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TRAF7 Sequesters c-Myb to the Cytoplasm by Stimulating Its Sumoylation[D]

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Small ubiquitin-related modifiers (SUMOs) are proteins that are posttranslationally conjugated to diverse proteins. The c-*myb* proto-oncogene product (c-Myb) regulates proliferation and differentiation of hematopoietic cells. PIASy is the only known SUMO E3 ligase for c-Myb. Here, we report that TRAF7 binds to c-Myb and stimulates its sumoylation. TRAF7 bound to the DNA-binding domain of c-Myb via its WD40 repeats. TRAF7 has an E3 ubiquitin ligase activity for self-ubiquitination, but TRAF7 also stimulated the sumoylation of c-Myb at Lys-523 and Lys-499, which are the same sites as those used for PIASy-induced sumoylation. TRAF7 inhibited *trans*-activation induced by wild-type c-Myb, but not by the sumoylation site mutant of c-Myb. The expression of both c-*myb* and *TRAF7* was down-regulated during differentiation of M1 cells. Endogenous TRAF7 localized to both the cytoplasm and nucleus of M1 cells. Consistent with this, significant amounts of sumoylated c-Myb were found in the cytoplasm of M1 cells, whereas nonsumoylated c-Myb was found predominantly in the nucleus. Overexpressed TRAF7 was localized in the cytoplasm of CV-1 cells, and sequestered c-Myb and SUMO1 in the cytosol, whereas PIASy was localized in the nucleus. Thus, TRAF7 negatively regulates c-Myb activity by sequestering c-Myb to the cytosol via sumoylation.

INTRODUCTION

Multiple posttranslational modifications, including phosphorylation, acetylation, and ubiquitination, have been shown to regulate the activity of transcription factors. Recently, a number of ubiquitin-like proteins have been found which become covalently linked to lysine residues in target proteins. One such group composed of SUMO1 and SUMO2/3 is distantly related to ubiquitin (Johnson, 2004). These 92–97 amino acid polypeptides are covalently conjugated to internal lysines of substrate proteins (Melchior, 2000; Muller *et al.,* 2001). Several proteins modified by SUMO1 have been identified, including RanGAP1 (Matunis *et al.,* 1996), PML (Muller *et al.,* 1998; Kamitani *et al.,* 1998; Duprez *et al.,* 1999), IκB (Desterro *et al.,* 1998), p53 (Gostissa *et al.,* 1999; Rodriguez *et al.,* 1999), LEF-1 (Sachdev *et al.,* 2001), and the c-*myb* proto-oncogene product (c-Myb; Bies *et al.,* 2002). Unlike ubiquitination, SUMO modification does not lead to substrate degradation. Instead, its functions appear to be substrate dependent; sumoylation can induce changes in the target's subcellular localization, its protein-protein interactions, or its protein-DNA interactions (Seeler and Dejean, 2003). SUMO modification augments the transcriptional activation potential of some transcription factors, such as p53 (Gostissa *et al.,* 1999; Rodriguez *et al.,* 1999). However, most often sumoylation antagonizes the activation potential of transcription factors or mediates repression (Ross *et al.,* 2002; Gill, 2004). In several cases, SUMO-mediated repression correlates with targeting of the protein to PML nuclear bodies (Sachdev *et al.,* 2001; Ross *et al.,* 2002; Schmidt and Muller, 2002).

The enzymatic reactions involved in SUMO modification are similar to those in ubiquitin modification and involve an E1-activating enzyme, consisting of an Aos1/Uba2 heterodimer and the E2-conjugating enzyme Ubc9 (Hochstrasser, 2001; Jackson, 2001). Ubc9 recognizes and binds to a SUMO consensus sequence, ψKX(D/E), where ψ is a hydrophobic amino acid and X is any amino acid, present in most target proteins (Bernier-Villamor *et al.,* 2002). Although the SUMO E1 and E2 enzymes are sufficient to modify most substrates in vitro, several SUMO E3 ligases, including the protein inhibitors of activated STATs (PIAS), the polycomb protein Pc2, and the nuclear pore component RanBP2, have been described recently (Johnson and Gupta, 2001; Kahyo *et al.,* 2001; Pichler *et al.,* 2002; Kagey *et al.,* 2003). The PIAS family, which is homologous to the yeast Siz family, have a conserved RING finger domain that regulates transactivation by conjugating SUMO-1 in molecules including p53 (Gostissa *et al.,* 1999; Rodriguez *et al.,* 1999), LEF-1 (Sachdev *et al.,* 2001), and SP3 (Ross *et al.,* 2002; Sapetschnig *et al.,* 2002). The nucleoporin RanBP2 is structurally unrelated to the PIAS/Siz family and sumoylates SP100 (Pichler *et al.,* 2002) and HDAC4 (Kirsh *et al.,* 2002) to regulate nuclear translocation.

The c-*myb* proto-oncogene is the cellular progenitor of the v-*myb* oncogenes carried by the chicken retroviruses AMV

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Abbreviations used: c-Myb, c-*myb* proto-oncogene product; DBD, DNA-binding domain; NLK, Nemo-like kinase; NPCs, nuclear pore complexes; NRD, negative regulatory domain; PIAS, protein inhibitors of activated STATs; SUMO, small ubiquitin-related modifier.

(avian myeloblastosis virus) and E26, which cause acute myeloblastic leukemia or erythroblastosis (Klempnauer *et al.*, 1982; Leprince *et al.*, 1983). The high level of c-*myb* expression in immature hematopoietic cells is down-regulated during terminal differentiation (Gonda and Metcalf, 1984). Analysis of c-*myb*-deficient mice indicated that c-*myb* is essential for proliferation of immature hematopoietic cells (Mucenski *et al.*, 1991), and analysis of tissue-specific c-*myb* knock-out mice revealed that c-*myb* is also required for T-cell development at several stages (Bender *et al.*, 2004). c-Myb is a transcriptional activator that recognizes the specific DNA sequence 5′-AACNG-3′ (Biedenkapp *et al.*, 1988; Ness *et al.*, 1989; Sakura *et al.*, 1989; Weston and Bishop, 1989). Some of the c-Myb target genes, including c-*myc*, are required for the G1/S transition in the cell cycle (Nakagoshi *et al.*, 1992), whereas several other target genes, including *mim-1*, *GBX2*, and *bcl-2*, are involved in lineage commitment in differentiation and suppression of apoptosis (Ness *et al.*, 1989; Frampton *et al.*, 1996; Taylor *et al.*, 1996; Kowenz-Leutz *et al.*, 1997). c-Myb has three functional domains that are responsible for DNA binding, transcriptional activation, and negative regulation (Sakura *et al.*, 1989). The DNA-binding domain (DBD) in the N-terminal region of c-Myb consists of three imperfect tandem repeats of 51–52 amino acids, each containing a helix-turn-helix variation motif (Ogata *et al.*, 1994).

c-Myb activity is regulated by interaction with various factors. Binding of the transcriptional coactivator CBP to the activation domain is essential for c-Myb-induced transactivation (Dai *et al.*, 1996). The negative regulatory domain (NRD) located in the carboxy (C)-terminal portion of the molecule normally represses c-Myb activity (Sakura *et al.*, 1989; Hu *et al.*, 1991; Kanei-Ishii *et al.*, 1992; Dubendorff *et al.*, 1992). The two corepressors TIF1β and BS69 directly bind to the two ΦXXΦΦ(Φ: hydrophobic amino acids) motifs in the NRD and inhibit c-Myb-induced *trans*-activation (Ladendorff *et al.*, 2001; Nomura *et al.*, 2004). Recently, two kinases, Homeodomain-interacting protein kinase 2 (HIPK2) and Nemo-like kinase (NLK), were shown to directly bind to c-Myb in response to Wnt-1 signals, leading to the phosphorylation and ubiquitination of c-Myb and its proteasome-dependent degradation (Kanei-Ishii *et al.*, 2004). The NRD also contains two sumoylation sites that are enhanced by PIASy (Bies *et al.*, 2002; Dahle *et al.*, 2003). A c-Myb mutant lacking the two sumoylation sites has enhanced *trans*-activating activity, suggesting that the sumoylation of c-Myb negatively regulates its activity. In addition, PIASy caused a shift in the nuclear distribution of c-Myb to the insoluble matrix fraction. Thus, sumoylation of c-Myb negatively regulates its *trans*-activating capacity.

In this study, we have identified TRAF7 as a novel SUMO E3 ligase for c-Myb. When c-Myb was expressed with TRAF7 in CV-1 cells, c-Myb was retained in the cytosol, suggesting that TRAF7 regulates the subcellular localization of c-Myb via sumoylation.

