

Site-specific Lys-63-linked Tumor Necrosis Factor Receptor-associated Factor 6 Auto-ubiquitination Is a Critical Determinant of I κ B Kinase Activation*

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Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) is a key mediator in proximal signaling of the interleukin-1/Toll-like receptor and the TNF receptor superfamily. Analysis of TRAF6-deficient mice revealed a fundamental role of TRAF6 in osteoclastogenesis; however, the molecular mechanism underlying TRAF6 signaling in this biological process is not understood. Recent biochemical evidence has indicated that TRAF6 possesses ubiquitin ligase activity that controls the activation of IKK and NF- κ B. Because these studies are primarily based on cell-free systems, the role of the ubiquitin ligase activity of TRAF6 and its auto-ubiquitination to initiate the NF- κ B pathway *in vivo* remain elusive. Here we show that an intact RING domain of TRAF6 in conjunction with the E2 enzyme Ubc13/Uev1A is necessary for Lys-63-linked auto-ubiquitination of TRAF6 and for its ability to activate IKK and NF- κ B. Furthermore, a RING mutant of TRAF6 abolishes its ability to induce receptor activator of NF- κ B-independent osteoclast differentiation and nuclear accumulation of the transcription factor NFATc1. Notably, we map the auto-ubiquitination site of TRAF6 to a single Lys residue, which if mutated renders TRAF6 unable to activate transforming growth factor- β -activated kinase 1 and IKK and to cause spontaneous osteoclast differentiation. Additionally, we provide biochemical and *in vivo* evidence that TRAF6 serves as an E3 to directly ubiquitinate NEMO. Reconstituting TRAF6-deficient cells with various TRAF6 mutants, we clearly demonstrate the requirement for the TRAF6 RING domain and site-specific auto-ubiquitination of TRAF6 to activate IKK in response to interleukin-1. These data establish a signaling cascade in which regulated site-specific Lys-63-linked TRAF6 auto-ubiquitination is the critical upstream mediator of IKK.

The pro-inflammatory cytokines interleukin-1 (IL-1)² and tumor necrosis factor (TNF) elicit a critical function in the innate immune response. Following receptor activation, these cytokines induce a cascade of signaling events leading to the activation of transcription factors such as NF- κ B and AP1 through upstream kinases, including I κ B kinase (IKK) and the mitogen-activated protein kinases (MAPKs; JNK, p38, and ERK). These events culminate in the expression and regulation of numerous pro-inflammatory genes (1, 2).

TNF receptor-associated factors (TRAFs) constitute a family of seven known adaptor proteins, and most of them participate in activation of the transcription factor NF- κ B and members of the MAPKs (3–5). Several TRAF proteins interact directly with the intracellular regions of various members of the TNF receptor family through a highly conserved motif at their C terminus termed the TRAF domain. In contrast, the N-terminal domain of the TRAFs is less well conserved but consists of zinc finger motifs and in some TRAFs a RING (really interesting new gene) domain, which has been identified in a number of E3 ubiquitin ligases (6).

Recently, the N-terminal RING domain of TRAF6 has been included in a growing family of ubiquitin (Ub) ligases, also known as E3s (7). RING-type E3s contain a series of Cys and His residues distinctly separated to constitute a novel zinc-binding domain. The first hint that TRAF6 possesses Ub ligase activity came from biochemical reconstitution experiments that examined TRAF6-dependent activation of the IKK (8, 9). IKK is a heterotrimeric complex composed of the kinase subunits IKK α and IKK β and the regulatory subunit NEMO (also known as IKK γ). The dimeric Ub-conjugating enzyme Ubc13/Uev1A and the TAB2-TAK1 kinase complex were shown to be required for TRAF6-mediated IKK activation *in vitro*. In a cell-free system, it appears that by facilitating the synthesis of unique Lys-63-linked poly-Ub chains, rather than the conventional Lys-48-

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² The abbreviations used are: IL-1, interleukin-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; TAB1, -2, and -3, TAK1-binding proteins 1, 2, and 3; GST, glutathione S-transferase; GFP, green fluorescent protein; TRAF, TNF receptor-associated factor; TAK1, transforming growth factor- β -activated kinase 1; IKK, I κ B kinase; RANK, receptor activator of NF- κ B; MEF, mouse embryonic fibroblast; WT, wild type; HEK, human embryonic kidney; NF- κ B, nuclear factor- κ B; ERK, extracellular signal-regulated kinase; NFAT, nuclear factor of activation factor; RANKL, RANK ligand; TRAP, tartrate-resistant acid phosphatase; MKK, MAPK kinase; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; Ub, ubiquitin; M-CSF, macrophage-colony stimulating factor; HA, hemagglutinin; IRES, internal ribosomal entry site; CMV, cytomegalovirus; BMM, bone marrow-derived monocyte.

