domain and one or more ubiquitin-associated (UBA) domains, has a role in proteasomal degradation. This is because both components interact with S5a/PSMD4, a subunit of the 19S proteasome activator (via the UBL domain), and the proteins are targeted for degradation by ubiquitination (via the UBA domain) [29]. Several UBL–UBA domain proteins have been well characterized, such as Rad23 [36-38], BAG-1 [39], Dsk2 [40]. Rad23 interacts with S5a through an N-terminal UBL domain, and the C terminus of Rad23 binds to the ubiquitinated Rad4 DNA repair protein and creates a link between the DNA repair and proteasome pathways [41]. BAG-1 has also been shown to play a role in the physical link between the molecular chaperones Hsc70/Hsp70 and the proteasome through a UBL domain at its N-terminal region [39]. Since NUB1 also possesses a ubiquitin-like domain at its N-terminal region and physically interacts with S5a/Rpn10 [21], we hypothesized that NUB1 might act as a linker between the TRF1 and the S5a subunit of the 19S proteasome activator through its UBL domain. Based on this hypothesis, we found that the association between TRF1 and S5a/PSMD4 was enhanced by the overexpression of NUB1 and reduced by the depletion of NUB1, suggesting that NUB1 could serve as a direct link between TRF1 and proteasomal degradation. Moreover, we found that although the level of TRF1 was decreased by NUB1, the ubiquitination of TRF1 was reduced by the overexpression of NUB1, implying that the overexpression of NUB1 induces the degradation of TRF1 by NUB1 prior to ubiquitin-mediated proteolysis. Therefore, we suggest that the NUB1-dependent-ubiquitin-independent pathway functions differently in a ubiquitin-independent manner for regulating the cellular abundance of TRF1.

In summary, our data provide evidence for the novel function of NUB1 in modulating

the cellular abundance of TRF1 through cooperation with proteasome. The key findings of this study suggest a new mechanism for the degradation of TRF1 by NUB1 through a ubiquitin-independent pathway. Although many important questions about the biological significance of the NUB1 function at the telomeres in regulating TRF1 remain unresolved, our data suggest that NUB1 represents a new pathway for controlling the stability of TRF1 by acting as a negative regulator of TRF1.

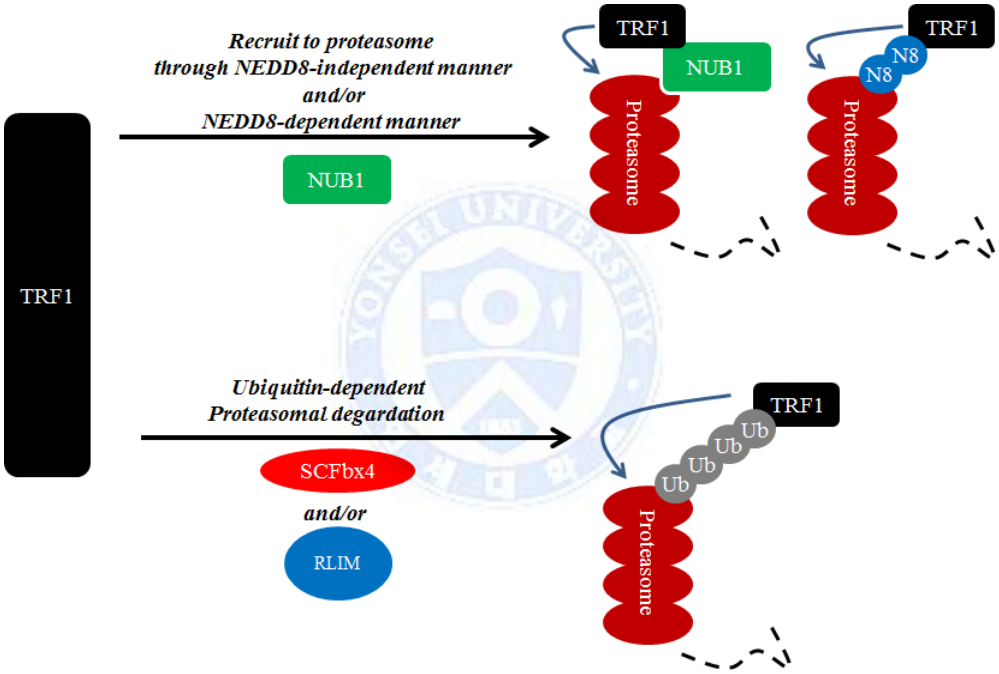

Figure 6. Model for NUB1-mediated proteolysis of TRF1

Acknowledgments

We thank Titia de Lange at rockefeller university for kindly providing the hTRF1(#370) antibody.

6. References

[1] A. Smogorzewska, T. de Lange, Regulation of telomerase by telomeric proteins, Annual review of biochemistry, 73 (2004) 177-208.

[2] T. de Lange, Protection of mammalian telomeres, Oncogene, 21 (2002) 532-540.

[3] J.D. Griffith, L. Comeau, S. Rosenfield, R.M. Stansel, A. Bianchi, H. Moss, T. de Lange, Mammalian telomeres end in a large duplex loop, Cell, 97 (1999) 503-514.

[4] M.A. Cerone, C. Autexier, J.A. Londono-Vallejo, S. Bacchetti, A human cell line that maintains telomeres in the absence of telomerase and of key markers of ALT, Oncogene, 24 (2005) 7893-7901.

[5] L. Chong, B. van Steensel, D. Broccoli, H. Erdjument-Bromage, J. Hanish, P. Tempst, T. de Lange, A human telomeric protein, Science, 270 (1995) 1663-1667.

[6] D. Liu, M.S. O'Connor, J. Qin, Z. Songyang, Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins, The Journal of biological chemistry, 279 (2004) 51338-51342.

[7] B. van Steensel, A. Smogorzewska, T. de Lange, TRF2 protects human telomeres from end-to-end fusions, Cell, 92 (1998) 401-413.

[8] B. van Steensel, T. de Lange, Control of telomere length by the human telomeric protein TRF1, Nature, 385 (1997) 740-743.

[9] A. Smogorzewska, B. van Steensel, A. Bianchi, S. Oelmann, M.R. Schaefer, G. Schnapp, T. de Lange, Control of human telomere length by TRF1 and TRF2, Molecular and cellular biology, 20 (2000) 1659-1668.

[10] J.R. Walker, X.D. Zhu, Post-translational modifications of TRF1 and TRF2 and their roles in telomere maintenance, Mechanisms of ageing and development, 133 (2012) 421-434.

[11] M.K. Kim, M.R. Kang, H.W. Nam, Y.S. Bae, Y.S. Kim, I.K. Chung, Regulation of telomeric repeat binding factor 1 binding to telomeres by casein kinase 2-mediated phosphorylation, The Journal of biological chemistry, 283 (2008) 14144-14152.

[12] Z.Q. Wu, X. Yang, G. Weber, X. Liu, Plk1 phosphorylation of TRF1 is essential for its binding to telomeres, The Journal of biological chemistry, 283 (2008) 25503-25513.

[13] T.H. Lee, A. Tun-Kyi, R. Shi, J. Lim, C. Soohoo, G. Finn, M. Balastik, L. Pastorino, G. Wulf, X.Z. Zhou, K.P. Lu, Essential role of Pin1 in the regulation of TRF1 stability and telomere maintenance, Nature cell biology, 11 (2009) 97-105.
[14] Q. Zhu, L. Meng, J.K. Hsu, T. Lin, J. Teishima, R.Y. Tsai, GNL3L stabilizes the TRF1 complex and promotes mitotic transition, The Journal of cell biology, 185 (2009) 827-839.
[15] B.D. Cook, J.N. Dynek, W. Chang, G. Shostak, S. Smith, Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres, Molecular and cellular biology, 22 (2002) 332-342.
[16] J.R. Donigian, T. de Lange, The role of the poly(ADP-ribose) polymerase tankyrase1 in telomere length control by the TRF1 component of the shelterin complex, The Journal of biological chemistry, 282 (2007) 22662-22667.
[17] T.H. Lee, K. Perrem, J.W. Harper, K.P. Lu, X.Z. Zhou, The F-box protein FBX4 targets PIN2/TRF1 for ubiquitin-mediated degradation and regulates telomere maintenance, The Journal of biological chemistry, 281 (2006) 759-768.
[18] Y.R. Her, I.K. Chung, Ubiquitin Ligase RLIM Modulates Telomere Length Homeostasis through a Proteolysis of TRF1, The Journal of biological chemistry, 284 (2009) 8557-8566.
[19] T. Kamitani, K. Kito, T. Fukuda-Kamitani, E.T. Yeh, Targeting of NEDD8 and its conjugates for proteasomal degradation by NUB1, The Journal of biological chemistry, 276 (2001) 46655-46660.
[20] K. Kito, E.T. Yeh, T. Kamitani, NUB1, a NEDD8-interacting protein, is induced by interferon and down-regulates the NEDD8 expression, The Journal of biological chemistry, 276 (2001) 20603-20609.
[21] K. Tanji, T. Tanaka, T. Kamitani, Interaction of NUB1 with the proteasome subunit S5a, Biochemical and biophysical research communications, 337 (2005) 116-120.
[22] R. Piterman, I. Braunstein, E. Isakov, T. Ziv, A. Navon, S. Cohen, A. Stanhill, VWA domain of S5a restricts the ability to bind ubiquitin and Ubl to the 26S proteasome, Molecular biology of the cell, 25 (2014) 3988-3998.

[23] T. Tanaka, H. Kawashima, E.T. Yeh, T. Kamitani, Regulation of the NEDD8 conjugation system by a splicing variant, NUB1L, The Journal of biological chemistry, 278 (2003) 32905-32913.
[24] G.E. Lee, E.Y. Yu, C.H. Cho, J. Lee, M.T. Muller, I.K. Chung, DNA-protein kinase catalytic subunit-interacting protein KIP binds telomerase by interacting with human telomerase reverse transcriptase, J Biol Chem, 279 (2004) 34750-34755.
[25] G. Rabut, M. Peter, Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series, EMBO reports, 9 (2008) 969-976.
[26] B. Li, S. Oestreich, T. de Lange, Identification of human Rap1: implications for telomere evolution, Cell, 101 (2000) 471-483.
[27] X.Z. Zhou, K.P. Lu, The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor, Cell, 107 (2001) 347-359.
[28] D. Voges, P. Zwickl, W. Baumeister, The 26S proteasome: a molecular machine designed for controlled proteolysis, Annual review of biochemistry, 68 (1999) 1015-1068.
[29] V. Su, A.F. Lau, Ubiquitin-like and ubiquitin-associated domain proteins: significance in proteasomal degradation, Cellular and molecular life sciences : CMLS, 66 (2009) 2819-2833.
[30] G. Schmidtke, B. Kalveram, E. Weber, P. Bochtler, S. Lukasiak, M.S. Hipp, M. Groettrup, The UBA domains of NUB1L are required for binding but not for accelerated degradation of the ubiquitin-like modifier FAT10, The Journal of biological chemistry, 281 (2006) 20045-20054.
[31] N. Rani, A. Aichem, G. Schmidtke, S.G. Kreft, M. Groettrup, FAT10 and NUB1L bind to the VWA domain of Rpn10 and Rpn1 to enable proteasome-mediated proteolysis, Nature communications, 3 (2012) 749.
[32] T.A. Soucy, P.G. Smith, M.A. Milhollen, A.J. Berger, J.M. Gavin, S. Adhikari, J.E. Brownell, K.E. Burke, D.P. Cardin, S. Critchley, C.A. Cullis, A. Doucette, J.J. Garnsey, J.L. Gaulin, R.E. Gershman, A.R. Lublinsky, A. McDonald, H. Mizutani, U. Narayanan, E.J. Olhava, S. Peluso, M. Rezaei, M.D. Sintchak, T. Talreja, M.P. Thomas, T. Traore, S. Vyskocil, G.S. Weatherhead, J. Yu, J. Zhang, L.R. Dick, C.F. Claiborne, M. Rolfe, J.B.

