Nuclear Tumor Necrosis Factor Receptor-associated Factor 6 in Lymphoid Cells Negatively Regulates c-Myb-mediated Transactivation through Small Ubiquitin-related Modifier-1 Modification

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Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an adaptor/scaffold protein that mediates several important signaling pathways, including the tumor necrosis factor-R:NF-κB pathway, involved in immune surveillance, inflammation, etc. Because most studies of TRAF6 function have focused primarily on its role as an adaptor molecule in signaling pathways in the cytoplasm, the potential functions of TRAF6 in other cellular compartments has not been previously investigated. Here, we demonstrate that TRAF6 resides not only in the cellular cytoplasm but is also found in the nuclei of both normal and malignant B lymphocytes. TRAF6 does not possess a nuclear localization signal but enters the nucleus through the nuclear pore complex containing RanGap1. Chromatin immunoprecipitation cloning experiments demonstrated that nuclear TRAF6 associates with c-Myb within the 5′-end of the c-Myb promoter. Further analysis showed that nuclear TRAF6 is modified by small ubiquitin-related modifier-1, interacts with histone deacetylase 1, and represses c-Myb-mediated transactivation. Thus, TRAF6 negatively regulates c-Myb through a novel repressor function in the nuclei of both normal and malignant B lymphocytes that could represent a novel control mechanism that maintains cell homeostasis and immune surveillance.

The tumor necrosis factor (TNF) receptor family (e.g. CD40 and BAFF-R) plays a key role in normal as well as neoplastic B-cell growth and survival (1, 2). TNF receptor engagement results in the assembly of a cascade of signaling molecules composed of critical proteins, including adaptor molecules such as TNF receptor-associated factor 6 (TRAF6) that are recruited to the cell membrane and into lipid raft microdomains (3). TRAF6 has been shown to act as an E3 ubiquitin ligase, allowing TRAF6 autoubiquitination, which further activates the IKK complex (4, 5), leading to activation of key transcription factors such as NF-κB, NFAT, and AKT, all of which are critical for cell growth, survival, and osteoclast differentiation. Although ubiquitination normally targets a protein for degradation, TRAF6-mediated ubiquitination is independent of proteasomal degradation (5–7), suggesting that the function of TRAF6 is broader than initially thought.

TRAF6 is one of seven closely related TRAF proteins and was isolated by screening of an expressed sequence tag expression library utilizing CD40 as bait (8). All TRAF protein sequences contain a C-terminal receptor-binding domain that mediates receptor binding. All of the TRAFs except for TRAF1 contain a variable number of zinc-finger motifs and a zinc ring domain (9). The zinc-binding domains seem to function principally in mediating interactions with other proteins such as kinases and transcription factors that are involved in the propagation and regulation of signaling cascades. TRAF6 is unique among the TRAFs in showing the least homology to the prototypical TRAF domain sequence and interacting with both the TNF-receptor family members as well as the Toll-like receptor family for signaling (10). TRAF6-deficient mice show defects in B-cell differentiation, lymph node organogenesis, osteoclastogenesis, and interleukin-1, lipopolysaccharide, and receptor activator of NF-κB signaling (11). Because most studies of TRAF6 have focused primarily on its function as an adaptor molecule in cytoplasmic signaling pathways, the role of TRAF6 in other cellular compartments has not been investigated.

Our recent studies have demonstrated that CD40, a TNF-receptor family member, is present in the nucleus of both normal and lymphoma B cells and also acts as a transcription factor (12, 13). Recent studies by others revealed the presence of a membrane growth factor receptor (14) and adaptor molecules (Daxx and TAB2) (15, 16) in the cell nucleus. These studies unveiled a new paradigm for cell signal transduction involving...
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cell membrane and adaptor proteins trafficking into the cell nucleus. However, the functional roles of these proteins in the nucleus and how they enter the nucleus have yet to be fully defined. Recent studies have suggested that sumoylation, a post-translational protein modification step very similar to protein ubiquitination, involving covalent attachment of small ubiquitin-related modifier (SUMO) to lysine residues, is linked to nucleocytoplasmic protein transport and transcriptional gene repression (17, 18).