linked poly-Ub chains that target proteins for degradation, TRAF6 activates TAK1 by an unknown mechanism. TAK1 then phosphorylates and activates IKK for NF- κ B activation. TAK1 also phosphorylates MKK6 and MKK7, which in turn activate the JNK and p38 MAPK pathways, respectively. Recent studies suggest that the TAK1-binding protein TAB2 and its homologue TAB3 contain a highly conserved C-terminal zinc finger domain that binds preferentially to Lys-63-linked poly-Ub chains and is required for TAK1 activation (10).

Among all of the TRAFs, TRAF6 plays a critical role in osteoclastogenesis as revealed by the severe osteopetrosis phenotype in TRAF6-deficient mice (11–13). However, the molecular mechanism by which TRAF6 exerts this biological activity is not well understood. Indeed, the model of TRAF6-mediated kinase activation is exclusively based on *in vitro* reconstitution experiments. Nonetheless, it supports a novel Ub-dependent TRAF6 signaling pathway. However, there are many conceptual questions remaining about the potential downstream targets of the TRAF6 Ub ligase activity and the possible role of these ubiquitination events in the activation of signaling cascades. In this report, we performed a series of comprehensive studies to show that one of the most important targets of TRAF6 Ub ligase activity is TRAF6 itself. We show that TRAF6 is auto-ubiquitinated via Lys-63 linkages, which depends on an intact RING and the dimeric E2 enzyme Ubc13/Uev1A. Furthermore, the RING domain of TRAF6 is critical for the activation of NF- κ B and for the induction of osteoclast differentiation through the stimulation of the transcription factor NFATc1. Strikingly, we identified a single critical Ub acceptor site residue (Lys-124) in TRAF6 that is required for TRAF6 auto-ubiquitination. In transient expression studies, mutation of this site abolished TRAF6-mediated NEMO ubiquitination, TAK1 and IKK activation, NF- κ B activation, and osteoclast differentiation. Moreover, in stable TRAF6-deficient cells mutation of Lys-124 of TRAF6 prevents TRAF6 ubiquitination and IKK activation after IL-1 stimulation. These data establish that regulated site-specific Lys-63-linked TRAF6 auto-ubiquitination is the critical upstream mediator of IKK activation, which may have general implications in other TRAF6-mediated signaling processes.

EXPERIMENTAL PROCEDURES

Cell Lines, Reagents, and Antibodies—The mouse macrophage cell line RAW264.7 (referred to as RAW) and HEK293 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured as previously described (14–16). Retroviral packaging cell line GP2-293 (Clontech, Palo Alto, CA) and mouse embryonic fibroblasts (MEFs) from wild-type and TRAF6^{-/-} mice were kindly provided by Dr. Tak Mak (University of Toronto). To generate conditioned medium expressing mouse M-CSF, a mouse fibroblastic cell line L929 (ATCC) was infected with pMX-FLAG-MCSF-IRES-GFP-puro, and a stable cell line (L929-MCSF) was generated by selection with puromycin. Monoclonal antibody to p-I κ B α was purchased from New England Biolabs (Ipswich, MA); goat anti-rabbit IgG-conjugated horseradish peroxidase was purchased from Bio-Rad; rabbit polyclonal antibodies against TAB2, TAK1, JNK1, NEMO, and TRAF6 and mono-