Bolen, S.P. Langston, An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer, Nature, 458 (2009) 732-736.
[33] Z. Zeng, W. Wang, Y. Yang, Y. Chen, X. Yang, J.A. Diehl, X. Liu, M. Lei, Structural basis of selective ubiquitination of TRF1 by SCFFbx4, Developmental cell, 18 (2010) 214-225.
[34] M.S. Hipp, B. Kalveram, S. Raasi, M. Groettrup, G. Schmidtke, FAT10, a ubiquitin-independent signal for proteasomal degradation, Molecular and cellular biology, 25 (2005) 3483-3491.
[35] G. Schmidtke, B. Kalveram, M. Groettrup, Degradation of FAT10 by the 26S proteasome is independent of ubiquitylation but relies on NUB1L, FEBS letters, 583 (2009) 591-594.
[36] H. Hiyama, M. Yokoi, C. Masutani, K. Sugasawa, T. Maekawa, K. Tanaka, J.H. Hoeijmakers, F. Hanaoka, Interaction of hHR23 with S5a. The ubiquitin-like domain of hHR23 mediates interaction with S5a subunit of 26 S proteasome, The Journal of biological chemistry, 274 (1999) 28019-28025.
[37] L. Chen, K. Madura, Rad23 promotes the targeting of proteolytic substrates to the proteasome, Molecular and cellular biology, 22 (2002) 4902-4913.
[38] R. Verma, R. Oania, J. Graumann, R.J. Deshaies, Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system, Cell, 118 (2004) 99-110.
[39] J. Luders, J. Demand, J. Hohfeld, The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome, The Journal of biological chemistry, 275 (2000) 4613-4617.
[40] M. Funakoshi, T. Sasaki, T. Nishimoto, H. Kobayashi, Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome, Proceedings of the National Academy of Sciences of the United States of America, 99 (2002) 745-750.
[41] C. Schauber, L. Chen, P. Tongaonkar, I. Vega, D. Lambertson, W. Potts, K. Madura, Rad23 links DNA repair to the ubiquitin/proteasome pathway, Nature, 391 (1998) 715-718.

Chapter III

NEDD8 modification promotes cytosolic localization of TRF1 and regulates TRF1 stability

1. Abstract

A telomere is a specialized heterochromatic structure found at the end of eukaryotic chromosomes. It is composed of telomeric DNA and a six-subunit protein complex, named shelterin, which is required for maintaining and protecting the telomere from nucleolytic attacks. Human TRF1 is one of the shelterin proteins directly involved in telomere length regulation. Post-translational modifications of TRF1 play important roles in modulating telomere length homeostasis by regulating the abundance of TRF1 at telomeres through the interactions with various proteins. Here, we demonstrate the novel post-translational modification of TRF1 called neddylation. We find that TRF1 is subject to NEDD8-conjugation *in vitro* and *in vivo*, and we also identify and characterize NEDP1, which is a human NEDD8-specific protease that efficiently abolishes the neddylation of TRF1. NEDD8 fusion to the C-terminus of TRF1 to mimic the effects of mono-neddylation is found to cause the cytoplasmic localization of TRF1 and reduce the stability of TRF1, suggesting that the neddylation of TRF1 is involved in the subcellular localization and cellular abundance of TRF1. Moreover, the level of TRF1 is increased and cytoplasmic localization is inhibited by MLN4924 treatment, which is a novel NEDD8-Activating Enzyme (NAE) inhibitor, implying that neddylation is a novel post-translational modification mechanism for controlling TRF1 at telomeres.

Keywords: hTRF1, NEDD8, Ubiquitin, Neddylation, Ubiquitination, post-translational modification

2. Introduction

Telomeres, the specialized nucleoprotein complexes found at the ends of linear chromosomes, are essential for chromosome integrity [1]. Properly capped telomeres protect chromosome termini from being recognized as DNA double strand breaks, and the dysfunction of telomeres has been implicated in numerous human diseases and disorders, such as diseases linked to aging and cancer [1, 2]. Mammalian telomeres consist of TTAGGG tandem repeats, and telomeric DNA is closely linked to the six-subunit protein complex named shelterin (TRF1, TRF2, POT1, TIN2, TPP1, and Rap1), which is required for maintaining the telomere structure [2-4]. TRF1 and TRF2 are double-stranded telomeric DNA-binding proteins, whereas POT1 binds to the single-stranded G-rich overhang. Other shelterin components are recruited to telomeres through the interactions with TRF1, TRF2, and POT1 [6,7]. The maintenance of functional telomeres requires telomerase, a unique ribonucleoprotein enzyme that contains telomerase reverse transcriptase (TERT), which can counteract the loss of terminal sequences during DNA replication [5]. However, other solutions to the end replication problem have been documented, generally involving a homologous recombination (HR) mechanism known as alternative lengthening of telomeres (ALT). Maintenance of the telomere length at human chromosome ends, either by telomerase or an alternative mechanism (ALT), is essential to the long-term replicative survival of cells [6].

Among the telomeric proteins, TRF1 and TRF2 directly bind to the double-stranded telomeric repeats and interact with a number of proteins to maintain telomere structure and length [7]. Both proteins contain a C-terminal DNA-binding motif that is closely related to the Myb domain and an internal conserved TRF2 homology domain that mediates dimerization [8]. TRF2 has an essential role in end protection and stabilizes a terminal loop structure called the t-loop [9]. TRF1 is a negative regulator of telomere lengthening by telomerase. Overexpression of TRF1 accelerates telomere shortening, whereas a loss of TRF1 from telomeres has been shown to induce inappropriate telomere elongation [10].

Post-translational modifications of TRF1 play a critical role in modulating telomere length homeostasis by regulating the abundance of TRF1 at telomeres through the interactions with various proteins [11]. We have previously identified casein kinase 2 (CK2) as a TRF1-interacting protein [12]. CK2-mediated phosphorylation is required for the efficient telomere binding of TRF1 and important for TRF1-mediated telomere length control. It has also been reported that the ability of TRF1 to bind telomeric DNA is increased by Polo-like kinase 1 (Plk1)-mediated phosphorylation [13]. In addition, ataxia telangiectasia mutated(ATM) interacts with and phosphorylates TRF1 in response to ionizing DNA damage. Tankyrase 1 has been shown to regulate telomere length through its interaction with TRF1 [14, 15]. Overexpression of Tankyrase 1 induces poly(ADP-ribosyl)ation of TRF1, resulting in dissociation of TRF1 from telomeres and allowing telomerase access to telomeres for telomere elongation [16]. The dissociated telomere-unbound TRF1, by either activation of Tankyrase 1 or inhibition of CK2, is subsequently degraded via ubiquitin-mediated proteolysis by E3 ubiquitin ligases, such as Fbx4 [17-19] and RLIM [20]. Thus, various post-translational modifications of TRF1—including poly(ADP-ribosyl)ation by Tankyrase 1, phosphorylation by CK2, and ubiquitination by Fbx4 or RLIM—may modulate telomere length homeostasis by determining the level of TRF1 at telomeres.

In this study, we identified a novel post-translational modification of TRF1, the conjugation of neural precursor cell expressed developmentally down-regulated 8 (NEDD8) called neddylation. NEDD8 is one of the ubiquitin-like proteins, and its conjugation to target occurs similarly to ubiquitination [21]. NEDD8 is activated by an E1 enzyme (APPBP1/Uba3), transferred to an E2-conjugating enzyme (Ubc12/UBE2M or UBE2F), and subsequently targeted to substrates that are recognized by an E3 ligase [22]. The best-characterized substrates for neddylation are the cullin family members, which is the largest class of RING ubiquitin E3 ligases (CRLs). Neddylation activates the CRLs, implying that it is closely related to ubiquitination [23, 24]. Recently, Recently, non-CRL neddylation targets, including several transcription factors (e.g., p53 [25-27], E2F [28, 29], NFkB [30, 31]), and numerous signaling pathways (e.g., receptor Tyr

kinase signaling [32], apoptosis [33], DNA damage [34] and nucleolar stress signaling [35, 36]) have also been studied, indicating that NEDD8 might have additional biological functions [37]. Moreover, NEDD8 and the neddylation enzymes are overexpressed in human cancers, suggesting that excessive neddylation is involved in cancer development [38, 39]. MLN4924, a potent and selective inhibitor of Nedd8-activating enzyme (NAE), has been identified to suppress the growth of human tumors, and it disrupts CRL-mediated protein turnover, leading to apoptosis in tumor cells [40]. To date, neddylation of TRF1 has not been reported.

3. Materials and Methods

3.1 Cell culture and plasmids construction

The human embryonic kidney cell line HEK293 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100units/ml penicillin, and 100μg/ml streptomycin in 5% CO2 at 37°C. The expression vectors for Flag-TRF1, Myc-SENP8, V5-NEDD8, HA-Ubiquitin were constructed by inserting the full-length cDNAs into pRK5-Flag, pRK5-Myc, pRK5-V5, and pRK5-HA (Stratagene), respectively. The expression vectors for EGFP-TRF1, EGFP-TRF1-NEDD8ΔGG, EGFP-TRF1-UbiquitinΔGG were constructed by inserting the full-length cDNAs into pEGFP-C2 (Clontech). The expression vectors for His-TRF1 and GST-SENP8 were constructed by cloning the full-length and truncated fragments from the TRF1 or SENP8 cDNA into pET28a (Clontech) and pGEX-4T-2 (GE Healthcare), respectively. The GST fusion proteins were expressed and purified by using a glutathione-Sepharose 4B according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). The His fusion proteins were expressed and purified by using a Ni-NTA agarose according to the manufacturer's instructions (Invitrogen)

3.2 GST Pull down, Immunoprecipitation, immunoblotting and antibodies

The expression vectors were transiently transfected into HEK293 cells using Lipofectamine-PLUS reagent according to the manufacturer's protocol (Invitrogen) for 24 h followed by lysis. For GST pull down assay, lysates were precleared with glutathione-Sepharose 4B (Amersham Biosciences, Piscataway, NJ) and incubated with glutathione-Sepharose beads containing GST fusion proteins for 2 h at 4℃. For immunoprecipitation, lysates were preincubated with protein A-Sepharose (Amersham Biosciences, Piscataway, NJ) and incubated with anti-Flag (Sigma) or anti-Myc (Santa Cruz Biotechnology) antibodies precoupled with protein A-Sepharose beads for 2 h at 4℃. Bound proteins were analyzed by SDS-PAGE, followed by immunoblotting. Immunoblotting were performed using anti-Flag (Sigma), anti-Myc (Santa Cruz Biotechnology), anti-V5 (Invitrogen), anti-NUB1 (Santa Cruz Biotechnology) and anti-

tubulin (Santa Cruz Biotechnology) antibodies as specified. All the immunoblots are representatives of at least three experiments that demonstrated the similar results.

3.3 *In vitro* Neddylation assay

In vitro neddylation was performed using *in vitro* neddylation kit according to the manufacturer's protocol (Enzo life science). For the *in vitro* TRF1 neddylation assay, 1ug of purified His-TRF1 was incubated with NEDD E1 (APPBP1-Uba3), E2 (UbcH12) and NEDD8 in a total reaction volume of 20ul (40mM Tris–HCl, pH 7.4, 5mM MgCl2, 2mM ATP and 2mM DTT). Samples were incubated at 37℃ for 1 h, and reactions were terminated with 2X non-reducing SDS-PAGE loading buffer (20mM Tris–HCl, pH 6.8, 100mM DTT, 2% SDS, 20% glycerol and 0.016% Bromophenol blue) before western blotting.