MATERIALS AND METHODS

Plasmid Constructions

TRAF7 cDNA clones were amplified by PCR using a mouse macrophage cDNA library (Stratagene, La Jolla, CA). To express TRAF7 in mammalian cells, TRAF7 cDNA was inserted downstream of the chicken cytoplasmic β-actin promoter, with or without an N-terminal FLAG tag (pact-FLAG-TRAF7 and pact-TRAF7). For in vitro translation of TRAF7, the TRAF7 cDNA was inserted into the pSPUTK vector (Stratagene). Various mutants of TRAF7 were generated using PCR-based method. The c-Myb expression plasmids that were used have been described previously (Sakura *et al.*, 1989).

Yeast Two-Hybrid Screening and In Vitro Binding Assays

Yeast two-hybrid screening was performed using a mouse embryonic cDNA library as described (Kanei-Ishii *et al.*, 2004). The mouse c-Myb lacking the transcriptional activation domain (amino acids 240–324) was used as bait. GST pulldown assays using GST-c-Myb were performed essentially as described previously (Dai *et al.*, 1996; Nomura *et al.*, 2004). The binding buffer was 50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 0.1% NP-40, and 150 mM KCl. To examine the binding of in vitro-translated c-Myb and FLAG-TRAF7, 293T cells (1×10^6 cells per 100-mm dish) were transfected with the FLAG-TRAF7 expression plasmid (8 μg) using Lipofectamine Plus (Invitrogen, Carlsbad, CA). Lysates were prepared from the transfected cells using RIPA buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail) and immunoprecipitated with an anti-FLAG antibody. The immunocomplexes were captured with protein G-Sepharose and sequentially washed with washing buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 M NaCl, 2% NP-40, and 10% glycerol) and then binding buffer. The FLAG-TRAF7 immunocomplexes bound to protein G-Sepharose were mixed with ^{35}S-c-Myb translated in vitro, and the bound proteins were analyzed by SDS-PAGE, followed by autoradiography.

Co-immunoprecipitation

To investigate the in vivo interaction of overexpressed proteins, 293T cells (1×10^6 cells per 100-mm dish) were transfected by Lipofectamine Plus (Invitrogen) with plasmids that express TRAF7 (4 μg) and c-Myb (2 μg). The transfected cells were then cultured for 40 h and lysed in Harlow buffer (250) (50 mM HEPES-KOH, pH 7.5, 250 mM NaCl, 0.2 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail; Roche, Indianapolis, IN). The lysates were immunoprecipitated with 1 μg of anti-c-Myb, which was raised against GST-CT5, or with anti-TRAF7 antibodies, and 30 μl of protein G-Sepharose (Pharmacia, Piscataway, NJ). The anti-TRAF7 rabbit polyclonal antibody was prepared using GST-TRAF7, which contains amino acids 1–170 (*Nco*I-*Xho*I). The immunocomplexes were washed three times with Harlow buffer (250). For coimmunoprecipitation of endogenous proteins, M1 cells were lysed with RIPA buffer, followed by sonication and dilution with 10 volumes of RIPA buffer lacking SDS to reduce SDS concentration to 0.01%. The lysates were immunoprecipitated with 1 μg of anti-TRAF7 antibody or control IgG, and 30 μl of protein G-Sepharose (Pharmacia). The immunocomplexes were washed five times with washing buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 0.01% SDS, and protease inhibitor cocktail). For immunoblotting, the immunoprecipitates or whole cell lysates were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were immunoblotted with anti-TRAF7 or anti-c-Myb antibodies, and the bound antibodies were visualized by horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using an ECL reagent (Amersham, Piscataway, NJ).

Northern Blotting

A multiple tissue mouse mRNA blot was purchased from Clontech (Palo Alto, CA). Total RNA was also prepared from IL-6-treated murine myeloid leukemia M1 cells at various times after stimulation and used for Northern blotting. The probe was the mouse TRAF7 coding sequence.

In Vivo Sumoylation Assay

CV-1 cells (1×10^6 cells per 100-mm dish) were transfected with the plasmids that express c-Myb (2 μg), TRAF7 (4 μg), His tag-linked SUMO1 (0.15 μg), and the internal control pact-β-gal (0.3 μg) using Lipofectamine Plus. Total plasmid amounts were adjusted to 6.45 μg by adding the empty plasmid. Cells were culture for 40 h after transfection, scraped into 800 μl of urea buffer (8 M urea, 0.1 M sodium phosphate, pH 8.0, 0.3 M NaCl, 10 mM *N*-ethylmaleimide), and sonicated mildly on ice. Proteins conjugating His-SUMO1 were purified with HIS-Select cobalt affinity resin (Sigma, St. Louis, MO). After several washes with urea buffer, the proteins were eluted with urea buffer containing 1 M imidazole. The purified proteins were subjected to SDS-PAGE followed by Western blotting with an anti-c-Myb (1–1) or anti-GMP1 (Zymed, South San Francisco, CA) antibody.

In Vitro Sumoylation Assay

293T cells (1×10^6 cells per 100-mm dish) were transfected with 8 μg of FLAG-TRAF7 or FLAG-c-Myb using Lipofectamine Plus. Forty hours after transfection, cells were scraped into RIPA buffer and immunoprecipitated with FLAG-M2 antibody. The immunocomplexes were captured with protein G-Sepharose and sequentially washed with washing buffer. The FLAG-tagged proteins were eluted with 150 ng/μl FLAG peptide. E1 (SAE1/SAE2) (ALEXSIS, Lausen, Switzerland), E2 (UbcH9; AG Scientific, San Diego, CA), SUMO1 (AG Scientific), FLAG-TRAF7, and FLAG-c-Myb were mixed in reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM ATP) and incubated for 2 h at 37°C. The reactions were analyzed by SDS-PAGE followed by Western blotting using anti-c-Myb (1–1) or anti-GMP-1 antibody.

CAT Reporter Assays

CV-1 cells (5×10^5 cells per 100-mm dish) were cotransfected by the $CaPO_4$ method with the *myc*-luciferase reporter (Nakagoshi *et al.*, 1992; 4 μg), the c-Myb expression plasmid (4 μg), the TRAF7 expression plasmid (0–8 μg), and the internal control plasmid pact-β-gal (0.5 μg). The total amount of plasmid DNA was adjusted to 16.5 μg by adding empty plasmid DNA. Twenty-four hours after transfection, cell lysates were prepared and used for CAT assays.

Subcellular Localization

M1 cells and DND39 cells, which were kindly donated by Dr. Y. Matsuo (Fujisaki Cell Center, Hayashibara Biochemical Labs), were grown in DMEM and RPMI medium containing 10% fetal bovine serum, respectively. CV-1 cells (8×10^4 per 6-well dish) were transfected with the plasmid expressing c-Myb, TRAF7, or FLAG-PIASy using the $CaPO_4$ method. CV-1 cells were also transfected with mixture of the FLAG-c-Myb, TRAF7 or FLAG-PIASy, and SUMO1 expression plasmids. Amounts of each plasmid are described in the legends to Figures 7 and 8. Forty hours after transfection, cells were fixed with 2% paraformaldehyde for 45 min. Transfected CV-1 cells and nontransfected M1 cells were permeabilized with either 0.004% digitonin for 2 min (12 min for M1 cells) or 0.2% Triton X-100 for 12 min and stained as described (Nomura *et al.*, 2004) with anti-c-Myb or anti-TRAF7 antibody. The signals for the different proteins were visualized by rhodamine- and FITC-conjugated secondary antibodies and analyzed by confocal microscopy.

Subcellular Fractionation

M1 cells (1×10^7 cells) were lysed in hypotonic buffer (20 mM HEPES-KOH, pH 8.0, 10 mM NaCl, 10 mM *N*-ethylmaleimide, and proteinase inhibitor cocktail). After a centrifugation, the supernatant was used as a cytoplasmic fraction, and the pellet was further extracted with nuclear extraction buffer (20 mM HEPES-KOH, pH 8.0, 420 mM NaCl, 10 mM *N*-ethylmaleimide, and proteinase inhibitor cocktail). After a centrifugation, the supernatant was used as a nuclear fraction. Concentrations of NaCl in the cytoplasmic and the nuclear fractions were adjusted to final concentration of 150 mM, Then, c-Myb (and sumoylated c-Myb) in both fractions was immunoprecipitated using an anti-c-Myb antibody (1-1) and analyzed by SDS-PAGE and Western blotting using an anti-c-Myb antibody (1-1) or an anti-GMP-1 antibody.