clonal antibodies for NFATc1 and Ub were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); and goat anti-mouse IgG-conjugated horseradish peroxidase was purchased from BD Biosciences Pharmingen. Rabbit polyclonal antibody against mouse TRAF6 and mouse monoclonal antibody against α -tubulin were purchased from Calbiochem. Rabbit polyclonal antibody to β -actin was purchased from Cytoskeleton (Denver, CO). Rabbit polyclonal antibody for TAB1 was purchased from Cell Signaling Technology (Beverly, MA), and mouse IgG was purchased from Invitrogen. Monoclonal antibody to HA was a generous gift from Dr. G. B. Mills (University of Texas M. D. Anderson Cancer Center). Monoclonal anti-FLAG, MG132, N-ethylmaleimide, Ponceau S, and a tartrate-resistant acid phosphatase (TRAP) kit were purchased from Sigma. Yeast E1, His-Ub, and Ub variants (WT, K48R, K63R, Lys-48 only, Lys-63 only, and KO) were purchased from Boston Biochem (Cambridge, MA). Recombinant mouse RANKL, GST-cJun-(1–79), GST-I κ B α -(1–54), and GST-TRAF6 were purified essentially as described (14, 16, 17). Recombinant IL-1 was purchased from PeproTech (Rocky Hill, NJ). His-tagged proteins Ubc13, Uev1A, and MKK6-K82A were expressed from pET45 in *Escherichia coli* and were purified using nickel-affinity chromatography.

Plasmids—Mammalian expression vectors for FLAG-TRAF6, HA-Ub, and HA-JNK were described previously (16–18). TRAF6 and its variants were subcloned into pGEX-4T1. All retroviral vectors were constructed in a modified pMX vector (19), which contained the gene of interest cloned upstream of an IRES-GFP with a puromycin-selectable maker. A region encoding residues 33–262 of mouse M-CSF (ATCC) was cloned in-frame with a FLAG tag in pCMV1-FLAG (Sigma), and then the entire coding sequence, including the preprotrypsin leader and FLAG tag of M-CSF, was cloned into pMX-IRES-puro. Human cDNAs for Ubc13, Uev1A, and MKK6 were amplified from a human thymus library (Stratagene, La Jolla, CA) using gene-specific PCR primers and inserted in-frame with an N-terminal FLAG tag in pcDNA3 and in-frame with a His tag in pET45. Expression vectors for HA-Ub were subcloned from pRK5-HA-Ub into pcDNA3.1. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene) and verified by DNA sequencing.

Transfection, Reporter Gene Assays, and Retroviral Infection—Transfection of HEK293 cells was performed essentially as described previously (16). Transfection of HEK293 cells with a 3 \times -NF- κ B-driven luciferase (0.5 μ g/well) and β -actin *Renilla* luciferase (10 ng/well) constructs and analysis of reporter activity were performed according to the manufacturer's instructions (Promega, Madison, WI). For production of retroviral supernatants, GP2-293 cells were co-transfected with the indicated retroviral vector and pVSV-g (Clontech) using FuGENE according to the manufacturer's instructions (Roche Applied Science). After overnight transfection, the medium was removed and fresh medium was added. Viral supernatant was collected after 48–72 h and used to infect RAW, HEK293, mouse bone marrow-derived or spleen-derived monocytes, and MEFs with the addition of Polybrene (8 μ g/ml). After 2 days, selection was initiated with puromy-

Signaling by TRAF6 Auto-ubiquitination

cin (2–4 $\mu\text{g}/\text{ml}$). In some cases, stable pools of cells or clones were maintained in puromycin (2 $\mu\text{g}/\text{ml}$).

In Vitro Ubiquitination Assay and Poly-Ub Chain Synthesis—Bound proteins used as E3 enzymes or substrates for *in vitro* ubiquitination were either immunopurified with anti-FLAG antibody and protein A/G-Sepharose beads (Pierce) from HEK293 cells transfected with the appropriate construct or bacteria-purified GST fusion proteins bound to glutathione-agarose beads (Sigma). The auto-ubiquitination assay was performed in 20- μl reaction volumes with the following components: 20 mM HEPES (pH 7.2), 10 mM MgCl_2 , 25 μM MG132, 1 mM dithiothreitol, 59 μM His-Ub, 50 nM E1, 850 nM E2 (Ubc13/Uev1A), 1 mM ATP, 30 μM creatine phosphate, and 1 unit of creatine kinase. The mixture was incubated at 30 °C for 1–2 h with gentle agitation. The beads were washed several times in Buffer C and then subjected to SDS-PAGE and immunoblotting with an Ub-specific antibody. The *in vitro* ubiquitination-ligase assay was carried out by incubating glutathione-agarose bound GST-TRAF6 or variants in 20 μl of reaction mixture described above for 2 h at 37 °C. After incubation, 10 μl of cold 20 mM Hepes (pH 7.4) was added to the reaction, and samples were centrifuged for 20 min at 4 °C. A volume (20 μl) of the supernatant was collected and subjected to SDS-PAGE and immunoblotting with an Ub-specific antibody.