3.4 *In vivo* Neddylation and ubiquitination assay

HEK293 cells transfected with Flag-TRF1, V5-Nedd8, Myc-SENP8 expression vectors were lysed in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% SDS, 10 mM NEM), followed by incubation with Benzonase (Sigma) at 37℃ for 20 min. Lysates were subjected to immunoprecipitation with anti-Flag M2 affinity resins (Sigma), followed by immunoblotting with anti-V5 antibody to illuminate nedd8-conjugated TRF1. HEK293 cells plated on 60-mm dishes were transfected with HA-TRF1, HA-TRF1 mutants (R425V or ΔMyb), FN-TNKS1, FN-TNKS1-DM and His-Ub or His-NEDD8 experssion vectors, as indicated. Cells were harvested after 24 h, and 10% of the cell suspension was kept for input and lysed in 2X SDS sample buffer. The remaining cells were lysed in 6 ml of buffer I (6M guanidinium-HCl [pH 8.0], 0.1M Na_2HPO_4/ NaH_2PO_4, 0.01M Tris-Cl, 10mM β-mercaptoethanol, and 5mM imidazole). 40ul of nickel-agarose beads were added, and lysates were rotated for O/N at 4℃. The beads were then sequentially washed for 5 min at room temperature in 1ml of buffer A (6M guanidinium-HCl [pH 8.0], 0.1M Na_2HPO_4/ NaH_2PO_4, 0.01M Tris-Cl, 10mM β-mercaptoethanol) and 1ml of buffer B (8M Urea [pH 8.0], 0.1M Na_2HPO_4/ NaH_2PO_4, 0.01M Tris-Cl, 10mM β-mercaptoethanol) and 1ml of buffer C (8M Urea [pH 6.3], 0.1M Na_2HPO_4/ NaH_2PO_4, 0.01M Tris-Cl, 0.2%

Triton X-100, 10mM β-mercaptoethanol) and 1ml of buffer D (8M Urea [pH 6.3], 0.1M Na_2HPO_4/ NaH_2PO_4, 0.01M Tris-Cl, 0.1% Triton X-100, 10mM β-mercaptoethanol).

3.5 Immunofluorescence and confocal microscopy

Cells were grown on glass coverslips, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized in 0.5% Triton X-100 in PBS for 20 min. Cells were then blocked in PBS containing 5% bovine serum albumin and incubated with anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4℃. After washing with PBS, cells were incubated with Alexa Fluor 546 goat anti-mouse immunoglobulin (Molecular Probes). DNA was stained with 4,6-diamino-2-phenylindole (DAPI) (Vectashield; Vector Laboratories, Burlingame, CA). Immunofluorescence images were captured using a confocal laser-scanning microscope (Carl Zeiss LSM700, Carl Zeiss, Inc., Jena, Germany).

3.6 Subcellular Fractionation

Cells were collected by centrifugation at 3000 x g for 4 min at 4℃ and suspended in 400ul buffer A (10mM HEPES [pH 7.5], 10mM KCl, 1mM DTT, 1mM PMSF, 0.05% NP40). After incubation on ice for 10 min. Nuclei were pelleted at 3000 x g for 5 min, and the supernatant (cytoplasmic extracts) was centrifuged at 13,000 x g for 20min. Nuclei were washed with 1ml of buffer A twice and suspended in 100ul of buffer B (20 mM HEPES [pH 7.5], 0.4M NaCl, 1mM DTT, 1mM PMSF. Nuclear extract was collected by centrifugation at 18,000 x g for 20 min.

3.7 RNA interference

The siRNA duplexes were transfected into HEK293 cells using Lipofectamine2000 transfection reagent (Invitrogen). The siRNA target sequences specific for NEDD8 were 5’-CATAATGAGGCATCATATA-3’. The scrambled sequence (5’-AATCGCATAGCGTATGCCGTT-3’) was used as a negative control and did not correspond to any known gene in the data bases

4. Results

4.1 TRF1 is target for NEDD8-conjugation system

TRF1 is subject to extensive post-translational modifications that in turn contribute to the regulation of their stability, binding activity, and localization [11]. Several previous studies have shown the role of post-translational modifications in controlling TRF1, such as phosphorylation [12, 13], ubiquitylation [18, 20] and SUMOlyation [41] in telomere maintenance and DNA damage response. However, NEDD8 conjugation of TRF1 has not yet been determined. To investigate whether TRF1 can be conjugated with Nedd8, we first performed *in vitro* neddylation assays with purified His-TRF1, Nedd8, NAE (E1), and Ubc12 (E2); reacted them with or without the Mg-ATP condition; and analyzed them by immunoblotting using endogenous TRF1 antibody and NEDD8 antibody. The results showed that TRF1 migrated as high-molecular weight bands when the Nedd8, NAE (E1), Ubc12 (E2), and Mg-ATP were present in the reaction (Figure 1A). To verify whether TRF1 is neddylated *in vivo*, HEK293 cells were co-transfected with Flag-TRF1 and V5-NEDD8 or V5-NEDD8ΔGG mutant and subjected to immunoprecipitation with anti-Flag antibody followed by anti-V5 antibody. The NEDD8-conjugated TRF1 was readily detected in the immunoprecipitates; however, these bands disappeared in the presence of a Nedd8ΔGG mutant, which abolishes the covalent conjugating ability of Nedd8 (Figure 1B). These immunoprecipitated proteins were not recognized by the ubiquitin antibody. In addition, neddylated-TRF1 was eliminated by treatment with MLN4924, a potent and specific inhibitor of NAE (Figure 1C). Furthermore, we confirmed the *in vivo* neddylation of TRF1 using endogenous NEDD8 antibody to illuminate Nedd8-modified TRF1. Neddylated-TRF1 disappeared by MLN4924 treatment and by depletion of NEDD8 using NEDD8 siRNA (Figure 1D). Taken together, we found the novel post-translational modification of TRF1, which is NEDD8-conjugation, called neddylation.

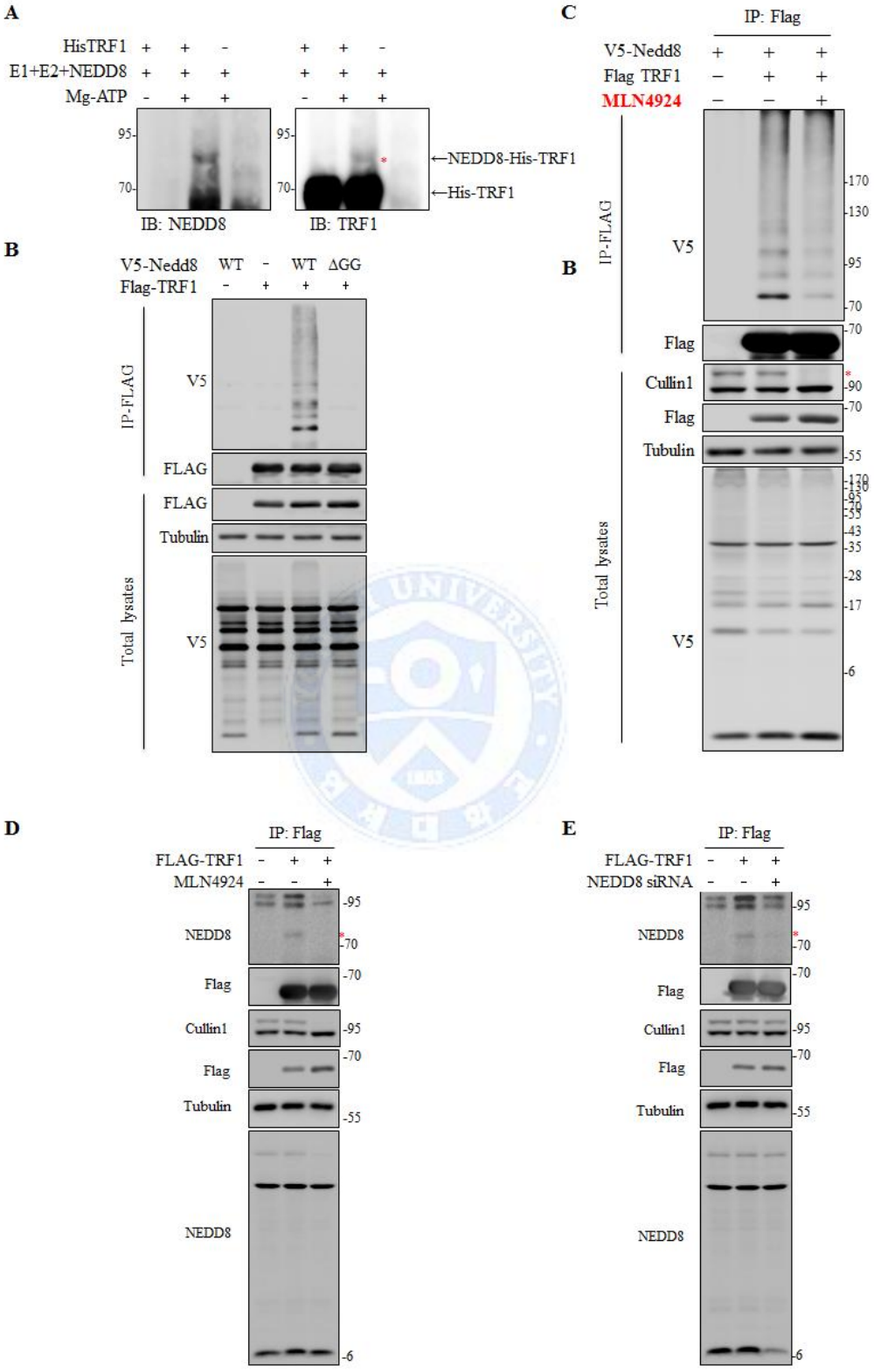
A
HisTRF1
E1+E2+NEDD8
Mg-ATP
95
70
IB: NEDD8
IB: TRF1
←NEDD8-His-TRF1
←His-TRF1
B
V5-Nedd8 WT - WT ΔGG
Flag-TRF1 - + + +
IP-FLAG
V5
FLAG
Total lysates
FLAG
Tubulin
V5
C
IP: Flag
V5-Nedd8
Flag TRF1
MLN4924
IP-FLAG
V5
Flag
Cullin1
Flag
Tubulin
Total lysates
V5
170
130
95
70
90
55
43
35
28
17
6
D
IP: Flag
FLAG-TRF1
MLN4924
NEDD8
Flag
Cullin1
Flag
Tubulin
NEDD8
E
IP: Flag
FLAG-TRF1
NEDD8 siRNA
NEDD8
Flag
Cullin1
Flag
Tubulin
NEDD8

Figure 1. TRF1 is target for NEDD8-conjugation. (A) Covalent neddylation of TRF1 *in vitro*. Purified His-TRF1 proteins were incubated with Nedd8 and Nedd8-E1/E2. Reactions were performed as described in the Materials and Methods. Samples were analysed by western blotting with anti-TRF1 and anti-NEDD8 antibodies. (B) *In vivo* TRF1 neddylation assay. HEK293 cells were co-transfected with Flag-TRF1 with V5-Nedd8 WT or ΔGG and subjected to immunoprecipitation with anti-Flag antibody, followd by immunoblotting with anti-V5 antibody to detect Nedd8 conjugated TRF1. (C) TRF1 neddylation was significantly attenuated by the NAE inhibitor MLN4924. HEK293 cells were co-transfected with Flag-TRF1 with V5-Nedd8 WT and treated with MLN4924 (1uM, 20h). Lysates subjected to immunoprecipitation with anti-Flag antibody, followd by immunoblotting with anti-V5 antibody. (D) *In vivo* TRF1 neddylation assay. HEK293 cells were transfected with Flag-TRF1 and treated with MLN4924 (1uM, 20h) and subjected to immunoprecipitation with anti-Flag antibody, followd by immunoblotting with anti-NEDD8 antibody to detect Nedd8 conjugated TRF1. (E) *In vivo* TRF1 neddylation assay. HEK293 cells were transfected with Flag-TRF1 and control siRNA or NEDD8 siRNA and subjected to immunoprecipitation with anti-Flag antibody, followd by immunoblotting with anti-NEDD8 antibody to detect Nedd8 conjugated TRF1.