RESULTS

Identification of TRAF7 as a c-Myb-binding Protein

To identify the regulator that binds to c-Myb, we performed a yeast two-hybrid screening of a mouse embryo library using the c-Myb mutant protein lacking its transcriptional activation domain (ΔTA) as the bait. Among 109 clones isolated, 2 clones encoded a protein containing WD40 repeats. Full-length clones of the protein were isolated and characterized. The cDNA encodes a 594-amino acid protein identical to the short form of TRAF7, which contains RING and zinc finger domains, a coiled-coil region, and WD40 repeats (Figure 1A; Xu *et al.*, 2004). TRAF7 was designated based on its high homology to RING and zinc finger domains of TRAF proteins (Xu *et al.*, 2004). However, TRAF7 lacks the conserved C-terminal domain found in TRAF1–6, and instead has several WD40 repeats in its C-terminal domain. Although TRAF7 functionally interacts with MEKK3, similar to other TRAF proteins, it is not known whether TRAF7 binds to members of TNF receptor family.

TRAF7 Binds to the DBD of c-Myb via Its WD40 Repeats

To examine whether c-Myb directly interacts with TRAF7, we performed GST pulldown assays using in vitro-translated TRAF7 and GST-c-Myb containing the full-length c-Myb. In vitro-translated full-length TRAF7 and the C-terminal 285-amino acid fragment of TRAF7 containing the WD40 repeats bound efficiently to GST-c-Myb (Figure 1A). In contrast, a TRAF7 fragment lacking the WD40 repeats failed to bind to GST-c-Myb. Thus, c-Myb directly binds to the WD40 repeats of TRAF7. Coimmunoprecipitation experiments were then performed to investigate in vivo interactions between these proteins. CV-1 cells were transfected with c-Myb and TRAF7 expression plasmids, and cell lysates were prepared from the transfected cells. Anti-c-Myb and anti-

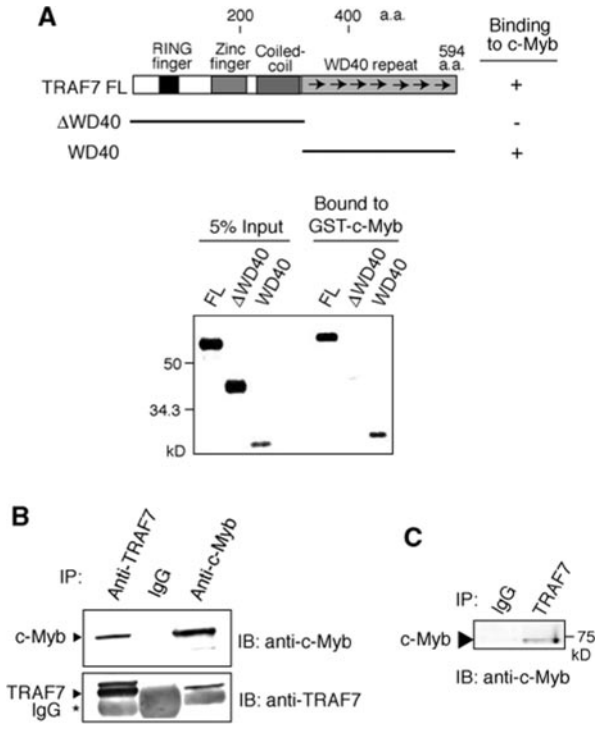

Figure 1. TRAF7 binds to c-Myb via its WD40 repeat. (A) GST pulldown assays. Top, the functional domain of TRAF7, and the deletion mutants used are shown schematically. The results of the binding assays are indicated on the right. The relative binding activities of the deleted forms of TRAF7 are designated + and − to indicate the binding of 9–20% and <1% of the input protein, respectively. Bottom, in the input lanes, three forms of ^{35}S-TRAF7 were synthesized in vitro and analyzed by SDS-PAGE followed by autoradiography. The ^{35}S-TRAF7 proteins indicated above each lane were mixed with a GST-c-Myb affinity resin, and the bound proteins were analyzed by SDS-PAGE followed by autoradiography. Less than 0.2% of the input TRAF7 proteins bound to the control resin bearing GST alone (unpublished data). (B) Coimmunoprecipitation of overexpressed TRAF7 and c-Myb proteins. Lysates were prepared from CV-1 cells transfected with a mixture of TRAF7 and c-Myb expression plasmids. Lysates were precipitated by anti-TRAF7 or anti-c-Myb antibody or control IgG, and the immunocomplexes were analyzed by Western blotting using anti-c-Myb or anti-TRAF7 antibody. (C) Endogenous c-Myb is coimmunoprecipitated with antibodies to TRAF7. Lysates from M1 cells were precipitated with the antibody shown above and the immunocomplexes were analyzed by Western blotting with the anti-c-Myb antibody.

TRAF7 antibodies coprecipitated TRAF7 and c-Myb, respectively, whereas control IgG did not (Figure 1B). To further confirm the in vivo association of c-Myb with TRAF7, coimmunoprecipitation of endogenous proteins in M1 cell lysates was performed (Figure 1C). Anti-TRAF7 antibodies coprecipitated c-Myb, whereas normal IgG did not.

To determine which domain of c-Myb interacts with TRAF7, we used various forms of in vitro-translated c-Myb and TRAF7-bound Sepharose resin for binding assays. To prepare TRAF7-bound Sepharose, 293T cells were transfected with the FLAG-tagged TRAF7 expression vector, and the cell lysates were immunoprecipitated with an anti-FLAG antibody. Immunocomplexes bound to protein G-Sepharose were then used for binding assays with c-Myb translated in vitro. The results demonstrate that the DBD of c-Myb is essential for the interaction with TRAF7 and that the R2 and

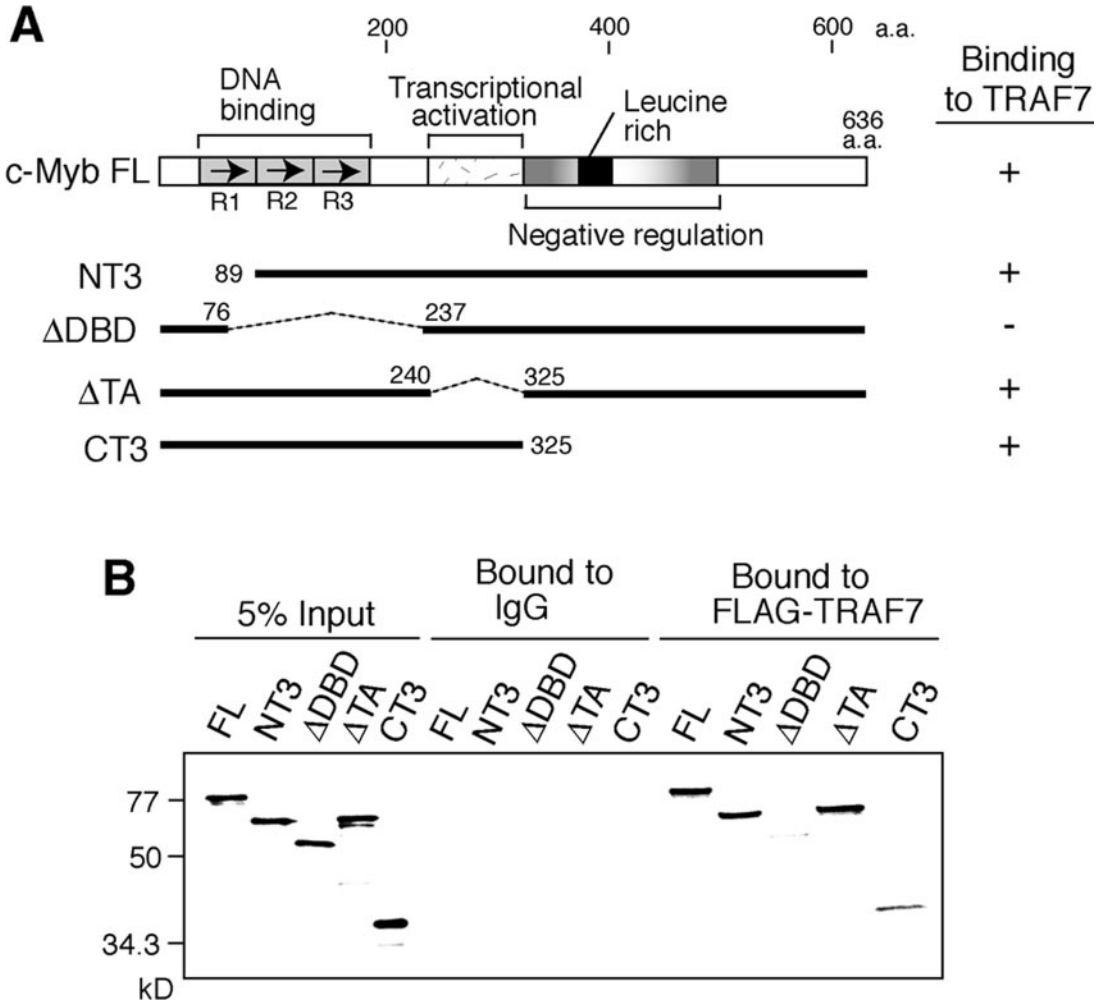

Figure 2. In vitro binding of c-Myb to TRAF7 via its DBD. (A) Summary of GST pulldown assays. The functional domain of c-Myb and the deletion mutants used are shown schematically. The results of binding assays shown in B are indicated on the right. The relative binding activities of various forms of c-Myb are designated + and − to indicate the binding of 9–20% and <1% of the input protein, respectively. (B) In vitro binding. In the input lanes, various forms of ^{35}S-c-Myb were synthesized in vitro and analyzed by SDS-PAGE, followed by autoradiography. FLAG-TRAF7 was expressed in 293T cells and immunoprecipitated with an anti-FLAG antibody or control IgG. The ^{35}S-c-Myb proteins indicated above each lane were mixed with the FLAG-TRAF7 bound to protein G-Sepharose or control IgG-Sepharose. The bound proteins were analyzed by SDS-PAGE, followed by autoradiography.