Molecular Modeling of TRAF6—We searched the Protein Data Bank for a potential structural template for the region of TRAF6 from the RING domain to the first zinc finger domain. The Rag1 dimerization domain (PDB ID 1RMD) aligned extremely well with this region of TRAF6, with only one single residue insertion in the entire alignment. Molecular modeling was then carried out using the program Swiss-Model (20). The structure figure was generated using the program PyMol.³

Osteoclast Differentiation—RAW cells were infected with the indicated retrovirus in 6-well plates overnight and then treated with puromycin for 2 days, after which cells were trypsinized, counted, and seeded in 24-well plates at varying densities (20,000–50,000/well). After 4–6 days, cells were fixed and stained for TRAP. Osteoclast formation was assessed by counting the total number of multinucleated (>3 nuclei), TRAP-positive cells present per well in quadruplicate. For mouse bone marrow-derived monocytes (BMMs), tibiae and femurs were excised from 6- to 8-week-old C57BL/6 mice, and the bone marrow cells were harvested. Whole bone marrow was cultured in α -minimal essential medium (10% serum) and plated in a 10-cm dish overnight. The next day the floating cells were collected, washed, and seeded with 10 ng/ml M-CSF. After 2 days, the cells were infected as described above for RAW cells. Spleen-derived monocytes from 6- to 8-week old C57BL/6 mice or from 10-day-old TRAF6-deficient mice (or a wild-type littermate) were cultured in α -minimal essential medium (10% serum) with 5 ng/ml mouse M-CSF (R&D, Minneapolis, MN) and plated in a 10-cm dish overnight. The next day the floating cells were collected, washed, and seeded with 5% L929-MCSF conditioned medium in α -minimal essential medium (10% serum). When the density of monocytes was sufficient for the experiment, the cells were then infected as described above.

Western Blotting, Immunoprecipitation, and in Vitro Kinase Assays—Cells were left unstimulated or stimulated as indicated in the figure legends and washed two times with phosphate-buffered saline. Depending on the experiment, cells were lysed in the following buffers: Buffer A (20 mM Tris, pH 7.4, 250 mM NaCl, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM EDTA, 1% Triton X-100, 2 mg/ml leupeptin, and 2 mg/ml aprotinin) or Buffer B (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM EDTA, 0.5% Nonidet P-40, 2 mg/ml leupeptin, and 2 mg/ml aprotinin) for 30 min on ice, and centrifuged at 13,000 rpm for 15 min. Protein was measured on the clarified lysates, and equal protein was then processed for Western blotting, immunoprecipitation, or kinases assays as previously described (14–16). The incorporation of ³²P into the substrate was quantitated with a PhosphorImager and represented as -fold activation compared with vector or time zero. Immunoprecipitation was carried out at 4 °C for 2–4 h with end-over-end rotation and washed three times in the indicated buffer followed by two washes in low salt buffer (20 mM Tris, pH 7.4, 25 mM NaCl, and 1 mM dithiothreitol). In some experiments, possible protein-protein interactions were prevented by immunoprecipitating in Buffer C (Buffer A with the addition of 0.1% SDS and 0.5% deoxycholate) or where indicated boiled in 1% SDS (50 μl) for 5 min followed by the addition of 1 ml of Buffer A, then centrifuged and re-immunoprecipitated with the appropriate antibody. To prevent the de-ubiquitination of some proteins, cells were lysed in Buffer A or B containing 10 mM *N*-ethylmaleimide.

RESULTS

TRAF6 Ubiquitination Is Auto-catalyzed and Dependent on Its Own RING Domain and the E2 Enzyme Ubc13/Uev1A—The RING domain of TRAF6 is believed to bind to an E2 Ub-conjugating enzyme and catalyze poly-ubiquitination of itself and potential downstream targets. As a prelude to understanding the RING-dependent Ub ligase activity of TRAF6, we constructed a mutant TRAF6 in which the highly critical Cys residue in its RING domain was mutated to Ala (TRAF6-C70A). When expressed in HEK293 cells along with HA-Ub, FLAG-tagged TRAF6 immunoprecipitates showed strong ubiquitination by immunoblotting with anti-HA, whereas TRAF6-C70A was not ubiquitinated (Fig. 1A). Utilizing an *in vitro* ubiquitination assay, immunopurified TRAF6, but not TRAF6-C70A, catalyzed its own ubiquitination in the presence of E1 and the dimeric E2 complex Ubc13/Uev1A (Fig. 1B, left). The auto-ubiquitination of TRAF6 is Lys-63-linked, because an Ub mutant containing only Lys-63 also supported TRAF6 auto-ubiquitination (Fig. 1B, right). To eliminate the possibility that this TRAF6 ubiquitination was mediated by an Ub ligase activity co-precipitating with TRAF6 from HEK293 cells, we expressed and purified TRAF6 from bacteria, which lacks an Ub system, and examined its auto-ubiquitination activity. Recombinant GST-TRAF6, but not GST-TRAF6-C70A, caused its own ubiquitination in the presence of Ubc13/Uev1A (Fig. 1C). Inclusion of different Ub mutants in the ubiquitination assay further indicated that this *in vitro* auto-ubiquitination of TRAF6 occurred through Lys-63 of ubiquitin as ubiquitination with Ub-K48, Ub-K63R, or an Ub mutant lacking all lysine res-