4.2 Deneddylation enzyme NEDP1 abolishes covalent neddylation of TRF1 *in vitro* and *in vivo*

To verify that these conjugations were neddylations, we performed in vitro neddylation assays and incubated the components with purified His-TRF1 and the GST-NEDP1, which is Nedd8-specific protease [42-44]. We found that NEDP1, which is Nedd8-specific protease, efficiently removed these conjugation bands, suggesting that these conjugations are neddylations (Figure 2A). We next investigated the interaction of TRF1 and NEDP1 using purified His-TRF1 and GST-NEDP1 by a GST pull-down assay. GST-NEDP1, but not the control GST, precipitated His-TRF1, indicating that TRF1 associates with NEDP1 *in vitro* (Figure 2B). We further examined the effect of NEDP1 on the neddylation of TRF1 *in vivo*. HEK293 cells were co-transfected with Flag-TRF1, V5-NEDD8, Myc-NEDP1, and Myc-NUB1 and subjected to immunoprecipitation with anti-Flag antibody followed by anti-V5 antibody to detect Nedd8-modified TRF1. NUB1 has been shown to directly interact with NEDD8, and the characterized function of NUB1 was recruiting NEDD8 and NEDD8 conjugates to the proteasome for degradation. Previously, we found NUB1 to be a TRF1-interacting protein; therefore, we also examined the neddylation of TRF1 by NUB1. We found that neddylated TRF1 was abolished by overexpression of NEDP1 and NUB1 (Figure 2C). Moreover, MG132 treatment prevented the reduction caused by NUB1 but did not block the reduction caused by NEDP1, suggesting that NUB1 recruited the neddylated TRF1 to the proteasome for degradation and NEDP1 detached NEDD8 from TRF1 (Figure 2D).

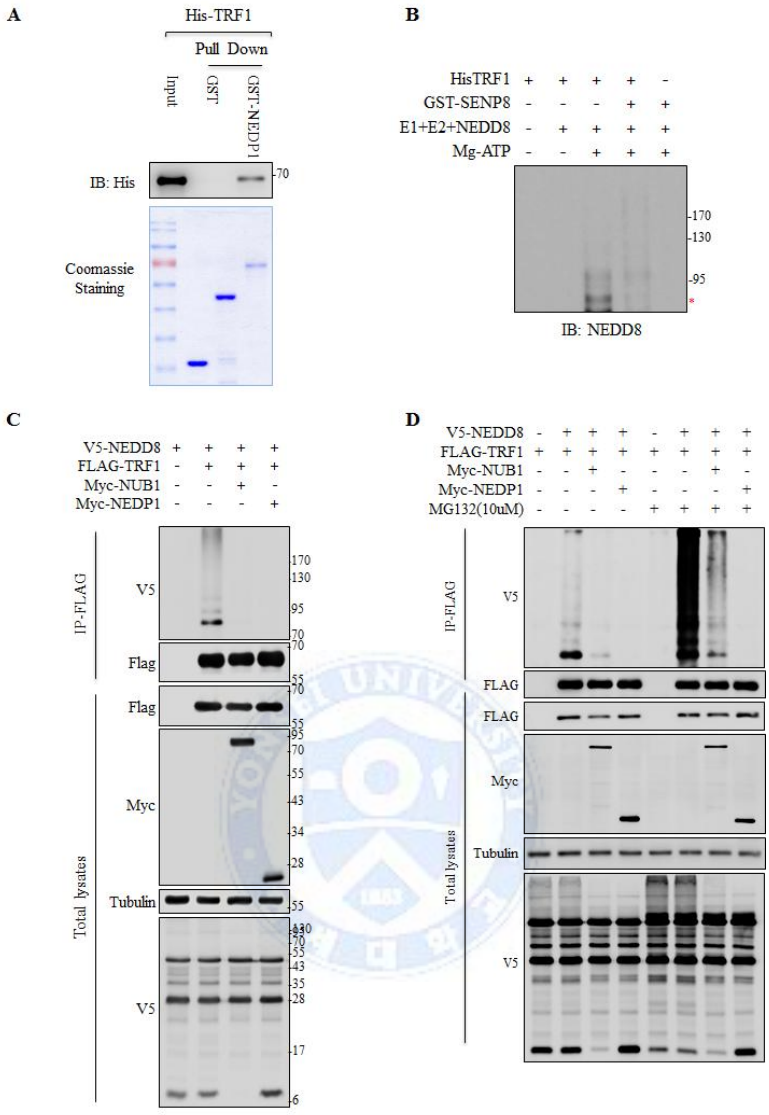

Figure 2. Deneddylation enzyme NEDP1 abolished covalent neddylation of TRF1 *in vitro* and *in vivo*. (A) Purified His-TRF1 proteins were incubated with Nedd8, Nedd8-E1/E2 and, with or without GST-NEDP1. Samples were analysed by western blotting with an anti-NEDD8 antibody. (B) NEDP1 specifically interacts with TRF1 in vitro and in vivo. GST, GST-NEDP1 were immobilized on glutathione-Sepharose and incubated with Purified His-TRF1. Bound proteins were detected by immunoblotting with anti-His antibody. (C) HEK293 cells were co-transfected with Flag-TRF1 and Myc-NEDP1 and subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-Myc antibody. (D) HEK293 cells were co-transfected with Flag-TRF1, V5-NEDD8 and Myc-NEDP1 and subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-V5 antibody to detect Nedd8 conjugated TRF1.

4.3 NEDD8-conjugation stimulates cytoplasmic localization of TRF1 and reduces stability of TRF1

Ubiquitin fusions have been demonstrated to mimic the mono-ubiquitination of many proteins. Although it remains unclear how accurately a C-terminal fusion represents the mono-ubiquitination of internal lysines, previous studies have shown that this alternative approach involves fusion of the required Ubl proteins, such as SUMO and NEDD8, to the C-terminus of the substrate protein to mimic the effects of mono-ubiquitination of a number of proteins, including p53 [26, 45], EGFR [32], DEDD [46]. Recently, the SUMOylation function of TRF1 was also reported by using the TRF1-SUMO fusion construct [41]. Mutation of the C-terminal glycines in the Ubl portion (ΔGG) inhibits the formation of isopeptide bonds between Ubls and target lysines and thus prevents the use of the fusion proteins in this way. Based on previous studies, we generated TRF1-NEDD8ΔGG and TRF1-UbquitinΔGG fusion constructs analogous to the previous methods. HEK293 cells were transfected with GFP-TRF1, GFP-TRF1-NEDD8ΔGG, and TRF1-UbΔGG fusion constructs, and the subcellular localization was analyzed. As shown in Figure 3A, GFP-TRF1 was predominantly localized to the nucleus, whereas NEDD8ΔGG or UbΔGG fusion at the C-terminus of TRF1 led to the cytoplasmic localization of TRF1. To confirm the immunofluorescence results, we performed subcellular fractionation and analyzed the results by immunoblotting. The fractionation showed that GFP-TRF1 was detected mainly in the nucleus; however, the NEDD8- or ubiquitin-fused TRF1 was found in the cytoplasm and accumulated by MG132 treatment (Figure 3B, C), implying that mono-neddylation or mono-ubiquitination promotes the cytoplasmic localization of TRF1 and is subsequently degraded by the proteasome. We further confirmed that NEDD8 or Ub fusion affects the stability of TRF1. HEK293 cells were transfected with GFP-TRF1, GFP-TRF1-NEDD8ΔGG, and TRF1-UbΔGG; treated with cycloheximide (100ug/ml) as indicated time to block new protein synthesis; and analyzed by immunoblotting with anti-GFP antibody. As shown in Figure 3D and graphically in Figure 3E, NEDD8, or ubiquitin fusion at the C-terminus of TRF1, reduced the half-life of TRF1 compared to the wild-type TRF1.

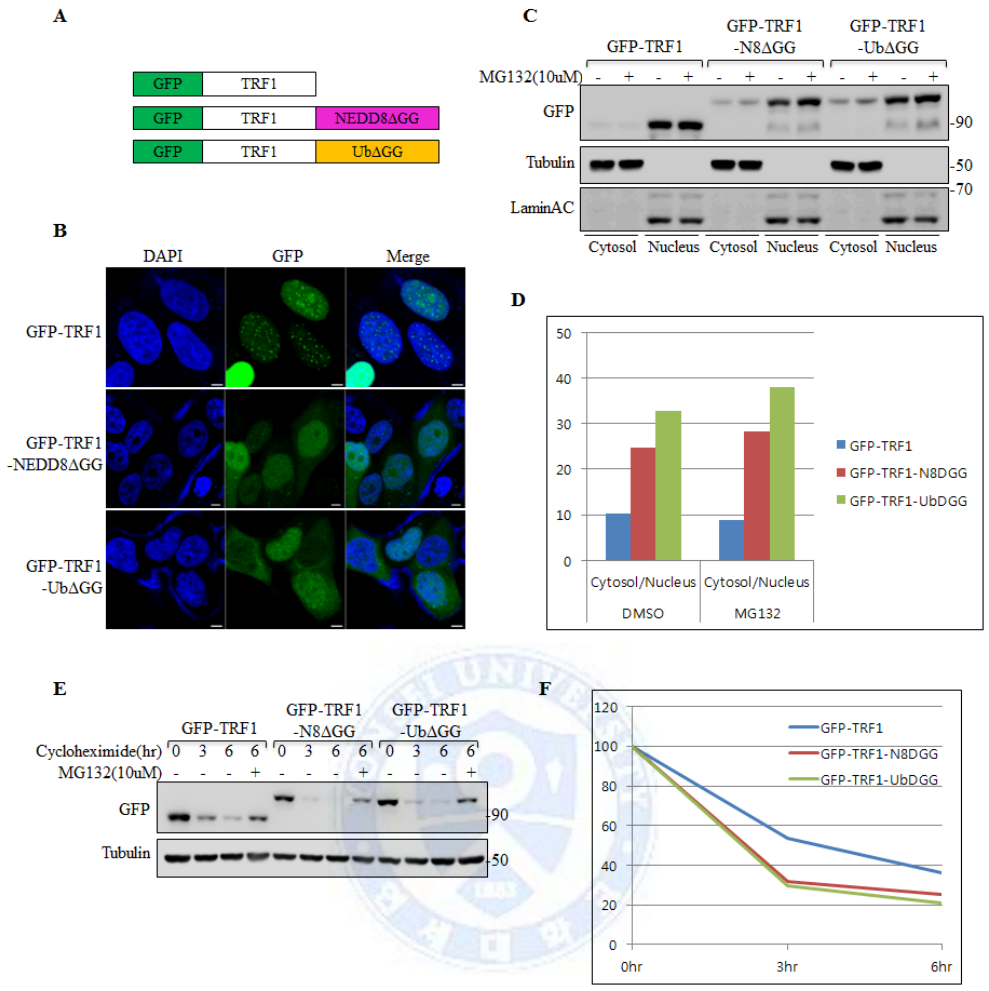

Figure 3. NEDD8-conjugation stimulates cytoplasmic localization of TRF1. (A) Schematic representation of the GFP-TRF1, GFP-TRF1-Nedd8ΔGG and GFP-TRF1-UbΔGG constructs used in this experiment. (B) MCF7 cells were transiently transfected with GFP-TRF1, GFP-TRF1-Nedd8ΔGG and GFP-TRF1-UbΔGG as indicated and GFP-fused proteins were monitored by confocal microscope. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue). (C) Cytoplasmic and nuclear fractions were analyzed by western blotting with anti-GFP antibodies. Lamin A/C and tubulin were used as nuclear and cytoplasmic markers, respectively. Bar graphs represent quantification of immunoblot images. (D) HEK293 cells were transfected with GFP-TRF1, GFP-Nedd8ΔGG and GFP-TRF1-UbΔGG expression vectors and treated with 100μg/ml cycloheximide together with, or without, 10μM MG132 for the indicated times. (E) Lysates were analyzed by immunoblotting with anti-GFP and anti-tubulin antibodies and performed quantitation of immunoblot signals

4.4 MLN4924 treatment enhances stability of TRF1 and inhibits cytoplasmic export of TRF1

Because MLN4924 efficiently abolished the NEDD8-conjugation of TRF1, we next examined its ability to regulate the level of TRF1. Interestingly, the level of TRF1 was increased by MLN4924 treatment (Figure 4A). In addition, the fractionation result showed that TRF1 levels greatly increased in the nucleus by MLN4924 treatment compared to the cytoplasm, and the cytoplasmic ratio per nucleus was significantly reduced compared to the DMSO treatment (Figure 4B, C). Taken together, inhibition of the NEDD8-conjugation system by MLN4924 treatment enhanced the stability of TRF1 and inhibited the cytoplasmic export of TRF1.