R3 repeats of the DBD are sufficient for binding (Figure 2). Thus, TRAF7 binds to the DBD of c-Myb via its WD40 repeats. TRAF7 bound to CT3, which lacks the C-terminal half containing the NRD. Therefore, oncogenic activation of c-*myb* by removal of the NRD is not due to the loss of interaction with TRAF7.

Expression Pattern of TRAF7 mRNA

To examine the tissue distribution of TRAF7 expression, we performed Northern blot analysis. The results suggest that mouse *TRAF7* mRNA is expressed in all tissues examined as transcripts of ~3.0 kb. Expression of *TRAF7* mRNA is relatively high in heart, liver, kidney, testis, prostate, thyroid, and salivary gland (Figure 3A). *TRAF7* mRNA was also expressed in the myeloid precursor cell line M1, which was derived from a spontaneous myeloid leukemia of SL mice (Ichikawa, 1969), and its expression was down-regulated during IL-6-induced terminal differentiation of the cells into macrophages (Figure 3B), similar to the pattern of c-*myb* (Figure 3B). Thus, the expression levels of both *TRAF7* and c-*myb* mRNA are high in undifferentiated M1 cells and are down-regulated during differentiation.

TRAF7 Stimulates the Sumoylation of c-Myb

TRAF2 and TRAF6 have been shown to function as ubiquitin ligases (E3) through their N-terminal RING domains (Deng *et al.*, 2000). TRAF7 was also demonstrated to be an E3 ubiquitin ligase capable of self-ubiquitination (Bouwmeester *et al.*, 2004). Therefore, we tested whether TRAF7 stimulates the ubiquitination of c-Myb. However, TRAF7 did not enhance the ubiquitination of c-Myb (unpublished data). The RING finger domain has been found not only in ubiquitin E3 ligases but also in SUMO E3 ligases such as PIAS (Gostissa *et al.*, 1999; Rodriguez *et al.*, 1999; Sachdev *et al.*, 2001). Therefore, we next tested whether TRAF7 induces sumoylation of c-Myb. 293T cells were transfected with the c-Myb expression plasmid, with or without TRAF7 and His-tagged SUMO1 expression plasmids, and the lysates were mixed with cobalt-Sepharose resin to purify the proteins linked to His-SUMO1. The bound proteins were then analyzed by

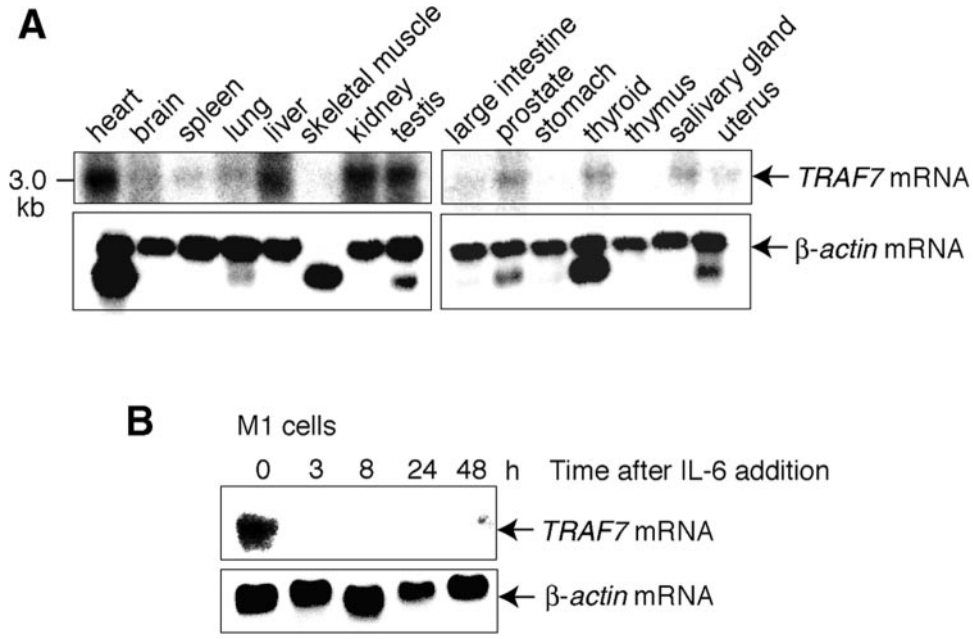

Figure 3. *TRAF7* is down-regulated during differentiation of M1 cells. (A) Expression of *TRAF7* mRNA in various tissues of mice. Northern blot analysis was performed using an RNA filter containing RNAs prepared from various tissues of mice. (B) Down-regulation of *TRAF7* expression during differentiation of M1 cells. M1 cells were treated with IL-6 to induce differentiation into macrophages and total RNAs were prepared at various times after stimulation and used for the Northern blot analysis.

Western blotting using an anti-Myb antibody. The three bands for sumoylated c-Myb were detected and their densities significantly increased in cells coexpressing TRAF7 (Figure 4A, left). In addition, similar results were also obtained using an anti-SUMO1 antibody in Western blotting (Figure 4A, right). Thus, TRAF7 stimulates the sumoylation of c-Myb in vivo.

The consensus sequence of the sumoylation site, ψKXE (ψ, hydrophobic amino acid; X, any amino acid), was identified by analyzing multiple sumoylation sites. Analysis of mouse c-Myb sequence using the SUMOplot Prediction Program indicated the presence of four putative sumoylation sites, Lys-499, Lys-523, Lys-92, and Lys-476, each of which had a probability score of more than 0.70. Lys-499 and Lys-523 were previously shown to be sumoylated by PIASy (Bies *et al.,* 2002; Dahle *et al.,* 2003). To determine the TRAF7-induced sumoylation sites in c-Myb, we expressed various c-Myb mutants of these putative sumoylation sites for in vivo sumoylation assays (Figure 4B). TRAF7-induced sumoylation was not observed in the 4KR mutant, in which all four putative sumoylation sites were mutated to Arg. TRAF7 also failed to sumoylate the 2KR mutant, in which Lys-499 and Lys-523 were mutated. The K499R mutant was still sumoylated by TRAF7 to some degree, whereas TRAF7 only slightly enhanced the sumoylation of the K523R mutant. These results indicate that TRAF7 sumoylates c-Myb primarily at Lys-499 and Lys-523.

To further confirm the sumoylation of c-Myb by TRAF7, we performed in vitro sumoylation assays. To prepare c-Myb and TRAF7, 293T cells were transfected with plasmids to express either FLAG-c-Myb or FLAG-TRAF7, and the cell lysates were immunoprecipitated with an anti-FLAG antibody in RIPA buffer and washed in the presence of 1.0 M NaCl and 2% NP40. c-Myb, E1 and E2 enzymes, and SUMO1 were incubated with or without TRAF7, and the immunoprecipitates were analyzed by Western blotting using an anti-Myb antibody (Figure 4C). In the absence of TRAF7, two bands migrating more slowly than intact c-Myb were generated. These bands were not observed when either E1, E2, or SUMO1 was absent. Addition of TRAF7 increased the densities of these two slower migrating bands, and also generated one additional band. These bands were also detected with an anti-SUMO1 antibody, indicating that the bands correspond to sumoylated c-Myb. Thus, TRAF7 can stimulate sumoylation of c-Myb both in vivo and in vitro.

TRAF7 Inhibits c-Myb-induced trans-Activation

To examine whether TRAF7 regulates c-Myb-induced transcriptional activation, we performed cotransfection assays using the CAT reporter containing the c-*myc* promoter (pc-*myc*-luc), which is a c-Myb target (Nakagoshi *et al.,* 1992; Figure 5). TRAF7 decreased c-Myb-induced *trans*-activation from the c-*myc* promoter to 50% of the control. On the other hand, TRAF7 (1–4 μg) did not inhibit *trans*-activation by the 2KR mutant of c-Myb, which cannot be sumoylated by TRAF7. However, higher amount of TRAF7 (8 μg) partly inhibited 2KR mutant-induced *trans*-activation. No effect of the high amount of TRAF7 on the 4KR-induced *trans*-activation suggests that overexpressed TRAF7 may induce the sumoylation of c-Myb at minor sites, leading to the inhibition of activity of 2KR mutant. Thus, TRAF7 inhibited the *trans*-activation induced by wild-type c-Myb, but not by the sumoylation site mutants.