³ W. L. DeLano (2002) DeLano Scientific, San Carlos, CA.

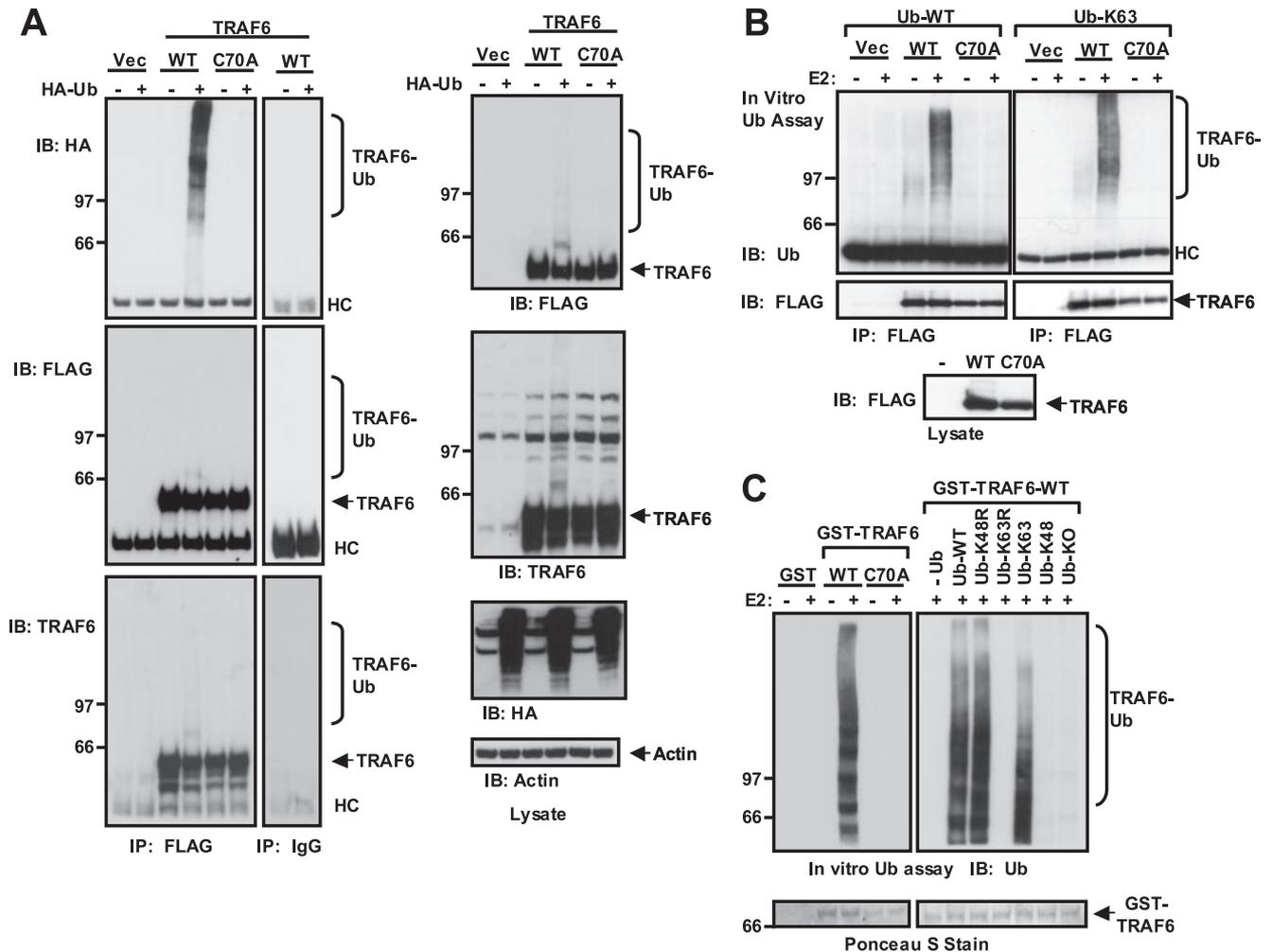


FIGURE 1. Lys-63-linked auto-ubiquitination of TRAF6 requires an intact RING domain and Ubc13/Uev1A. *A*, the RING domain of TRAF6 is required for auto-ubiquitination *in vivo*. HEK293 cells were transfected with the indicated plasmids encoding FLAG-TRAF6 or TRAF6-C70A in the absence (–) or presence (+) of HA-Ub (1 μ g). Cells were lysed in Buffer A and immunoprecipitated with anti-FLAG or mouse IgG in Buffer C. Bound proteins were subjected to SDS-PAGE and immunoblotted first with anti-HA, and then the membrane was stripped and reprobed with the indicated antibody (*left panels*). Cell lysates were immunoblotted with the indicated antibodies (*right panels*). *B*, *in vitro* Lys-63-linked auto-ubiquitination of TRAF6 requires Ubc13/Uev1A. HEK293 cells were transfected with the indicated plasmids encoding FLAG-TRAF6 or TRAF6-C70A, and cells were lysed (Buffer A) and immunoprecipitated with anti-FLAG in Buffer C followed by an *in vitro* ubiquitination assay with Ub (*left panel*) or Ub-Lys-63 (*right panel*) in the absence (–) or presence (+) of Ubc13/Uev1A (E2). The extent of TRAF6 ubiquitination was determined by immunoblotting with anti-Ub (*top*). The membrane was stripped and reprobed with anti-FLAG (*middle*). Cell lysates were immunoblotted with anti-FLAG (*bottom*). *C*, bacteria-expressed TRAF6 is auto-ubiquitinated through Lys-63-linked poly-Ub. The indicated GST fusion proteins were subjected to an *in vitro* ubiquitination assay in the absence (–) or presence (+) of Ubc13/Uev1A with Ub-WT (*left panel*) or the indicated Ub mutants (*right panel*). After the ubiquitination assay, the GST fusion proteins bound to glutathione-agarose were washed with Buffer C and then subjected to SDS-PAGE and immunoblotted with anti-Ub. The membrane was stained with Ponceau S (*bottom*).