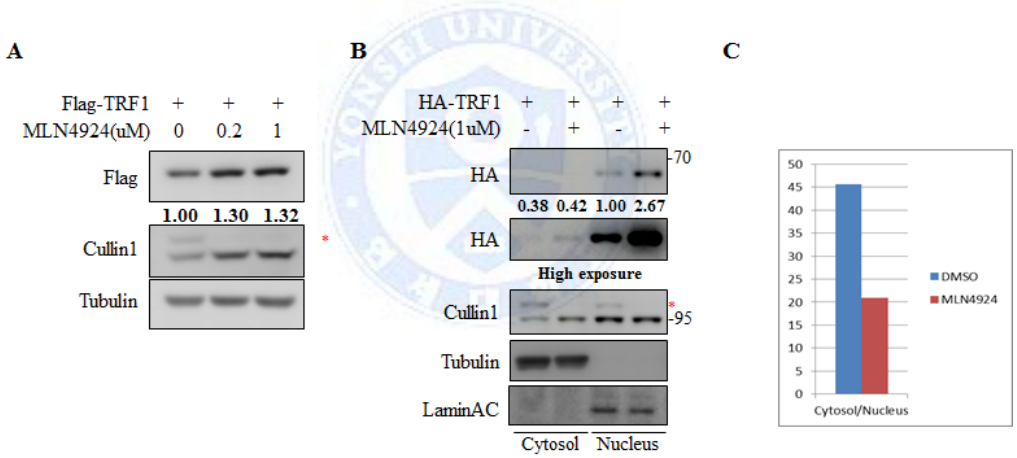

Figure 4. MLN4924 stablized the TRF1 levels. (A) HEK293 cells were transiently transfected with Flag-TRF1 and treated with 1uM of MLN4924 for 20hrs. Lysates were analyzed by immunoblotting with anti-Flag and anti-tubulin antibodies. (B) HEK293 cells were transiently transfected with HA-TRF1 and treated with 1uM of MLN4924 for 20hrs. Cytoplasmic and nuclear fractions were analyzed by western blotting with anti-HA antibodies. Lamin A/C and tubulin were used as nuclear and cytoplasmic markers, respectively. (C) Bar graphs represent quantification of immunoblot images.

4.5 Neddylation and ubiquitination of TRF1 is increased after release from telomere

It has been reported that TRF1 is degraded by ubiquitin-mediated proteolysis after its release from telomeres [17]. Either NEDD8ΔGG or UbΔGG fusion at the C-terminus of TRF1 causes a clear reduction in the half-life of TRF1 and promotes the cytoplasmic localization of TRF1. We further confirmed TRF1 neddylation using the R425V mutant and the Myb domain-deleted TRF1 construct. The R425V mutant was shown to disrupt the binding of TRF1 to the telomere [47], and the Myb domain of TRF1 was shown to be responsible for telomere binding [17, 18]. HEK293 cells were co-transfected with HA-TRF1 or HA-TRF1 mutants (R425V or ΔMyb) and His-NEDD8 or His-Ub expression vectors. Thereafter, cell lysates containing His Ub-modified or His NEDD8-modified proteins were precipitated under denaturing conditions by Ni-NTA pull-down, and the neddylated TRF1 and ubiquinated TRF1 were analyzed by immunoblotting using anti-HA antibody. We found that neddylation of TRF1 is increased on the telomere unbound TRF1 mutant but does not occur on the Myb deletion mutant, and ubiquitination is also increased on TRF1 R425V mutants and abolished on Myb deletion mutants as reported previously, suggesting that NEDD8 conjugation of TRF1 is increased after release from telomere (Figure 5A, B). Furthermore, to examine whether the dissociation of TRF1 from telomere contributes to the neddylation of TRF1 *in vivo,* HEK293 cells were co-transfected with HA-TRF1 and His-NEDD8, His-Ub and Flag-tankyrase1 (FN-TNKS1), or poly(ADP-ribose) polymerase (PARP) activity dead mutant of Flag-tankyrase1 (FN-TNKS1 DM) expression vectors. Lysates were precipitated under denaturing conditions by Ni-NTA pull-down, and the neddylated-TRF1 and ubiquinated-TRF1 analyzed by immunoblotting using anti-HA antibody. It has been reported that the poly(ADP-ribosyl)ation of TRF1 by overexpression of TNKS1 removes TRF1 from telomeres, resulting in TRF1 ubiquitination and degradation by the proteasome [14, 48]. We found that, like ubiquitination, overexpression of Tankyrase1 increased the neddylation of TRF1, whereas PARP-dead mutant of Tankyrase1 does not affect to the neddylation of TRF1 or ubiquitination of TRF1 (Figure 5C, D). Taken together, these results strongly indicate that neddylation of TRF1 is increased after release from telomere.

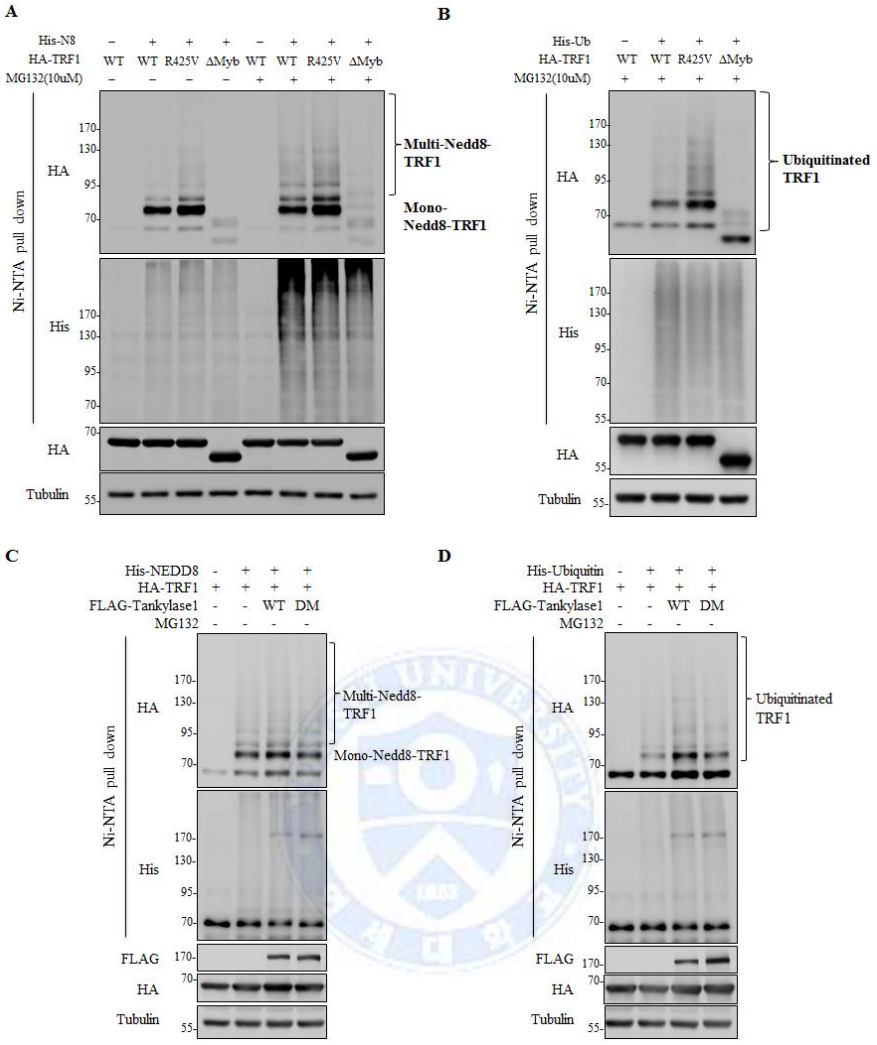

Figure 5. Neddylation and ubiquitination of TRF1 is increased after release from telomere. (A) HEK293 cells were co-transfected with His tagged NEDD8, HA-TRF1(WT), HA-TRF1(R425V) and HA-TRF1(ΔMyb) and performed Ni-NTA pull-down under denature condition followed by immunoblotting with anti-HA antibody. (B) HEK293 cells were transfected with His tagged Ubiquitin, HA-TRF1(WT), HA-TRF1(R425V) and HA-TRF1ΔMyb and performed Ni-NTA pull-down under denature condition followed by immunoblotting with anti-HA antibody. (C) HEK293 cells were co-transfected with His tagged NEDD8, HA-TRF1 and Flag-Tankylase1(WT) or Flag-Tankylase1(DM) and performed Ni-NTA pull-down under denature condition followed by immunoblotting with anti-HA antibody. (D) HEK293 cells were co-transfected with His tagged Ubiquitin, HA-TRF1 and Flag-Tankylase1(WT) or Flag-Tankylase1(DM) and performed Ni-NTA pull-down under denature condition followed by immunoblotting with anti-HA antibody.

4.6 Neddylation and ubiquitination is linked for regulating the cellular abundance of TRF1

Because TRF1 neddylation and ubiquitination occur in very similar ways, next we focus on possible linkage between neddylation and ubiquitination on TRF1. HEK293 cells were co-transfected with V5-NEDD8 or HA-Ubiquitin, Flag-TRF1, Myc-Fbx4, Myc-NEDP1, and Myc-USP22 [49], which is TRF1-specific ubiquitin protease, as indicated. Cell lysates were immunoprecipitated with anti-Flag antibodies and the neddylated-TRF1 and ubiquinated-TRF1 analyzed by immunoblotting using anti-V5 antibody and anti-HA antibody, respectively. Interestingly, deubiquitinase USP22 abolished the ubiquitination but not neddylation of TRF1, and deneddylase NEDP1 abolished the neddylation but not ubiquitination. Moreover, Fbx4 promotes the ubiquitination and the neddylation of TRF1, suggesting that neddylation may be linked with ubiquitination (Figure 6A, B). To further confirm whether the Fbx4 contributes to the neddylation of TRF1, HEK293 cells were co-transfected with His-NEDD8, HA-TRF1, Myc-Fbx4, Myc-SENP8, and Myc-UBE2M. UBE2M, which is NEDD8-specific E2 enzyme, was used as a control. Lysates were precipitated under denaturing conditions by Ni-NTA pull-down and the neddylated-TRF1 analyzed using anti-HA antibody. We found that Fbx4 promotes the neddylation of TRF1 and that overexpression of NEDP1 clearly abolished the neddylated TRF1 caused by Fbx4, suggesting that Fbx4 also stimulates the neddylation of TRF1 (Figure 6C). Additionally, we found that the ubiquitination of TRF1 is not affected by MLN4924 or depletion of NEDD8, and that NEDD8 overexpression significantly reduced the ubiquitination of TRF1 (Figure 6D).

4.7 Identification of NEDD8-conjugation site on TRF1

To examine the exclusive function of TRF1 neddylation, we tried to identify the NEDD8-conjugation site on TRF1. We found that neddylation of TRF1 is increased on the telomere unbound TRF1 mutant but does not occur on the Myb deletion mutant (Figure 5A). There are eight lysine residues on the Myb domain of TRF1. To examine which lysine residue(s) could be neddylated, we mutated each lysine into arginine. However, none of these mutations could abolish the TRF1 neddylation (Figure 7A). To further validate that Myb domain lysine residues are neddylated, we mutated all eight lysine residues into arginine sequentially. However, even though all eight lysine residues were mutated into arginine, TRF1 could still be neddylated (Figure 7B), suggesting that Myb domain deletion might affect conformation of TRF1, resulting in different consequences between domain study and mutagenesis study. Further study is required to identify the NEDD8-conjugation sites for TRF1.