Subcellular Localization of TRAF7 and c-Myb in M1 Cells

To understand the role of TRAF7, we investigated the subcellular localization of endogenous TRAF7 in M1 cells. Because M1 cells have little cytoplasm, cytoplasmic localization can be difficult to verify. Therefore, we used two different methods to permeabilize M1 cells for immunostaining. When M1 cells were permeabilized using digitonin, which disrupts the cellular membranes but not the nuclear membrane, cytoplasmic β-actin, but not the nuclear lamin B, was detected (Figure 6B). In these digitonin-treated cells, TRAF7 and c-Myb signals were detected, and appeared to be nonuniformly distributed (Figure 6A), suggesting that both proteins were localized in the cytosol. When cells were permeabilized using Triton X-100, which also disrupts the nuclear membrane, not only cytoplasmic β-actin but also nuclear lamin B were detected (Figure 6B). In these Triton X-100-treated cells, the TRAF7 and c-Myb signals were al-

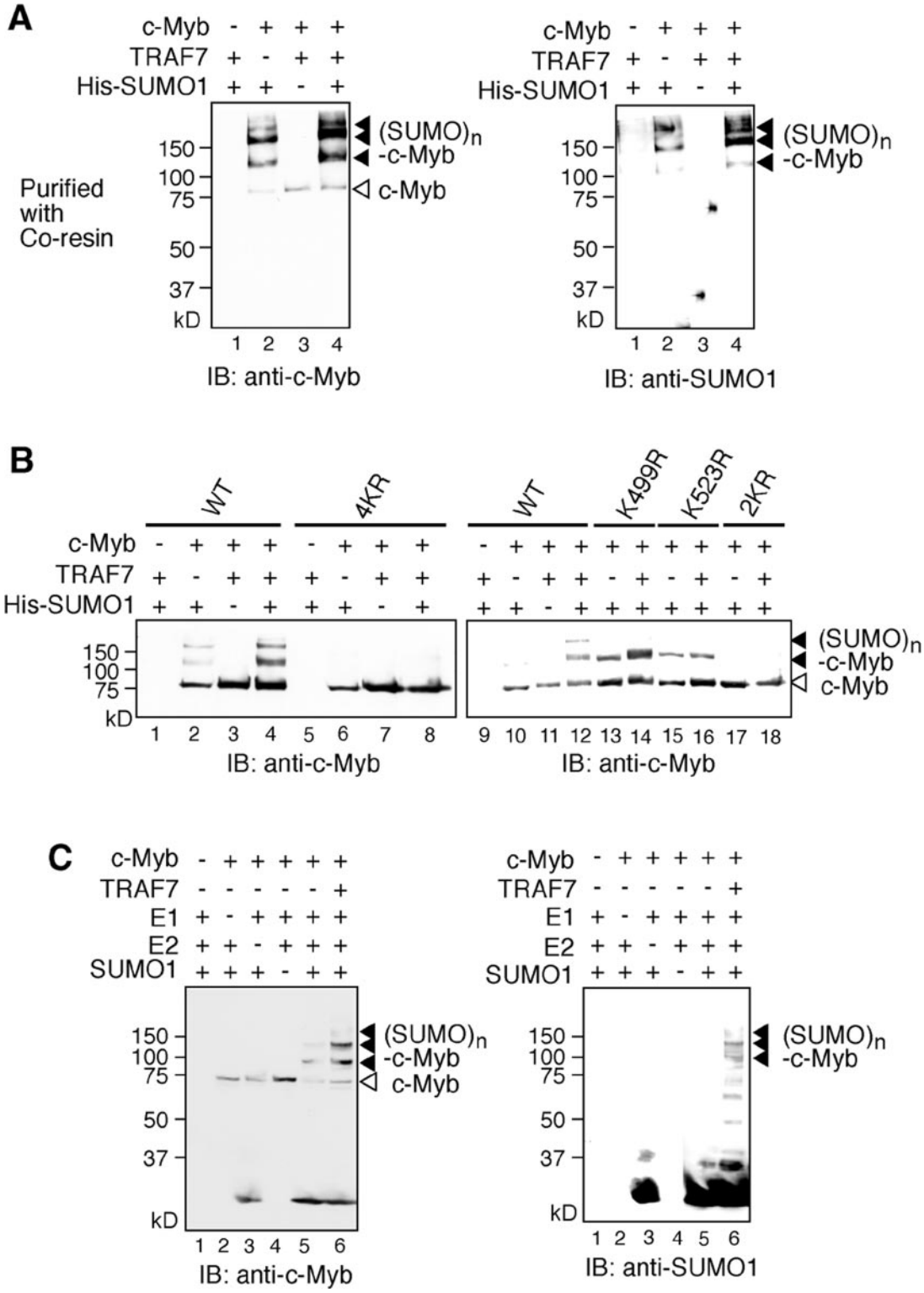

Figure 4. *TRAF7* stimulates the sumoylation of c-Myb. (A) Stimulation of in vivo sumoylation of c-Myb by TRAF7. CV-1 cells were transfected with plasmids expressing c-Myb, TRAF7, and His-tagged SUMO1, as shown above each lane. Whole cell lysates were mixed with cobalt resin to purify proteins modified with His-tagged SUMO1, and the bound proteins were analyzed by Western blotting with anti-c-Myb (left panel) or anti-SUMO1 (right panel) antibody. (B) TRAF7 sumoylates c-Myb at two sites. CV-1 cells were transfected with plasmids expressing various forms of c-Myb, TRAF7, and His-tagged SUMO1, as shown above each lane. Sumoylated c-Myb was detected as described above. (C) In vitro sumoylation of c-Myb by TRAF7. 293T cells were transfected with c-Myb or TRAF7 expression plasmids, and both proteins were immunoprecipitated. These proteins were incubated with E1, E2, and SUMO1, and the reactions were analyzed by Western blotting with anti-c-Myb or anti-SUMO1 antibody.

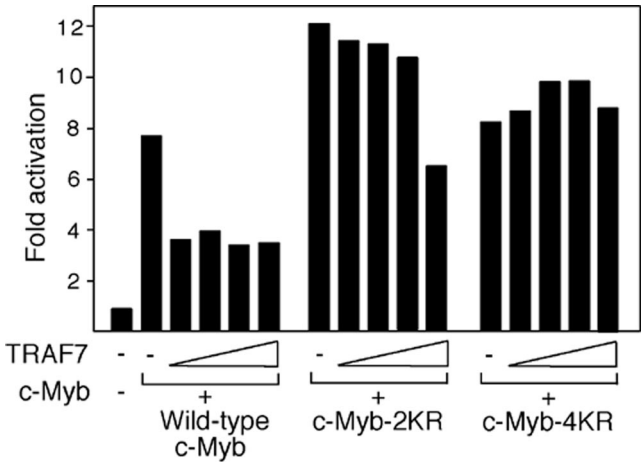

Figure 5. TRAF7 inhibits c-Myb-dependent transcriptional activation. CV-1 cells were transfected with a mixture of the CAT reporter bearing the c-*myc* promoter (pc-*myc*-CAT) and either the wild-type, 2KR mutant, or 4KR mutant c-Myb expression plasmid or the control plasmid, with or without increasing amounts (0, 1, 2, 4, or 8 μg) of the TRAF7 expression plasmid. CAT activity was then measured. All CAT cotransfection experiments were repeated at least three times, and typical results are shown. The differences between each set of experiments were within 20%.

most uniformly distributed (Figure 6A), indicating that both proteins were in the nucleus. Thus, the results of immunostaining of M1 cells suggested that TRAF7 and c-Myb are localized in both the cytoplasm and nucleus. We also used a human Burkitt's B lymphoma cell line, DND39 (Miyazaki *et al.,* 2002), for immunostaining (Supplementary Figure S1). TRAF7 and c-Myb are localized in both the cytoplasm and nucleus also in DND39 cells.

To further confirm these results, the cytoplasmic and nuclear fractions of M1 cells were separated and used for immunoprecipitation using an anti-c-Myb antibody, followed by Western blotting with anti-c-Myb or anti-SUMO1 antibody (Figure 6C). Similar amounts of sumoylated c-Myb, which had the molecular weight of ~100 kDa, were localized in both the cytoplasm and the nucleus, whereas unmodified c-Myb was enriched in the nucleus. The sumoylated c-Myb detected in M1 cells had a molecular weight (~100 kDa) similar to that of in vitro-sumoylated c-Myb shown in Figure 4C. Western blotting to detect lamin B and β-actin indicated that the cytoplasmic and nuclear fractions were not contaminated by each other (Figure 6D). These results suggest that c-Myb is retained in the cytoplasm by sumoylation.