idues (Ub-KO) was not observed (Fig. 1C). Collectively, these data show that TRAF6 catalyzes its own Lys-63-linked ubiquitination in a manner that depends on its RING domain and Ubc13/Uev1A.

An Intact RING Domain of TRAF6 Is Required for NF- κ B Activation and Biological Signaling—We next asked whether the RING domain of TRAF6, which is required for its auto-ubiquitination, is also essential for TRAF6 to activate signaling in cells. Transient expression of TRAF6, but not TRAF6-C70A, in HEK293 cells activated an NF- κ B-dependent luciferase reporter (Fig. 2A) and JNK activity (data not shown). Additionally, a catalytic mutant of Ubc13 (Ubc13-C87A) dose-dependently blocked TRAF6-mediated NF- κ B activation (Fig. 2B). Taken together, these results suggest that auto-ubiquitination of TRAF6 in conjunction with Ubc13 is linked to its ability to activate downstream signaling pathways (8).

Because TRAF6 plays a pivotal role in osteoclast differentiation, we explored the biological function of the RING domain of TRAF6 to support osteoclastogenesis in an osteoclast progenitor cell type, RAW, and in primary mouse BMM. We first established that retroviral expression of TRAF6, but not TRAF6-C70A, in either RAW cells or mouse BMM induced spontaneous formation of multinucleated, TRAP-positive osteoclasts in the absence of RANKL (Fig. 2C). The osteoclasts formed by infecting with TRAF6 were functional based upon bone resorption and actin ring formation assays (Fig. 2D). Furthermore, expression of TRAF6, but not TRAF6-C70A, induced nuclear accumulation of NFATc1, a transcription factor important in terminal differentiation of osteoclast progenitors (21) (Fig. 2E). Taken together, these results indicate that an intact RING domain of TRAF6 is required for its auto-ubiquitination and to activate downstream components of the IKK