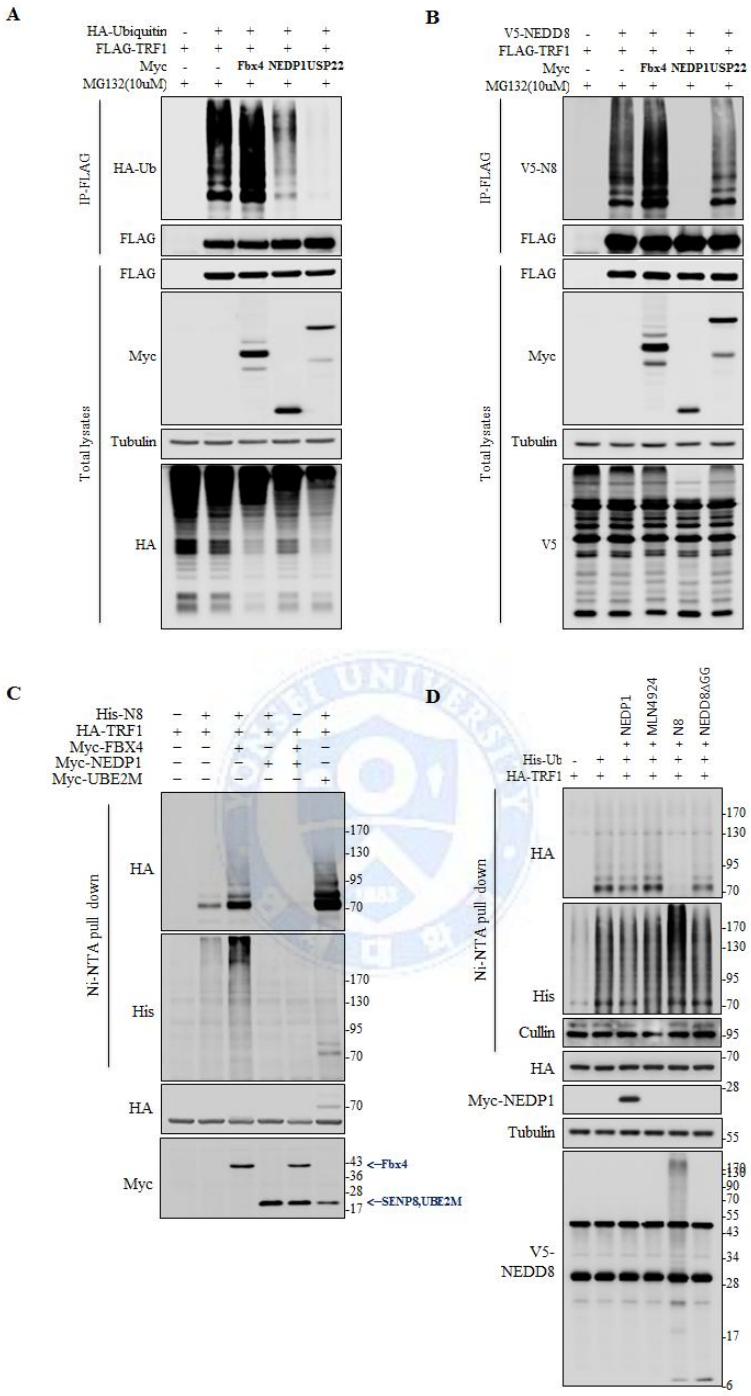

Figure 6. Possible linkage between neddylation and ubiquitination of TRF1 (A) *In vivo* ubiquitination assay. HEK293 cells were co-transfected with Flag-TRF1, HA-Ubiquitin and Myc-Fbx4 or Myc-NEDP1 or Myc-USP22 and subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-HA

antibody to detect ubiquitinated TRF1 (B) *In vivo* neddylation assay. HEK293 cells were co-transfected with Flag-TRF1, V5-NEDD8 and Myc-Fbx4 or Myc-NEDP1 or Myc-USP22 and subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-V5 antibody and anti-HA antibody to detect Nedd8 conjugated TRF1 and ubiquitinated TRF1, respectively. (C) *In vivo* neddylation assay under denaturation condition. HEK293 cells were co-transfected with HA-TRF1, His-NEDD8, Myc-Fbx4,Myc-NEDP1, and Myc-UBE2M. and lysates were precipitated by Ni-NTA pull-down and the neddylated-TRF1 analyzed using anti-HA antibody (D) *In vivo* ubiquitination assay under denaturation condition. HEK293 cells were co-transfected with HA-TRF1, His-Ub, Myc-NEDP1, V5-NEDD8, V5NEDD8ΔGG, and 1uM od MLN4924 for 4hrs as indicated. Lysates were precipitated by Ni-NTA pull-down and the neddylated-TRF1 analyzed using anti-HA antibody

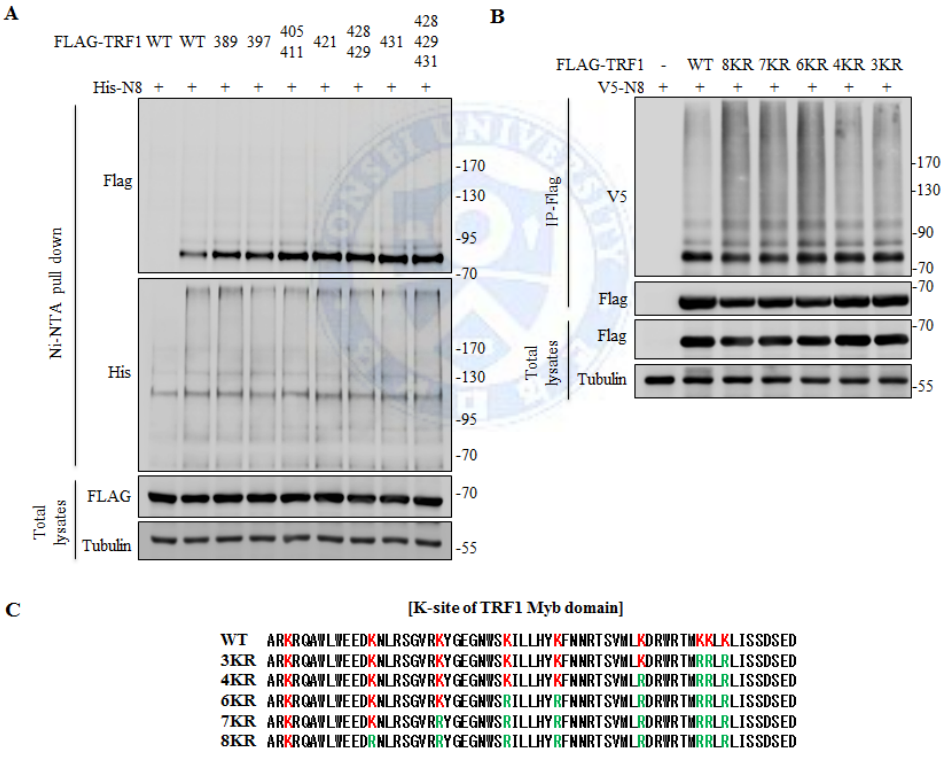

Figure 7. Identification of NEDD8-conjugation site on TRF1 (A) *In vivo* neddylation assay under denaturation condition. HEK293 cells were co-transfected with various Flag-tagged point mutants of TRF1 and, His NEDD8. Lysates were precipitated by Ni-NTA pull-down and the neddylated-TRF1 analyzed using anti-Flag antibody (B) *In vivo* neddylation assay. HEK293 cells were co-transfected with various Flag-tagged multiple lysine(K)-mutants of TRF1, V5-NEDD8 subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-V5 antibody to detect neddylated TRF1.

5. Discussion

TRF1 directly binds to telomeric DNA and plays crucial roles in the maintenance of telomere length and structure. The post-translational modification of TRF1 is important for modulating telomere length homeostasis by regulating the cellular abundance of TRF1. Recent biochemical studies have revealed the existence of numerous modifying enzymes, which contribute to the regulation of stability, binding activity, and localization of TRF1 [11]. Phosphorylation of TRF1 is mainly involved in the telomeric binding activity and stability, modulated by many different kinases such as AKT, ATM, CDK1, CK2, and Plk1. Overexpression of AKT led to an increase in the level of TRF1 and a decrease in telomere length [50] and ATM negatively regulates TRF1 binding to telomeric DNA, which in turn promotes telomerase-dependent telomere lengthening [51]. Plk1-mediated phosphorylation and CK2-mediated phosphorylation promotes interaction between TRF1 and telomeric DNA [12, 13]. MMS21-mediated SUMOylation has been reported to facilitate the TRF1 association with telomeric DNA needed for telomere recombination in ALT cells [52]. Poly(ADP-ribosyl) ation of TRF1 by Tankyrase1 removes TRF1 from telomeric DNA, after which dissociated TRF1 is ubiquitinated by Fbx4 and RLIM to promote ubiquitin-mediated proteolysis [19, 20]. These post-translational modifications are very close to each other for controlling the TRF1, and they play crucial roles in the maintenance of telomere length and structure.

Here, we identified the novel post-translational modification of TRF1 (i.e., neddylation). Although cullin family proteins are the best-validated target for neddylation, recent studies have reported that several non-cullin proteins are also neddylated and, in turn, regulate the diverse cellular processes [37]. In this paper, it is found for the first time that TRF1 is a target for NEDD8-conjugation *in vitro* and *in vivo* and the treatment of MLN4924, which is an NAE inhibitor [39], efficiently reduces the neddylation of TRF1. NEDP1 is a Cys protease that selectively binds to NEDD8 resulting in removal of NEDD8 from neddylated substrates [42, 44]. We also identified that NEDP1 directly interacts with TRF1 in vitro and clearly abolished the neddylation of TRF1 in vitro and in

vivo. To date, NEDD8-conjugation of TRF1 has not been reported; therefore, we further confirmed the function of neddylation on TRF1.

NEDD8-conjugation is similar to ubiquitination. To examine the biological function of neddylation, we generated NEDD8 GG- or ubiquitin GG-fused TRF1 constructs for mimicking the mono-neddylated or mono-ubiquitinated TRF1, based on the previous studies [26, 41]. Interestingly, we found that the expression of these C-terminal fusion proteins in the cytoplasm and accumulated by MG132 treatment, implying that neddylation led to cytoplasmic localization of TRF1 and was degraded by proteasome. Moreover, the stability of TRF1 was also decreased by fusion of NEDD8 or ubiquitin, which suggests that mono-neddylation as well as mono-ubiquitination are involved in the stability and subcellular localization of TRF1. These results remain unclear because the C-terminal fusion is not quite accurate compared with actual neddylation that occurs in internal lysine on TRF1. Additionally, we found that the TRF1 levels are greatly increased by MLN4924 treatment, especially in nuclear fraction, indicating that the inhibition of neddylation stabilizes the level of TRF1 and prevents the cytosolic localization of TRF1.

As NEDD8 and the neddylation enzymes are overexpressed in human cancers, NEDD8-mediated protein turnover is a novel and effective anticancer strategy [39]. Earlier studies have shown that the activity of CRLs is directly regulated by modification with NEDD8, resulting in controlling the ubiquitination and subsequent proteasomal degradation of numerous CRL target proteins [53, 54]. Inhibition of NAE by MLN4924 led to diverse cellular dysfunctions, such as DNA re-replication and damage that, in turn, fails to repair, leading to apoptosis or senescence [55, 56]. MLN4924 also inhibits the neddylation of non-cullin substrates; however, the biological consequences have not been studied. In this study, we found that neddylation of TRF1 is inhibited and expression level dramatically increased by MLN4924 treatment. The cellular abundance of TRF1 was directly involved in telomere length regulation, suggesting that neddylation plays crucial roles in regulation of TRF1.