Here, we demonstrate that TRAF6, a cytoplasmic adaptor protein, enters the nucleus through the nuclear pore complex involving RanGAP-1, a Ran GTPase-activating enzyme. Further studies show that nuclear TRAF6 is modified by SUMO-1, interacts with histone deacetylase (HDAC) 1, and has the ability to repress c-Myb-mediated transactivation in B lymphocytes.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Human large B-cell lymphoma cell lines (MZ, McA, LR, MS, LP, and EJ) were established from diagnostic biopsy tissue or effusions from patients as described elsewhere (16). The cells were cultured in RPMI medium (Invitrogen) containing 10% fetal calf serum (HyClone, Logan, UT). Normal human B lymphocytes were purified from donors’ buffy coats by using the human B-cell enrichment mixture from StemCell Technologies (Vancouver, Canada).

**Antibodies and Small Interfering RNA Oligonucleotides**—The following primary antibodies were used: polyclonal and monoclonal TRAF6, c-Myb, SUMO-1, RanGAP-1, and HDAC1 (Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescein isothiocyanate and Texas-Red-labeled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Small-interfering RNA (siRNA) oligonucleotides for TRAF6 were purchased from Ambion (Austin, TX).

**Subcellular Fractionation of B Lymphoid Cells**—Cellular fractionation procedures were performed as described previously (12).

**Chromatin Immunoprecipitation PCR and Cloning Assays**—Chromatin immunoprecipitation (ChIP) cloning assays were performed using the ChIP assay kit and protocol provided by Millipore (Billerica, MA). Cells were cross-linked with 1% formaldehyde in culture medium for 10 min at 37 °C, washed with cold 1× phosphate-buffered saline, resuspended in SDS cell lysis buffer (provided with kit) for 10 min on ice, and sonicated three times at 10-s intervals. Samples were subjected to centrifugation for 10 min at 13,000 rpm at 4 °C, and the supernatants were diluted with ChIP dilution buffer. To reduce non-specific background, samples were precleared with salmon sperm DNA and protein A-agarose (50% slurry) for 30 min at 4 °C with agitation. Primary antibodies were added to the samples that were incubated overnight at 4 °C. The slurry was added to each sample, which was then incubated for an additional hour. The protein A-antibody-DNA complexes were washed and eluted according to the manufacturer’s protocol and then reverse cross-linked by heating at 65 °C for 4 h. DNA fragments were purified by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Purified DNA from immunoprecipitations and DNA inputs were used for PCR amplification with PCR beads from Amersham Biosciences and oligonucleotide primers specific for the c-Myb promoter (forward, 5′-TTA GTG AGC GGT GAT GTG TG-3′; reverse, 5′-AAT TCC CGC ACA GAA GAT TG-3′; 225 bp). The PCR conditions were as follows: the cDNA template was denatured at 95 °C for 1 min, annealed at 48 °C for 30 s, and extended at 72 °C for 1 min per cycle for 35 cycles. The PCR product was visualized on a 2.0% agarose gel.

For cloning purposes, DNA fragments were repaired in the reaction containing 1 mM dNTPs and T4 DNA polymerase at 37 °C for 30 min to create blunt ends. DNA fragments were purified by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The immunoprecipitated DNA fragments were dephosphorylated by calf intestine phosphatase and cloned into PCR-Blunt II Topo vectors (Invitrogen). Each clone was subjected to DNA sequencing and subsequent Blast computer analysis to identify the DNA sequence from the Entrez Genome Project data base of the National Institutes of Health.

**Co-immunoprecipitation Procedures**—Antibodies were cross-linked to Dynabeads protein A (Dynal Biotech, Oslo, Norway) according to the manufacturer’s directions. Cell lysates were precleared with IgG Dynabeads protein A for 30 min at 4 °C before incubation with antibody-linked Dynabeads overnight at 4 °C. The immunoprecipitated Dynabead complexes were washed five times with immunoprecipitation buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 0.5% Nonidet P-40, 0.5% glucopyranoside, 1 µg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride). Proteins were eluted by boiling in protein loading buffer and then processed for Western blot analysis.