TRAF7 Sequesters c-Myb in the Cytoplasm

We next examined the effect of overexpressed TRAF7 on the subcellular localization of c-Myb in transfected CV-1 cells (Figure 7). When either wild-type c-Myb, 2KR mutant or TRAF7 alone was overexpressed in CV-1 cells, both forms of c-Myb were found almost uniformly distributed throughout the nucleoplasm, whereas TRAF7 was in the cytoplasm (Figure 7, A and B, and Supplementary Figure S2A). It is interesting that TRAF7 appears to be localized in the specific regions of cytoplasm or uniformly in the cytoplasm, depending on each cell. TRAF7 also accumulated noticeably at the periphery of the nuclear membrane. To examine whether TRAF7 affects the subcellular localization of c-Myb, CV-1 cells were transfected the wild-type or the 2KR mutant of c-Myb and the TRAF7 expression plasmid. Transfected cells were permeabilized using digitonin or Triton X-100 and stained with antibodies against c-Myb or TRAF7. When wild-type c-Myb was coexpressed with TRAF7, significant amounts of c-Myb were detected in the cytoplasm (Figure 7C and Supplementary Figure S2B). In contrast, the subcellular localization of the 2KR mutant was not affected by TRAF7 (Figure 7C and Supplementary Figure S2C). In these experiments, digitonin disrupted the cellular membranes but not the nuclear membrane, because the nuclear lamin B was not detected in the digitonin-permeabilized cells (Figure 7D).

To further investigate whether sumoylation of c-Myb is important for cytoplasmic sequestration of c-Myb, we examined the colocalization of TRAF7 with SUMO1 and c-Myb. When c-Myb was coexpressed with only SUMO1, both c-Myb and SUMO1 were detected only in the nucleus (Figure 8A). When c-Myb and SUMO1 were coexpressed with TRAF7, however, significant amounts of c-Myb and SUMO1 were localized not only in the nucleus but also in the cytoplasm (Figure 8, B and C, and Supplementary Figure S3, A and B). The 2KR mutant of c-Myb, which cannot be sumoylated by TRAF7, was detected only in the nucleus when it was coexpressed with TRAF7 and SUMO1 (Figure 8D). SUMO1 was also localized not only in the nucleus but also in the cytoplasm when expressed with TRAF7 and 2KR mutant of c-Myb (Figure 8C and Supplementary Figure S3C), probably because TRAF7 also sequesters SUMO1 or sumoylated-proteins other than c-Myb in the cytoplasm. These results further support that the cytoplasmic retention of c-Myb requires its sumoylation by TRAF7.

Previously, PIASy was reported to stimulate sumoylation of c-Myb (Dahle *et al.,* 2003). To directly compare the subcellular localization of two sumo E3 ligases of c-Myb, PIASy, and TRAF7, we also examined the subcellular localization of PIASy. When PIASy was expressed alone in CV-1 cells, it was localized almost uniformly in the nucleoplasm (Figure 8E). PIASy was reported to be detected in the nuclear bodies which are the dot-like domain (Sachdev *et al.,* 2001), whereas it was also shown to be localized almost uniformly in the nucleoplasm in other types of cells (Chun *et al.,* 2003). Thus, PIASy appears to be localized in the nuclear dotlike domain or uniformly in the nucleoplasm. When c-Myb and SUMO1 were coexpressed with PIASy, all c-Myb and SUMO1 were detected only in the nucleus, and neither c-Myb nor SUMO1 was localized in the cytoplasm (Figure 8, F and G). Thus, the subcellular localization of TRAF7 and PIASy exhibit a striking difference, although both stimulate sumoylation of c-Myb.

DISCUSSION

In the present study, we identified TRAF7 as a c-Myb-binding protein. TRAF7 enhances the sumoylation of c-Myb, causing c-Myb to be retained in the cytoplasm. Thus, TRAF7 negatively regulates c-Myb-induced *trans*-activation via its sumoylation.

In CV-1 cells, overexpressed TRAF7 localized primarily in the cytosol, and coexpression of TRAF7 with c-Myb stimulated the sumoylation of c-Myb and its retention in the cytoplasm. Thus, TRAF7 inhibits wild-type c-Myb-induced *trans*-activation by blocking the nuclear entry of c-Myb via sumoylation. Endogenous sumoylated c-Myb was detected in the cytosol of M1 cells. The cytoplasmic distribution of c-Myb in human hematopoietic cells has also been reported (Bading *et al.,* 1988), but the mechanism is unknown. Our present study demonstrates that sumoylation by TRAF7 contributes to the cytoplasmic retention of c-Myb. Multiple reports have suggested that SUMO modification could be

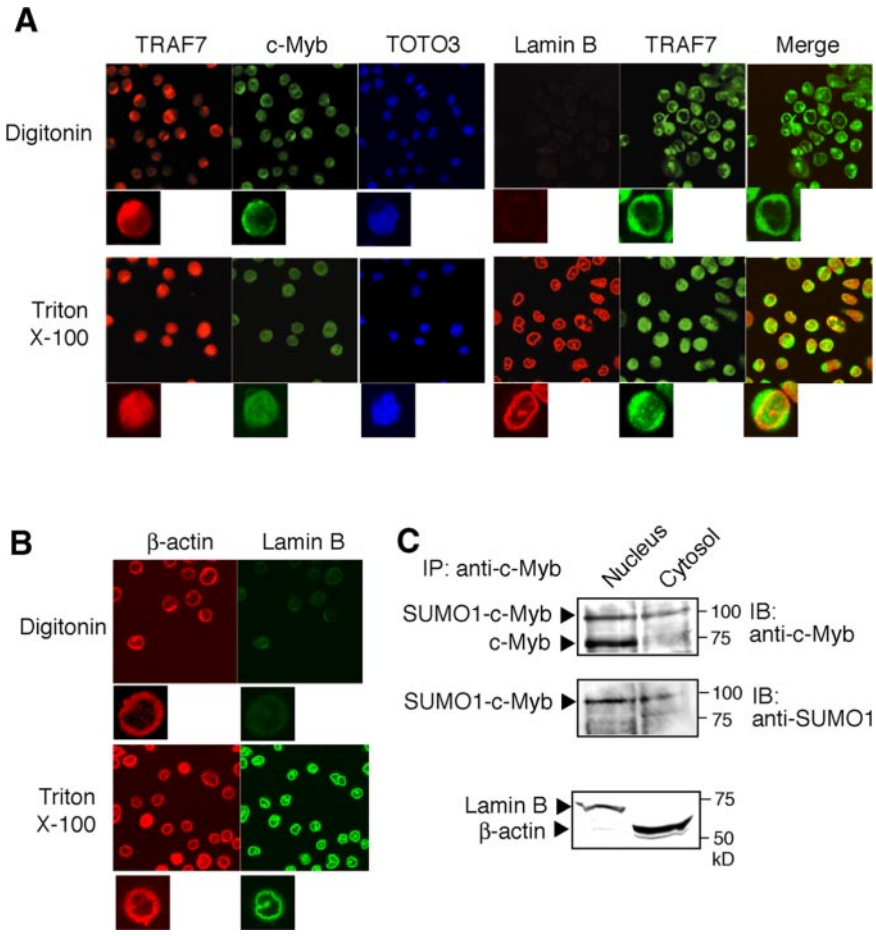

Figure 6. Endogenous TRAF7 and c-Myb are localized in both cytoplasm and nucleus. (A) Immunostaining of endogenous TRAF7 and c-Myb. M1 cells were permeabilized with digitonin (top panels) or Triton X-100 (bottom panels), immunostained with antibodies against the protein indicated above the panel, and visualized by FITC- and rhodamine-conjugated secondary antibodies, using confocal microscopy. DNA was stained with TOTO3. Typical staining pattern of single cell is shown below with higher magnification. In the left three panels, TRAF7, c-Myb, and DNA are shown in red, green, and blue, respectively. In the right three panels, TRAF7 and lamin B are shown in green and red, respectively. TRAF7 and lamin B are superimposed (the right-most panels). Transfection of empty vector alone did not give rise any signals (unpublished data). (B) Control experiments for permeabilization. M1 cells were permeabilized with digitonin (top panels) or Triton X-100 (bottom panels), immunostained with anti-β-actin or anti-lamin B antibodies, and visualized as described above. β-Actin and lamin B are shown in red and green, respectively. Typical staining pattern of single cell is shown below with higher magnification. (C) Subcellular fractionation of TRAF7 and c-Myb. M1 cells were lysed in hypotonic buffer, and the cytosol and nuclear fractions were separated. Proteins of both fractions were immunoprecipitated using anti-c-Myb antibody, and subjected to Western blotting using anti-TRAF7 or anti-SUMO1 antibody. (D) β-tubulin and lamin B were detected as controls.

coupled to the transport of proteins between the nucleus and the cytoplasm. RanBP2 (also referred as to Nup358) has SUMO E3-like activity (Pichler *et al.*, 2002), suggesting that SUMO modification may occur at nuclear pore complexes (NPCs) because proteins are transported between the nucleus and the cytoplasm. In addition, Ubc9 localizes to both the cytoplasmic and nucleoplasmic fibrils of the NPC (Zhang *et al.*, 2002). SENP2, a SUMO protease that is able to remove SUMO from modified proteins, localizes to the nucleoplasmic face of the NPC (Zhang *et al.*, 2002). TRAF7 may be one of the E3 ligases that is enriched on the outer nuclear membrane to regulate nuclear entry of transcription factors such as c-Myb.