Previous reports have shown that the TRF1 is degraded by ubiquitin-mediated

proteolysis after release from telomeres by tankyrase1-mediated poly(ADP-ribosyl)ation [17]. We found that neddylation of TRF1 was also increased by tankyrase1 overexpression, similar to ubiquitination. Ubiquitination of TRF1 is mediated by the Fbx4, which is a subunit of the Skp1-Cul1/Rbx1-F-box protein) ubiquitin E3 ligases. Unexpectedly, we found that Fbx4 also promotes the TRF1 neddylation, suggesting that Fbx4, as a TRF1-specific adaptor, stimulates both ubiquitination and neddylation of TRF1. Several reports have shown that neddylation is linked with ubiquitination. However, knowledge of a link between the two effects on TRF1 remains unclear. Thus, further work is required to assess whether TRF1 neddylation has a physiological significance and a biological function.

The dynamic regulation of TRF1 at telomeres by post-translational modifications and their interacting proteins, such as TIN2, Tankyrase1, and Fbx4, plays an important role in telomere homeostasis maintenance [11]. Dissociation of TRF1 from telomeres by Tankyrase1 allows the telomerase access to the chromosome ends for telomere extension; therefore, unbound TRF1 is subsequently degraded by ubiquitin-mediated degradation [17]. On the other hand, TIN2 protects TRF1 from ubiquitination and recycles the TRF1 back to the telomeres through deubiquitination. Therefore, TRF1 is required for efficient telomere DNA replication [57]. The present findings suggest that novel post-translational modification of TRF1, called neddylation for controlling the stability and subcellular localization, provides a potential mechanism for telomere maintenance.

Acknowledgments

We thank Titia de Lange at rockefeller university for kindly providing the hTRF1(#370) antibody.

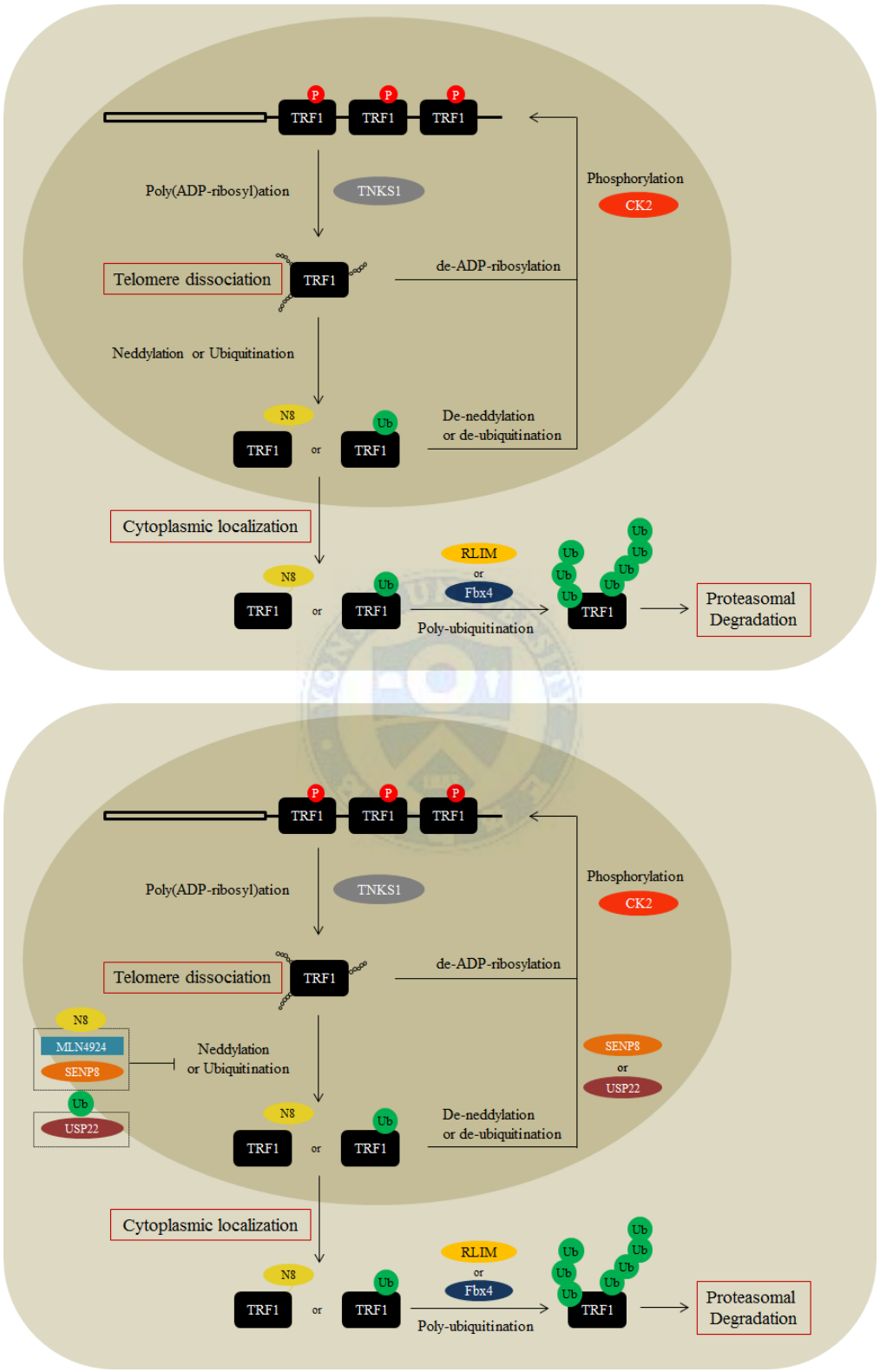

Figure. 8. Model for regulation of TRF1 by Neddylation

6. References

[1] A. Smogorzewska, T. de Lange, Regulation of telomerase by telomeric proteins, Annual review of biochemistry, 73 (2004) 177-208.

[2] D. Liu, M.S. O'Connor, J. Qin, Z. Songyang, Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins, The Journal of biological chemistry, 279 (2004) 51338-51342.

[3] J.D. Griffith, L. Comeau, S. Rosenfield, R.M. Stansel, A. Bianchi, H. Moss, T. de Lange, Mammalian telomeres end in a large duplex loop, Cell, 97 (1999) 503-514.

[4] J.A. Stewart, M.F. Chaiken, F. Wang, C.M. Price, Maintaining the end: roles of telomere proteins in end-protection, telomere replication and length regulation, Mutation research, 730 (2012) 12-19.

[5] C.W. Greider, E.H. Blackburn, Identification of a specific telomere terminal transferase activity in Tetrahymena extracts, Cell, 43 (1985) 405-413.

[6] M.A. Cerone, C. Autexier, J.A. Londono-Vallejo, S. Bacchetti, A human cell line that maintains telomeres in the absence of telomerase and of key markers of ALT, Oncogene, 24 (2005) 7893-7901.

[7] A. Smogorzewska, B. van Steensel, A. Bianchi, S. Oelmann, M.R. Schaefer, G. Schnapp, T. de Lange, Control of human telomere length by TRF1 and TRF2, Molecular and cellular biology, 20 (2000) 1659-1668.

[8] D. Broccoli, A. Smogorzewska, L. Chong, T. de Lange, Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2, Nature genetics, 17 (1997) 231-235.

[9] B. van Steensel, A. Smogorzewska, T. de Lange, TRF2 protects human telomeres from end-to-end fusions, Cell, 92 (1998) 401-413.

[10] B. van Steensel, T. de Lange, Control of telomere length by the human telomeric protein TRF1, Nature, 385 (1997) 740-743.

[11] J.R. Walker, X.D. Zhu, Post-translational modifications of TRF1 and TRF2 and their roles in telomere maintenance, Mechanisms of ageing and development, 133 (2012) 421-434.

[12] M.K. Kim, M.R. Kang, H.W. Nam, Y.S. Bae, Y.S. Kim, I.K. Chung, Regulation of

telomeric repeat binding factor 1 binding to telomeres by casein kinase 2-mediated phosphorylation, The Journal of biological chemistry, 283 (2008) 14144-14152.
[13] Z.Q. Wu, X. Yang, G. Weber, X. Liu, Plk1 phosphorylation of TRF1 is essential for its binding to telomeres, The Journal of biological chemistry, 283 (2008) 25503-25513.
[14] J.R. Donigian, T. de Lange, The role of the poly(ADP-ribose) polymerase tankyrase1 in telomere length control by the TRF1 component of the shelterin complex, The Journal of biological chemistry, 282 (2007) 22662-22667.
[15] S. Smith, I. Giriat, A. Schmitt, T. de Lange, Tankyrase, a poly(ADP-ribose) polymerase at human telomeres, Science, 282 (1998) 1484-1487.
[16] S. Smith, T. de Lange, Tankyrase promotes telomere elongation in human cells, Current biology : CB, 10 (2000) 1299-1302.
[17] W. Chang, J.N. Dynek, S. Smith, TRF1 is degraded by ubiquitin-mediated proteolysis after release from telomeres, Genes & development, 17 (2003) 1328-1333.
[18] Z. Zeng, W. Wang, Y. Yang, Y. Chen, X. Yang, J.A. Diehl, X. Liu, M. Lei, Structural basis of selective ubiquitination of TRF1 by SCFFbx4, Developmental cell, 18 (2010) 214-225.
[19] T.H. Lee, K. Perrem, J.W. Harper, K.P. Lu, X.Z. Zhou, The F-box protein FBX4 targets PIN2/TRF1 for ubiquitin-mediated degradation and regulates telomere maintenance, The Journal of biological chemistry, 281 (2006) 759-768.
[20] Y.R. Her, I.K. Chung, Ubiquitin Ligase RLIM Modulates Telomere Length Homeostasis through a Proteolysis of TRF1, The Journal of biological chemistry, 284 (2009) 8557-8566.
[21] A.G. van der Veen, H.L. Ploegh, Ubiquitin-like proteins, Annual review of biochemistry, 81 (2012) 323-357.
[22] G. Rabut, M. Peter, Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series, EMBO reports, 9 (2008) 969-976.
[23] F.G. Whitby, G. Xia, C.M. Pickart, C.P. Hill, Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes, The Journal of biological chemistry, 273 (1998) 34983-34991.

[24] T. Hori, F. Osaka, T. Chiba, C. Miyamoto, K. Okabayashi, N. Shimbara, S. Kato, K. Tanaka, Covalent modification of all members of human cullin family proteins by NEDD8, Oncogene, 18 (1999) 6829-6834.

[25] D.P. Xirodimas, M.K. Saville, J.C. Bourdon, R.T. Hay, D.P. Lane, Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity, Cell, 118 (2004) 83-97.

[26] S. Carter, O. Bischof, A. Dejean, K.H. Vousden, C-terminal modifications regulate MDM2 dissociation and nuclear export of p53, Nature cell biology, 9 (2007) 428-435.

[27] W.M. Abida, A. Nikolaev, W. Zhao, W. Zhang, W. Gu, FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity, The Journal of biological chemistry, 282 (2007) 1797-1804.

[28] S.J. Loftus, G. Liu, S.M. Carr, S. Munro, N.B. La Thangue, NEDDylation regulates E2F-1-dependent transcription, EMBO reports, 13 (2012) 811-818.

[29] I. Aoki, M. Higuchi, Y. Gotoh, NEDDylation controls the target specificity of E2F1 and apoptosis induction, Oncogene, 32 (2013) 3954-3964.

[30] R.E. Amir, K. Iwai, A. Ciechanover, The NEDD8 pathway is essential for SCF(beta -TrCP)-mediated ubiquitination and processing of the NF-kappa B precursor p105, The Journal of biological chemistry, 277 (2002) 23253-23259.

[31] F. Gao, J. Cheng, T. Shi, E.T. Yeh, Neddylation of a breast cancer-associated protein recruits a class III histone deacetylase that represses NFkappaB-dependent transcription, Nature cell biology, 8 (2006) 1171-1177.