**Confocal Microscopic Analysis**—Cells were cytopun onto poly-L-lysine-coated glass slides, fixed with 100% cold methanol for 5 min, and air-dried. Nonspecific protein binding was prevented by blocking the cells with 5% fetal calf serum in phosphate-buffered saline. Cells were stained with the appropriate primary antibodies (1:200 dilution) for 2 h at room temperature or overnight at 4 °C. After three washes with phosphate-buffered saline, the slides were stained with the appropriate antidonkey secondary antibody (labeled with fluorescein isothiocyanate, Cy2, Cy3, Texas-Red, or Cy5; 1:200 dilution) for 45 min and washed with phosphate-buffered saline. Coverslips were applied with Slow Fade reagent (Molecular Probes, Eugene, OR). The cells were visualized using an Olympus laser scanning confocal microscope. Images were captured with a 60× objective using the appropriate filter sets.

**Plasmids and Site-directed Mutagenesis**—TRAF6 expression vectors in pGEX-4T1 and pcR3-FLAG have been previously described (5). Site-directed mutagenesis was performed using the QuikChange multisite-directed mutagenesis kit from Stratagene (La Jolla, CA). All mutations in reporter constructs were verified by DNA sequencing. The c-Myb expression vector was a gift from Dr. Alan Gewirtz (University of Pennsylvania School of Medicine, Philadelphia, PA). The EW5-luc plasmid (3×-c-Myb binding sites) was provided by Dr. Scott A. Ness (The University of New Mexico, Albuquerque, NM). The pCMVSPORT-SUMO1 expression vector was purchased from Open Biosystems (Huntsville, AL).
**RESULTS**

**Identification of Nuclear TRAF6 in Lymphoma B Cells**—While studying the nuclear role of CD40 in lymphoma B cells, we identified the presence of TRAF6 protein in the nuclei of cells from five different aggressive B-cell lymphoma cell lines by nuclear cell fractionation and Western blotting (Fig. 1A). TRAF5, another member of the TRAF protein family, on the other hand, was detected primarily in the cytoplasmic fraction and was used as a negative control. TRAF2 and TRAF3 were also found in the nucleus, but at lower relative concentrations in comparison to TRAF6 (data not shown). TRAF6 is also present in the nuclei of both resting (Go) and activated T and B lymphocytes (Fig. 1B; for more detail on B cells, see supplemental Fig. S2). To further determine the subcellular localization of TRAF6 in lymphoma B cells, we used indirect immunofluorescence confocal microscopy as well as nucleoplasm fractionation analysis. As shown in Fig. 1C, confocal microscopic analysis demonstrated that TRAF6 protein was expressed not only in the plasma membrane and cellular cytoplasm but also in the nucleus of lymphoma B cells. TRAF6 protein expression was not widely distributed throughout the nucleus, but it was expressed unequivocally in punctate nuclear body protein complexes in the nuclear compartment, demonstrated using stack images from confocal microscopic analysis (see supplemental Fig. S1). Control TRAF5 was detected primarily in the cytoplasm (Fig. 1C). Cell fractionation studies in two different lymphoma cell lines confirmed that TRAF6 was also present in the nucleoplasm fraction, suggesting that TRAF6 may interact with chromatin (Fig. 1D).

**Characterizing Nuclear TRAF6 in Lymphoma B Cells**—TRAF6 has been shown to function as an E3 ubiquitin ligase and is capable of autoubiquitination. To determine if TRAF6 was ubiquitinated in lymphoma B cells, co-immunoprecipitation analysis was performed using anti-TRAF6 polyclonal antibody with cytoplasmic and nuclear extracts purified from a lymphoma cell line (MS). As shown in Fig. 2A, TRAF6 is endogenously autoubiquitylated in the cytoplasmic fraction but not in the nuclear fraction, suggesting that TRAF6 may have a role other than ubiquitination in the nucleus. Subsequent ChIP cloning analysis using anti-TRAF6 poly-