Endogenous TRAF7 in M1 cells is found not only in the cytosol but also in the nucleus, whereas overexpressed TRAF7 in CV-1 cells is primarily localized to the cytosol. M1 cells could have some factor which transports TRAF7 into the nucleus by modifying or binding to TRAF7. In the nucleus of M1 cells, TRAF7 appears to be almost uniformly distributed in the nucleoplasm. On the other hand, PIASy is found associated with the nuclear bodies (Sachdev *et al.*, 2001) or almost uniformly in the nucleoplasm (Chun *et al.*, 2003). Although overexpressed TRAF7 in CV-1 cells is mainly localized in the cytoplasm, we cannot exclude the possibility that small amounts of TRAF7 are also present in the nucleus. Because TRAF7 and PIASy sumoylate c-Myb at the same sites, it is important to analyze whether TRAF7 and PIASy have any difference in regulating c-Myb activity in the nucleus.

We have observed that coexpression of TRAF7 inhibited the transactivation by wild-type c-Myb, but not by the sumoylation site mutants of c-Myb (Figure 5). In these experiments, we prepared the lysates for CAT assays 24 h after transfection. When we measured the CAT activity 48 h after transfection, however, TRAF7 affected similarly both the wild-type and 2KR-dependent transactivation (unpublished data). These results suggest that accumulated TRAF7 nonspecifically affects the Myb-dependent transactivation by regulating other proteins. It should be noted that multiple reports demonstrated that the repression of activity of multiple transcription factors by sumo E3 ligase, such as PIAS, does not depend on the sumoylation sites (see review by Verger *et al.*, 2003). For instance, PIAS1 and PIASy act as sumo E3 ligase of p53 and LEF-1, respectively, and repress the activity of both wild-type and the sumoylation site mutant of p53 and LEF-1 (Sachdev *et al.*, 2001; Schmidt and Muller, 2002). Although the mechanism of these observations remains unknown at present, it is possible that PIAS may also indirectly affect the transactivation by p53 and LEF-1 by regulating some other factors that bind to p53 and LEF-1.

c-Myb is sumoylated by TRAF7 at Lys-523 and Lys-499, which are the same sites as those previously shown to be sumoylated by PIASy. PIASy is found in the nucleus (Sachdev *et al.*, 2001; Chun *et al.*, 2003), whereas TRAF7 is found only in the cytoplasm in transfected CV-1 cells. Both TRAF7 and PIASy inhibit c-Myb-induced *trans*-activation. PIASy was speculated to inhibit transactivation by multiple tran-

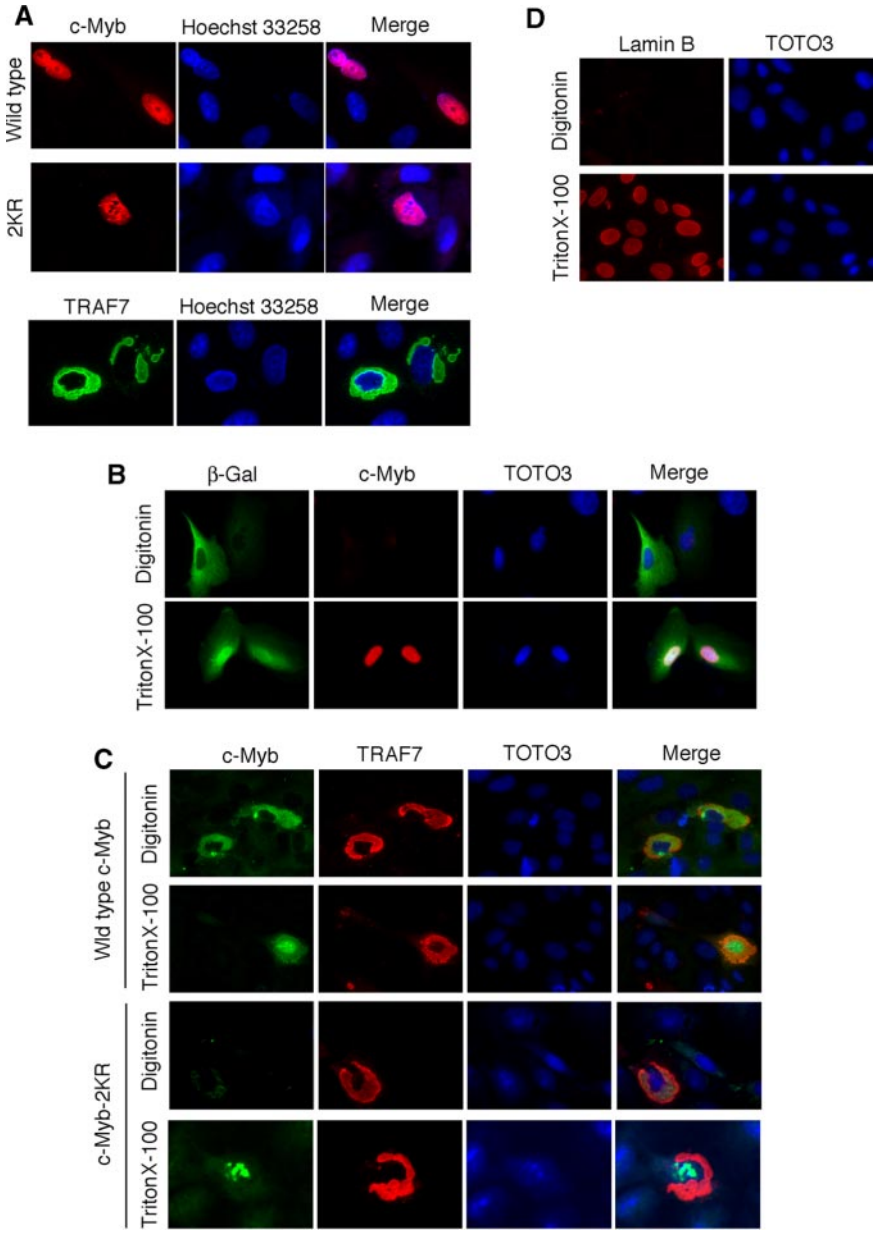

Figure 7. Overexpressed TRAF7 in CV-1 cells sequesters c-Myb in the cytoplasm. (A) Localization of overexpressed TRAF7 in the cytoplasm. CV-1 cells were transfected with plasmids expressing the proteins shown above each panel. The cells were permeabilized with Triton X-100 and immunostained with antibodies against each protein, visualized by rhodamine-or FITC-conjugated secondary antibodies, and analyzed by confocal microscopy. DNA was stained with Hoechst 33258. c-Myb, TRAF7, and DNA are shown in red, green, and blue, respectively. Transfection of empty vector alone did not give rise any signals (unpublished data). (B) Localization of overexpressed c-Myb in the nucleus. CV-1 cells were transfected with the wild-type c-Myb expression plasmid (2 μg) and the β-galactosidase expression plasmid (2 μg). Transfected cells were permeabilized with digitonin (top panels) or Triton X-100 (bottom panels), immunostained with antibodies against the proteins shown above, visualized by FITC- and rhodamine-conjugated secondary antibodies, and analyzed by confocal microscopy. DNA was stained with TOTO3. β-Galactosidase, c-Myb, and DNA are shown in green, red, and blue, respectively. In the right-most panels, the signals for both proteins and DNA are superimposed. (C) TRAF7 sequesters c-Myb in the cytoplasm. CV-1 cells were transfected with the wild-type or 2KR mutant c-Myb expression plasmid (2 μg) and the TRAF7 expression plasmid (4 μg). Transfected cells were permeabilized with digitonin (top panels) or Triton X-100 (bottom panels), immunostained with antibodies against the proteins shown above, visualized by FITC- and rhodamine-conjugated secondary antibodies, and analyzed by confocal microscopy. DNA was stained with TOTO3. TRAF7, c-Myb, and DNA are shown in red, green, and blue, respectively. In the right-most panels, the signals for both proteins and DNA are superimposed. More examples of immunostaining are shown in Supplementary Figure S2, B and C. (D) Control experiments for permeabilization. CV-1 cells were permeabilized with digitonin (top panels) or Triton X-100 (bottom panels), immunostained with the anti-lamin B antibody, and visualized as described above. DNA was stained with TOTO3. Lamin B and DNA are shown in red and blue, respectively.

scription factors, including c-Myb, by recruiting sumoylated transcription factors into the nuclear matrix, and probably into PML nuclear bodies (Sachdev *et al.*, 2001; Ross *et al.*, 2002; Schmidt and Muller, 2002; Dahle *et al.*, 2003). In contrast, cytosolic TRAF7 sequesters c-Myb outside the nuclear membrane and inhibits the c-Myb-induced *trans*-activation. Thus, PIASy and TRAF7 sumoylate c-Myb at the same sites, but the mechanisms by which they regulate c-Myb activity are different. However, we cannot completely exclude the possibility that inhibition of c-Myb activity by TRAF7 may be an effect of sumoylation per se, because the cytoplasmic sequestration of TRF7-sumoylated c-Myb is not complete.