[32] S. Oved, Y. Mosesson, Y. Zwang, E. Santonico, K. Shtiegman, M.D. Marmor, B.S. Kochupurakkal, M. Katz, S. Lavi, G. Cesareni, Y. Yarden, Conjugation to Nedd8 instigates ubiquitylation and down-regulation of activated receptor tyrosine kinases, The Journal of biological chemistry, 281 (2006) 21640-21651.

[33] T. Nagano, T. Hashimoto, A. Nakashima, U. Kikkawa, S. Kamada, X-linked inhibitor of apoptosis protein mediates neddylation by itself but does not function as a NEDD8-E3 ligase for caspase-7, FEBS letters, 586 (2012) 1612-1616.

[34] T. Ma, Y. Chen, F. Zhang, C.Y. Yang, S. Wang, X. Yu, RNF111-dependent neddylation activates DNA damage-induced ubiquitination, Molecular cell, 49 (2013)

897-907.
[35] B. Mahata, A. Sundqvist, D.P. Xirodimas, Recruitment of RPL11 at promoter sites of p53-regulated genes upon nucleolar stress through NEDD8 and in an Mdm2-dependent manner, Oncogene, 31 (2012) 3060-3071.
[36] J. Zhang, D. Bai, X. Ma, J. Guan, X. Zheng, hCINAP is a novel regulator of ribosomal protein-HDM2-p53 pathway by controlling NEDDylation of ribosomal protein S14, Oncogene, 33 (2014) 246-254.
[37] R.I. Enchev, B.A. Schulman, M. Peter, Protein neddylation: beyond cullin-RING ligases, Nature reviews. Molecular cell biology, 16 (2015) 30-44.
[38] Y. Jiang, L. Jia, Neddylation Pathway as a Novel Anti-Cancer Target: Mechanistic Investigation and Therapeutic Implication, Anti-cancer agents in medicinal chemistry, (2015).
[39] S.T. Nawrocki, P. Griffin, K.R. Kelly, J.S. Carew, MLN4924: a novel first-in-class inhibitor of NEDD8-activating enzyme for cancer therapy, Expert opinion on investigational drugs, 21 (2012) 1563-1573.
[40] T.A. Soucy, P.G. Smith, M.A. Milhollen, A.J. Berger, J.M. Gavin, S. Adhikari, J.E. Brownell, K.E. Burke, D.P. Cardin, S. Critchley, C.A. Cullis, A. Doucette, J.J. Garnsey, J.L. Gaulin, R.E. Gershman, A.R. Lublinsky, A. McDonald, H. Mizutani, U. Narayanan, E.J. Olhava, S. Peluso, M. Rezaei, M.D. Sintchak, T. Talreja, M.P. Thomas, T. Traore, S. Vyskocil, G.S. Weatherhead, J. Yu, J. Zhang, L.R. Dick, C.F. Claiborne, M. Rolfe, J.B. Bolen, S.P. Langston, An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer, Nature, 458 (2009) 732-736.
[41] J.K. Hsu, T. Lin, R.Y. Tsai, Nucleostemin prevents telomere damage by promoting PML-IV recruitment to SUMOylated TRF1, The Journal of cell biology, 197 (2012) 613-624.
[42] H.M. Mendoza, L.N. Shen, C. Botting, A. Lewis, J. Chen, B. Ink, R.T. Hay, NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins, The Journal of biological chemistry, 278 (2003) 25637-25643.
[43] L.N. Shen, H. Liu, C. Dong, D. Xirodimas, J.H. Naismith, R.T. Hay, Structural basis

of NEDD8 ubiquitin discrimination by the deNEDDylating enzyme NEDP1, The EMBO journal, 24 (2005) 1341-1351.

[44] Y. Chan, J. Yoon, J.T. Wu, H.J. Kim, K.T. Pan, J. Yim, C.T. Chien, DEN1 deneddylates non-cullin proteins in vivo, Journal of cell science, 121 (2008) 3218-3223.

[45] S. Carter, K.H. Vousden, p53-Ubl fusions as models of ubiquitination, sumoylation and neddylation of p53, Cell cycle, 7 (2008) 2519-2528.

[46] J.C. Lee, G.X. Wang, O. Schickling, M.E. Peter, Fusing DEDD with ubiquitin changes its intracellular localization and apoptotic potential, Apoptosis : an international journal on programmed cell death, 10 (2005) 1483-1495.

[47] T. Ohishi, Y. Muramatsu, H. Yoshida, H. Seimiya, TRF1 ensures the centromeric function of Aurora-B and proper chromosome segregation, Molecular and cellular biology, 34 (2014) 2464-2478.

[48] B.D. Cook, J.N. Dynek, W. Chang, G. Shostak, S. Smith, Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres, Molecular and cellular biology, 22 (2002) 332-342.

[49] B.S. Atanassov, Y.A. Evrard, A.S. Multani, Z. Zhang, L. Tora, D. Devys, S. Chang, S.Y. Dent, Gcn5 and SAGA regulate shelterin protein turnover and telomere maintenance, Molecular cell, 35 (2009) 352-364.

[50] Y.C. Chen, S.C. Teng, K.J. Wu, Phosphorylation of telomeric repeat binding factor 1 (TRF1) by Akt causes telomere shortening, Cancer investigation, 27 (2009) 24-28.

[51] S. Kishi, X.Z. Zhou, Y. Ziv, C. Khoo, D.E. Hill, Y. Shiloh, K.P. Lu, Telomeric protein Pin2/TRF1 as an important ATM target in response to double strand DNA breaks, The Journal of biological chemistry, 276 (2001) 29282-29291.

[52] P.R. Potts, H. Yu, The SMC5/6 complex maintains telomere length in ALT cancer cells through SUMOylation of telomere-binding proteins, Nature structural & molecular biology, 14 (2007) 581-590.

[53] V.N. Podust, J.E. Brownell, T.B. Gladysheva, R.S. Luo, C. Wang, M.B. Coggins, J.W. Pierce, E.S. Lightcap, V. Chau, A Nedd8 conjugation pathway is essential for proteolytic targeting of p27Kip1 by ubiquitination, Proceedings of the National Academy of Sciences

of the United States of America, 97 (2000) 4579-4584.
[54] G. Bornstein, D. Ganoth, A. Hershko, Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate, Proceedings of the National Academy of Sciences of the United States of America, 103 (2006) 11515-11520.
[55] J.J. Lin, M.A. Milhollen, P.G. Smith, U. Narayanan, A. Dutta, NEDD8-targeting drug MLN4924 elicits DNA rereplication by stabilizing Cdt1 in S phase, triggering checkpoint activation, apoptosis, and senescence in cancer cells, Cancer research, 70 (2010) 10310-10320.
[56] M.A. Milhollen, U. Narayanan, T.A. Soucy, P.O. Veiby, P.G. Smith, B. Amidon, Inhibition of NEDD8-activating enzyme induces rereplication and apoptosis in human tumor cells consistent with deregulating CDT1 turnover, Cancer research, 71 (2011) 3042-3051.
[57] A. Sfeir, S.T. Kosiyatrakul, D. Hockemeyer, S.L. MacRae, J. Karlseder, C.L. Schildkraut, T. de Lange, Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication, Cell, 138 (2009) 90-103.

국문 요약

샤프론 단백질에 의한 텔로머라아제의 핵 이동과 텔로미어 결합단백질인 TRF1의 조절에 관한 연구

연세대학교 대학원
융합오믹스 의생명과학과
정유영

텔로미어는 TTAGGG라는 단순한 반복 DNA배열로 구성되는 진핵생물 염색체의 말단영역으로, 6종류의 말단소체 결합단백질로 둘러싸여있으며 염색체 말단을 DNA 손상 반응 단백질의 인식으로부터 피하기 위해 T-loop을 형성한다. 진핵세포는 선형 유전체를 가지고 있는데, 각 세포 주기마다 말단의 뉴클레오티드를 잃어버리게 되면서 점점 짧아져 불안정하게 된다. 따라서 진핵세포에서는 염색체 말단에 이러한 반복 DNA배열 구조를 통해 말단 복제문제를 해결하고 있다. 텔로미어의 길이가 일정 수준보다 짧아지게되면, 세포 노화 상태 또는 세포사멸이 일어나게 된다. 인간의 체세포는 텔로미어의 연장을 위한 메커니즘이 없어서 여러 번의 세포분열 후 더 이상 증식을 할 수 없게되지만 암세포는 무한한 세포 성장을 위해 텔로미어 길이를 늘리게 되는데, 이는 말단소체복원효소(telomerase)에 의해서 일어나게 된다. 따라서 텔로미어와 말단소체복원효소에 대한 연구는 인간의 노화와 암에 대한 기작 및 치료를 위한 좋은 연구대상이다. 본 논문에서는 말단소체복원효소의 핵으로의 이동과 활성조절에 대한 연구와, 텔로미어 결합단백질 중에서 TRF1의 안정성 조절과 전사후 변형(post-translational modification)에 대한 연구에 대해 기술하였다.

우리는 말단소체복원효소와 상호작용하는 새로운 단백질인 FKBP52를 발견하였다. FKBP52는 스테로이드 호르몬 수용기 단백질을 핵으로 이동시키는 과정에 관여하는 것으로 잘 알려져 있는데, 이를 기반으로 FKBP52 단백질이 말단소체복원효소의 핵 이동에도 연관이 있을것이라 가설을 세우고 연구를 진행하

였다. 말단소체복원효소의 조립 및 활성 조절에는 다양한 샤프론 단백질들(예, Hsp90, p23)이 중요하게 작용하는데, FKBP52는 이 샤프론중에 하나인 hsp90와 직접 결합하고 또한 세포질 내의 단백질이동에 관여하는 dynein/dynactin moter 단백질과도 직접적으로 결합하고 있다. 이 연구를 통해 우리는 FKBP52 단백질이 다른 샤프론 단백질들과 함께 말단소체복원효소의 세포질에서부터 핵으로의 이동하는 과정에 중요하게 작용하는 것을 발견하였고, 또한 말단소체복원효소의 활성조절에도 중요하게 작용하는것을 확인하였다.

또한 우리는 말단소체 결합단백질인 TRF1과 상호 작용하는 새로운 단백질로써 NUB1을 발견하였고, NUB1이 직접적으로 TRF1과 단백질 분해효소(proteasome)와의 연결에 관여함으로써 TRF1의 안정성 조절에 작용하고 있음을 확인하였다. NUB1 단백질은 전사후 변형기작중의 하나인 NEDD8 단백질의 접합과정을 저해하는 것으로 잘 알려져 있는데, 이를 바탕으로 우리는 TRF1에 NEDD8이 접합되는지 확인하였고, Neddylation이라는 TRF1의 새로운 전사후 변형 메커니즘을 확인하였다. 또한 우리는 TRF1에 NEDD8이 접합 되었을 때, TRF1 단백질의 세포내 위치가 세포질로 이동하는 것을 확인하였고, 안정성이 감소하는 것 또한 확인하였다. 그리고 세포내 NEDD8 접합 시스템을 저해하였을 때, TRF1 단백질의 안정성이 매우 증가하는 것을 확인하였다. 또한 TRF1의 neddylation이 텔로미어 DNA로부터 떨어졌을 때 촉진되는 것을 확인하였는데, 이는 이전연구인 TRF1의 ubiquitination과 유사한 양상으로 나타남을 확인하였다. TRF1의 neddylation과 ubiquitination의 상호작용에 대해서는 추가적인 연구가 필요하다. 본 연구를 통해 우리는 말단소체복원효소의 핵이동에 관여하는 단백질인 FKBP52와, 텔로미어 결합단백질인 TRF1의 세포내 안정성 조절 및 새로운 전사후 변형기작의 발견으로 텔로미어의 유지 및 통제에 대한 새로운 메커니즘을 제안하고자 한다.

중심단어: 텔로미어(telomere), 말단소체복원효소(telomerase), FKBP52, Hsp90, dynein/dynactin, TRF1, NUB1, 단백질 분해효소, 전사후 변형(post-translational modification)