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**FIGURE 1. Identification of nuclear TRAF6 in lymphoma B cells.** A, cytoplasmic (25 μg) and nuclear extracts (25 μg) from five large B-cell lymphoma cell lines were subjected to Western blotting for TRAF6 and TRAF5. Actin and Oct-1, cytoplasmic and nuclear markers, respectively, were used as controls. B, nuclear extracts (25 μg) from resting T-lymphocytes (GoT), PMA/ion-activated T-lymphocytes (ActT), resting B-lymphocytes (GoB), CD40L/IgM-activated B-lymphocytes (ActB), and a large B-cell lymphoma cell line (MS) were subjected to Western blotting for TRAF6 and lamin B (nuclear marker). C, confocal microscopy analysis of TRAF6 (top panels) and TRAF5 (bottom panels) in large B-cell lymphoma MS cells. Topro-3 was used as a nuclear marker. D, plasma membrane (PM), cytoplasm (C), endoplasmic reticulum (ER), nuclear envelope (NE), and nucleoplasm fractions isolated by extensive cellular fractionation from two large B-cell lymphoma cell lines (MS and McA) were subjected to Western blotting for TRAF6, p62 (ER/NE marker), Oct-1 (nuclear marker), and actin (cytoplasmic marker). Each lane was loaded with 25 μg of protein from each fraction.
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Interaction of TRAF6 with c-Myb Results in Repression of Its Transactivation Activity—The 185-bp DNA fragment of the c-Myb promoter, which was immunoprecipitated with TRAF6 antibody using ChIP cloning assays, contains a consensus c-Myb binding site. The other clones obtained were also analyzed for transcription factor binding sites that identified 87% of the sequences as containing at least one consensus c-Myb binding site (data not shown). These findings suggest that TRAF6 interacts with DNA, possibly indirectly, probably through binding to the c-Myb protein. Immunoprecipitation assays clearly showed that TRAF6 interacts with c-Myb (Fig. 3A) that was confirmed by confocal microscopic analysis (Fig. 3B). Because the Gal4 assays had demonstrated that TRAF6 has transcriptional repression activity, we examined whether TRAF6 could be involved in regulating c-Myb-mediated transcriptional activity. When B-cell lymphoma MS cells were co-transfected with a 3×-c-Myb binding site reporter plasmid (EW5-luc) in conjunction with a c-Myb expression vector and with increasing amounts of FLAG-TRAF6 expression vector, overexpression of TRAF6 repressed c-Myb-mediated transcriptional activity in a dose-dependent manner (Fig. 3C). To further substantiate the TRAF6-mediated transcriptional repression of c-Myb, normal B-lymphocytes were co-transfected with a TRAF6 expression vector along with the reporter EW5-luc, followed by CD40 ligand and anti-IgM stimulation. Normal B-cell activation by CD40 and IgM induced c-Myb transcriptional activity (Fig. 3D). As predicted, TRAF6 also repressed c-Myb transcriptional activity induced by CD40 and IgM stimulation in normal B-lymphocytes (Fig. 3D), further confirming that TRAF6 negatively regulates c-Myb-mediated transcription.

We next questioned whether endogenous TRAF6 represses c-Myb transcriptional activity. To test this hypothesis, we inhibited endogenous TRAF6 protein expression using an RNA interference approach. Transfection of a TRAF6 siRNA into lymphoma B cells resulted in a decrease of cellular TRAF6 protein without altering the endogenous level of actin protein (Fig. 3E). Under these conditions, TRAF6 siRNA transfection enhanced c-Myb-mediated transactivation (Fig. 3E), further confirming the negative regulatory effect of nuclear TRAF6 on c-Myb transcriptional activity.

clonal antibody (Fig. 2B) revealed a total of 200 clones, one of which contained a 185-bp fragment belonging to the proximal 5′-end of the c-Myb oncogene promoter. ChIP analysis followed by reverse transcription-PCR confirmed the interaction of TRAF6 with the c-Myb promoter (Fig. 2C). c-Myb is known to bind to its own promoter, in the same region as TRAF6 (Fig. 2C). The other 199 clones contained DNA that did not match any known promoter sequence. However, the majority of the sequences matched genomic DNA that is in the proximity of both known and unknown genes (data not shown). The interaction of TRAF6 with DNA, either directly or indirectly, suggests that TRAF6 might have a role in gene transcriptional regulation. To determine if TRAF6 has transcriptional activation activity, yeast two-hybrid assays were performed by fusing full-length TRAF6 to the Gal4 DNA-binding domain and then transfecting the resulting construct into lymphoma cells. Unexpectedly, the chimeric protein showed decreased transactivation activity (Fig. 2D), suggesting that the TRAF6 protein might act as a negative transcriptional regulator in the nuclei of lymphoma B cells.