TRAF7 was previously demonstrated to be an E3 ubiquitin ligase capable of self-ubiquitination (Bouwmeester *et al.*, 2004). However, we have shown that TRAF7 stimulates the sumoylation of c-Myb. Thus, TRAF7 can stimulate both ubiquitination and sumoylation. Recently, it was reported that the Mdm2 RING finger E3 ubiquitin ligase can also promote NEDD8 modification of p53 (Xirodimas *et al.*, 2004). Thus, one RING finger protein may be able to have the activity of multiple members of the ubiquitin ligase family.

TRAF7 was designated as a TRAF family protein based on the high homology with the RING and zinc finger domains of TRAF proteins (Xu *et al.*, 2004). However, the C-terminal domain conserved in TRAF1–6 is replaced by the WD40 repeats in TRAF7. TRAF2 and TRAF6 have been most extensively studied, and these proteins associate, directly or indirectly, with members of the TNF receptor family to mediate signals from these receptors. TRAF2 and TRAF6 have been shown to function as ubiquitin ligases (E3) through their N-terminal RING domains (Deng *et al.*, 2000). Although ubiquitination normally targets a protein for deg-

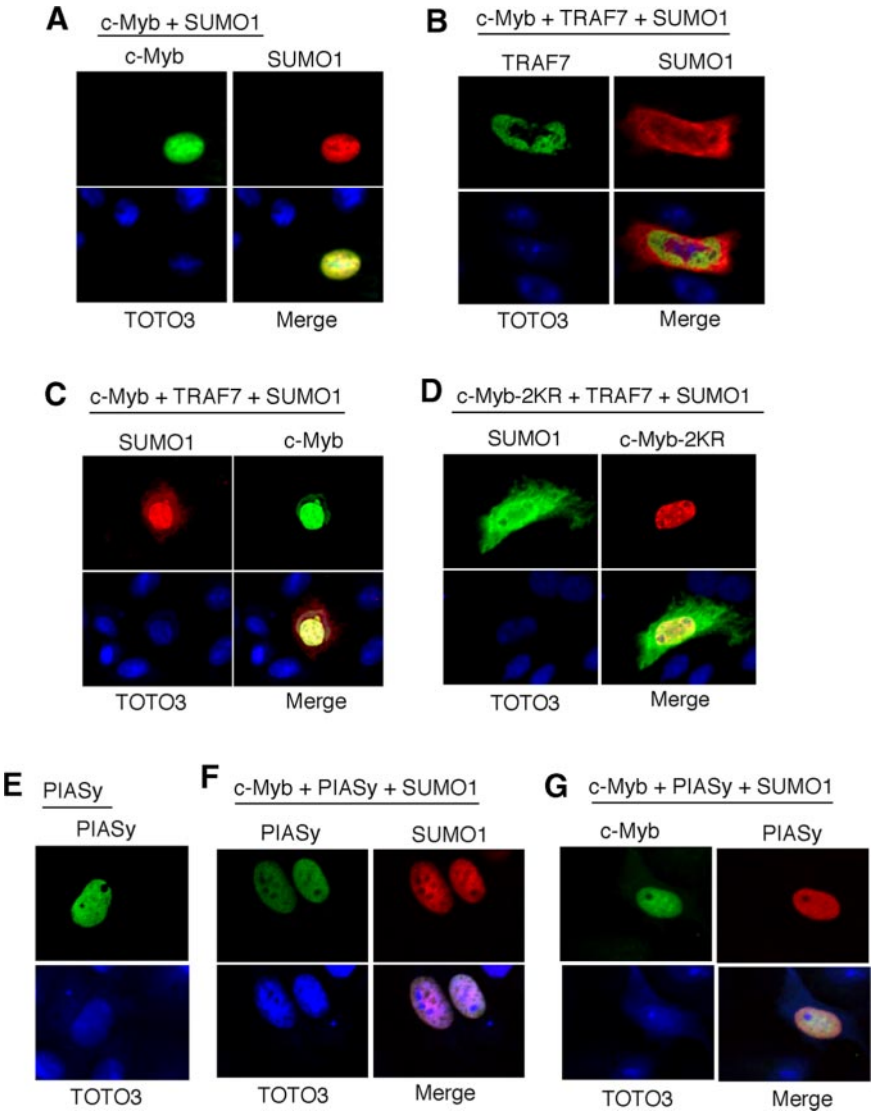

Figure 8. TRAF7 sequesters SUMO1 in the cytoplasm. (A) Colocalization of c-Myb and SUMO1 in the nucleus in the absence of TRAF7. CV-1 cells were transfected with plasmids expressing c-Myb (2 μg) and SUMO1 (1 μg). The cells were permeabilized with Triton X-100 and immunostained with antibodies against each protein, visualized by FITC- or rhodamine-conjugated secondary antibodies, and analyzed by confocal microscopy. DNA was stained with TOTO3. c-Myb, SUMO1, and DNA are shown in green, red, and blue, respectively. The signals for c-Myb, SUMO1, and DNA are superimposed in the bottom right panel. (B and C) TRAF7 sequesters SUMO1 and c-Myb in the cytoplasm. CV-1 cells were transfected with plasmids expressing c-Myb (2 μg), SUMO1 (1 μg), and TRAF7 (4 μg), permeabilized with Triton X-100, and immunostained with antibodies against each protein, visualized by FITC- or rhodamine-conjugated secondary antibodies, and analyzed by confocal microscopy. DNA was stained with TOTO3. In B, TRAF7, SUMO1, and DNA were shown in green, red, and blue, respectively. In C, c-Myb, SUMO1, and DNA were shown in green, red, and blue, respectively. The signals for the proteins and DNA were superimposed in the bottom right panels. (D) TRAF7 sequesters SUMO1, but not the 2KR mutant of c-Myb in the cytoplasm. CV-1 cells were transfected with plasmids expressing the 2KR mutant of c-Myb (2 μg), SUMO1 (1 μg), and TRAF7 (4 μg), and immunostaining was performed as described above. DNA was stained with TOTO3. 2KR mutant of c-Myb, SUMO1, and DNA are shown in red, green, and blue, respectively. The signals for c-Myb, SUMO1, and DNA are superimposed in the bottom right panel. (E) Nuclear localization of PIASy. CV-1 cells were transfected with the PIASy expression plasmid (2 μg), and immunostaining was performed as described above. DNA was stained with TOTO3. PIASy and DNA are shown in green and blue, respectively. (F and G) Nuclear localization of c-Myb and SUMO1 in the presence of PIASy. CV-1 cells were transfected with plasmids expressing c-Myb (2 μg), SUMO1 (1 μg), and PIASy (4 μg), and immunostaining was performed as described above. DNA was stained with TOTO3 (blue). In F, PIASy and SUMO1 were shown in green and red, respectively. In G, c-Myb and PIASy were shown in green and red, respectively. The signals for the proteins and DNA were superimposed in the bottom right panels.

radation, TRAF2- and TRAF6-mediated ubiquitination leads to the activation of downstream kinases through a proteasome-independent mechanism. Specifically, the TRAF ubiquitin ligases catalyze the synthesis of a unique polyubiquitin chain linked through lysine 63 (K63) of ubiquitin. The interaction of TRAF2 with the TNF receptor 2 requires the C-terminal domain conserved in TRAF1–6 (Takeuchi *et al.*, 1996), which TRAF7 lacks, suggesting that TRAF7 cannot interact with members of the TNF receptor family. However, TRAF proteins form a trimer via the coiled-coil region (Park *et al.*, 1999), which is a present in TRAF7. Thus, we are presented with the interesting possibility that the signals from members of the TNF receptor family induce TRAF7-dependent sumoylation via trimers containing TRAF7 and other TRAF family members. The present results demonstrating the TRAF7-induced sumoylation of c-Myb may lead to an understanding of the molecular mechanisms by which c-Myb activity is regulated.

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