**FIGURE 2. Characterizing nuclear TRAF6 in lymphoma B cells.** A, cytoplasmic (cyto) and nuclear extracts (NE) from large B-cell lymphoma MS cells were used to immunoprecipitate endogenous TRAF6 using the polyclonal TRAF6 antibody, followed by Western blotting for TRAF6 (top) and ubiquitin (bottom). Rabbit IgG was used as a negative control. B, schematic diagram of ChIP cloning procedure. TRAF6 ChIP cloning isolated a total of 200 clones after five experiments, one of which contained a 185-bp sequence that matched the c-Myb promoter. C, ChIP-PCR analysis in large B-cell lymphoma MS cells to confirm the association of TRAF6 with the c-Myb promoter. c-Myb was used as a positive control and IgG as a negative control. D, lymphoma cells (MS) were transfected with pBind empty vector or pBind-TRAF6. After 24 h, cell extracts were analyzed for luciferase activity. pBind-id, pACT, and pMyOD were used as positive controls. Luciferase activities were normalized with the pBind empty vector alone. Data shown are representative of three independent experiments.
TRAF6 Is Modified by SUMO-1 in Neoplastic B Cells—Recent findings suggest that sumoylation, a protein modification process that requires conjugation of the small ubiquitin-like protein SUMO, is associated with gene transcriptional repression. Of the SUMO proteins, only SUMO-1 contains a nuclear localization signal that functions by transporting the cargo protein into the nucleus. Confocal microscopy assays demonstrated that TRAF6 co-localizes with SUMO-1 in the cytoplasm as well as in the nucleus (Fig. 4A). The nuclear interaction between TRAF6 and SUMO-1 appears to occur in nuclear body complexes (indicated by arrows) (see supplemental Fig. S1 for confocal stacking images). Immunoprecipitation analysis, carried out with TRAF6 antibody to confirm TRAF6-SUMO-1 interactions, demonstrated that, when immunoblotted with SUMO-1 monoclonal antibody, three protein bands of molecular masses 40, 80, and 90 kDa appeared in the nuclear extracts but were not present in cytoplasmic extracts or IgG controls (Fig. 4B). Because the TRAF6 protein is ~62 kDa, the 90-kDa protein band could represent SUMO-1-modified TRAF6, and the 40- and 80-kDa bands could represent SUMO-1-modified proteins that interact with TRAF6. Further analysis by Western blotting (Fig. 4C) and co-immunoprecipitation (Fig. 4D) confirmed that the 80-kDa band is RanGap1, a Ran GTPase-activating enzyme, fused with SUMO-1, and this fusion protein co-localized with TRAF6 at the nuclear membrane (Fig. 4E), suggesting that TRAF6 actively enters the nucleus through the nuclear pore complex. The 40-kDa protein has not as yet been identified.

To demonstrate that TRAF6 is modified by SUMO-1, we performed in vitro sumoylation assays by incubating recombinant GST-TRAF6 fusion proteins with reaction mixtures containing recombinant sumoylation components. A slower migrating protein band reacted with anti-SUMO-1 antibody in the reaction that contained TRAF6 and wild-type SUMO-1 but not in the reactions without E1/E2 enzymes or wild-type SUMO-1 or in the reaction with mutated SUMO-1 (Fig. 5A). These results indicate that the slower migrating band is in fact, a sumoylated full-length TRAF6 species. To confirm this result, endogenous TRAF6 proteins were co-immunoprecipitated from purified cytoplasmic extracts of a lymphoma cell line (MS) and incubated with sumoylation components as indicated in Fig. 5B.
TRAF6-containing immunoprecipitates were analyzed by Western blotting, using an anti-SUMO-1 or anti-TRAF6 antibody. A slower migrating band was observed in the sample with TRAF6 and wild-type SUMO-1 but was not present in the sample without wild-type SUMO-1 or with recombinant SENP1 (a SUMO protease) (Fig. 5B). Modified TRAF6 species could be cleaved by the catalytic domain of recombinant SENP-1, further confirming that TRAF6 is modified by SUMO-1. We next sought to examine whether TRAF6 is modified by SUMO-1 endogenously in vivo and to identify the amino acid residue(s) of TRAF6 involved in SUMO1 conjugation. A sumoylation site prediction software, based on properties sequential forward selection (20), reveals four SUMO consensus lysine sites (124, 142, 319, and 453) on TRAF6 with high scores and one SUMO lysine site (124) that was refined based on the NDSM (negatively charged amino acid-dependent sumoylation motif) (21). These sites were individually mutated on the TRAF6 expression vector, by converting the lysine amino acid residues into arginines. MS cells were co-transfected with the FLAG-tagged wild-type (wt)-TRAF6 or TRAF6 mutants, along with SUMO1 expression vector. A slower migrating band of ~90 kDa, which occurred only in wt-TRAF6 and K319R mutant, suggested that TRAF6 is modified by SUMO-1 at lysines 124, 142, and 453 (Fig. 5C). TRAF6 mutants (K124R, K142R, and K453) were unable to suppress c-Myb-mediated transcriptional activation (Fig. 5D), indicating that the sumoylation of TRAF6 at lysines 124, 142, and 453 is required for its repressive activity. Nuclear TRAF6 also interacts with HDAC1 (Fig. 5E), suggesting that TRAF6 can recruit HDAC proteins into transcription repressor complexes in gene promoters for its inhibitory role in the nucleus.

**DISCUSSION**

Many components of signal transduction networks, including membrane and adaptor proteins, are now known to localize to different cellular compartments, where they exert diverse functions (22, 23). The finding that TRAF proteins localize in the nucleus is not unprecedented, because previous studies have demonstrated that TRAF proteins such as TRAF2 (24) and TRAF4 (25) can be identified in the cell nucleus, although their functional roles in the nucleus have not as yet been defined. Min et al. (24) suggested that nuclear TRAF2 directly regulates transcriptional activity via a mechanism independent of its role in cytoplasmic signal transduction. Our findings show that nuclear TRAF6 may function differently than cytoplasmic TRAF6, possibly because TRAF6 ubiquitination occurs in the cytoplasm and sumoylation in the nucleus. How nuclear TRAF6 sumoylation correlates with TRAF6 ubiquitination in the cytoplasm and whether these mechanisms are dependent of each other are important issues. Recent findings have suggested that in some proteins such as...
IκBα, ubiquitination competes with sumoylation (26), and in certain circumstances, important proteins (e.g. NEMO) are ubiquitinated, and then sumoylated (27). In fact, both IκBα and IKKs, as well as CD40, all components of the CD40:NF-κB canonical pathway, are found in the nucleus, raising the possibility that the entire CD40-TRAF6-IKK-NF-κB signaling pathway, like the PI3K-AKT-PTEN signaling pathway (28), functions in the nuclear compartment.

Sumoylation has emerged recently as an important step in post-translational protein modification that regulates the functions of numerous proteins, mainly transcription factors, involved in many cellular processes (29). Post-translational modification by SUMO has diverse effects on substrate activity, but, in most cases described so far, sumoylation of transcriptional regulators correlates with inhibition of gene transcription (30). Recent studies provide new insights into the mechanisms by which sumoylation regulates transcription and suggest that one consequence of sumoylation is promotion of the interaction of transcription factors with co-repressors of transcription (17). Our finding that nuclear TRAF6 is modified by SUMO-1, repressing transcriptional activity, implies that TRAF6 has a function very similar to that of the protein Daxx, which initially was identified as a cytoplasmic signaling adaptor molecule but has been reported recently to function as a transcriptional repressor in the nuclear compartment (15). Unlike Daxx, TRAF6 does not contain an intrinsic nuclear localization signal, suggesting either that TRAF6 binds to another nuclear localization signal-bearing protein to be transported as molecular complex or that a novel mechanism regulates its nuclear import. Our results suggest that TRAF6 enters the nucleus, through the nuclear pore complex containing RanGap1, a RanGTPase-activating enzyme, where TRAF6 is then further modified by sumoylation, which confers its repressor function on gene transcription. TRAF6 is present in the nuclei of un-stimulated normal B cells and does not exhibit increased nuclear translocation in response to CD40L stimulation. This suggests that TRAF6 exists as a distinct pool in the nucleus that constitutively shuttles between the nucleus and the cytoplasm, as do the NF-κB upstream signaling kinases IκBα, NIK, and IKK in unstimulated cells in the absence of exogenous stimuli (31–33), or alternatively, that an unknown stimulus provides the signal for TRAF6 nuclear translocation. It is also possible that stress conditions can induce nuclear TRAF6 translocation, similar to IKK nuclear translocation and sumoylation modification observed under genotoxic stress induction (27). It will be of considerable importance to determine the one or more mechanisms that induce nuclear translocation of TRAF6 in lymphocytes in future studies.
The finding that TRAF6 is a negative regulator is also not unprecedented. Recent studies have suggested that TRAF6 is a T-cell-intrinsic negative regulator required for the maintenance of immune homeostasis, through the suppression of the PI3K-AKT pathway (34). However, the mechanism of suppression of PI3K-AKT activation by TRAF6 has yet to be further elucidated. We also have found that TRAF6 is expressed in the nucleus of T lymphocytes, suggesting that nuclear TRAF6 expression is not restricted to B lymphocytes. These data support our findings suggesting that TRAF6 can function as a negative transcriptional regulator, expanding the function of TRAF6 as an adaptor molecule in the cytoplasm. The negative regulatory mechanism of nuclear TRAF6 in lymphocytes could represent a novel control system to keep innate immunity and inflammation in check by suppressing key signaling pathways, such as c-Myb, a downstream target of the interleukin-2-PI3K-AKT pathway (35).

How TRAF6 represses c-Myb-mediated transactivation activity is still unknown. Our ChIP cloning experiments suggested that TRAF6 interacts with the c-Myb promoter DNA, through its association with c-Myb, an important transcription factor that has been shown to bind to its own promoter (36). c-Myb protein contains a transactivation domain and a negative regulatory domain, indicating that c-Myb can either activate or repress target genes (37). We suggest that TRAF6 represses c-Myb transactivation activity through one of two potential mechanisms: disassembling the protein complex between c-Myb and its partner transcription factors from binding to DNA or recruiting a repressor complex. Our confocal images show that TRAF6 and SUMO-1 co-localized in punctated nuclear protein complexes. Therefore, the latter mechanism seems more likely, because ChIP analysis demonstrated that TRAF6 and c-Myb endogenously bind to genomic DNA, and immunoprecipitation analysis showed that TRAF6 associates with HDAC1. Perhaps sumoylated TRAF6 can recruit corepressor proteins, such as HDAC1, to form nuclear promyelocytic leukemia (PML) bodies (38), similar to Sp100 or Daxx (15, 39). Although this result does not directly demonstrate dependence on HDAC for transcriptional repression activity by TRAF6, it is consistent with previous reports suggesting that sumoylation promotes transcriptional repression through recruitment of HDACs (40). On the contrary, sumoylated TRAF6 may enhance c-Myb sumoylation and repress c-Myb transactivation, by acting as a SUMO ligase similar to TRAF7 (41).

Until recently, TRAF molecules like TRAF6 were considered to be primarily cytoplasmic adaptor molecules, binding TNFR and Toll-like receptor-mediated signaling pathways (10). Our findings not only extend the role of TRAF6 in cellular signal transduction but also broaden the range of important physiological processes that are regulated by sumoylation. The physiological importance of negative regulation of c-Myb transactivation by nuclear TRAF6 and how it relates to the disease process in lymphomas are still unclear. c-Myb is an oncogene that is highly expressed in hematopoietic malignancies (42), but its exact oncogenic function is still mostly undefined (43). A recent study demonstrated, however, that c-Myb suppresses the oncogenic activity of the proto-oncogene c-Rel (44). Therefore, c-Myb can function as an oncogene or a tumor suppressor gene. Putting these findings together, our model predicts that TRAF6 can function as a negative regulator by suppressing transcriptional regulators to maintain cell signaling homeostasis in the nucleus, in addition to its role as a positive regulator of the NF-kB pathway in the cytoplasm (Fig. 6).

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