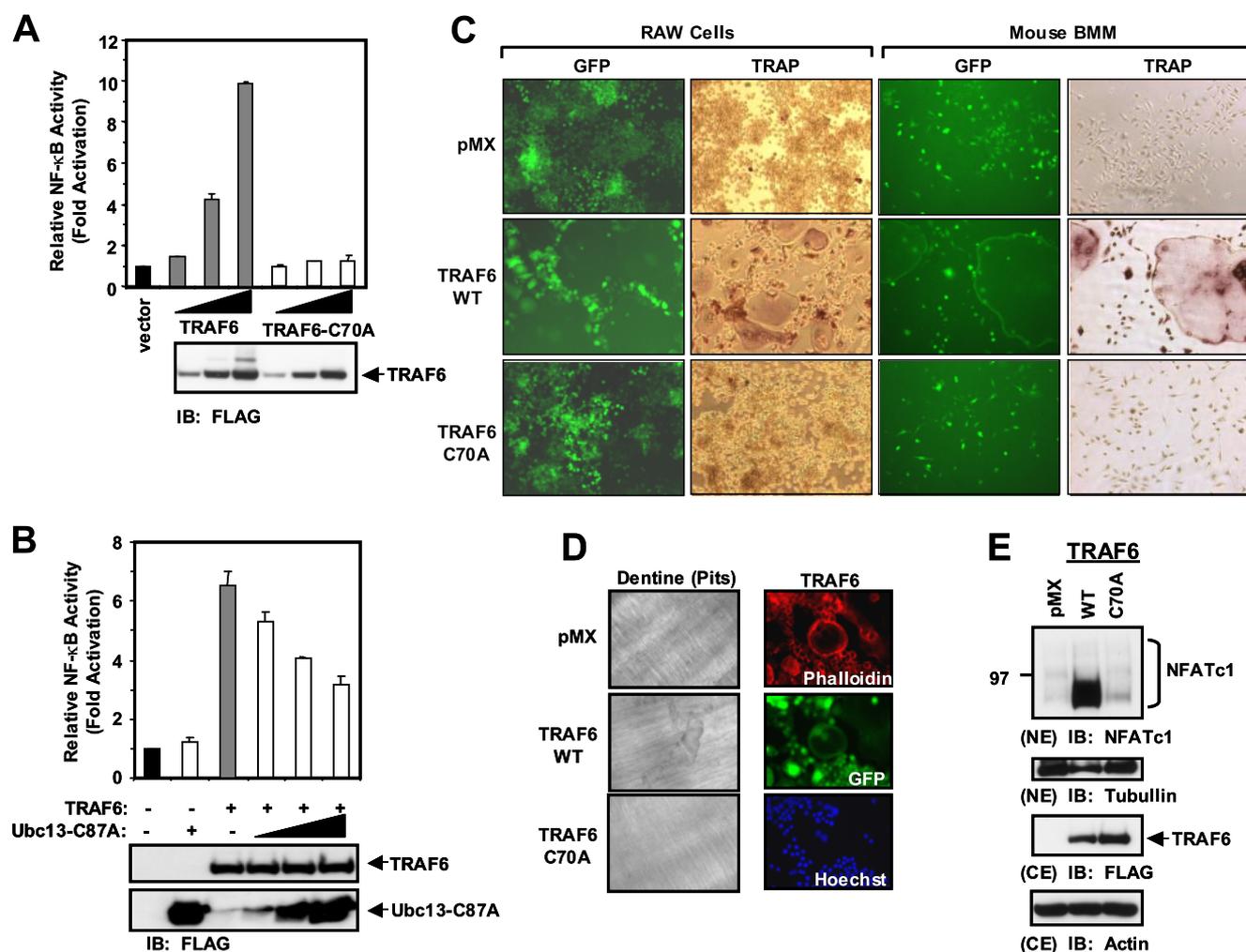


FIGURE 2. The TRAF6 RING structure is required for signaling. *A*, the RING domain structure of TRAF6 is required for NF- κ B activation. HEK293 cells were co-transfected with empty vector or with increasing amounts of FLAG-TRAF6 or FLAG-TRAF6-C70A together with an NF- κ B-luciferase reporter. Thirty-six hours after transfection, cells were harvested for luciferase activity and processed according to the manufacturer's protocol (Promega). Expression of the transfected TRAF6 and TRAF6-C70A constructs was determined by immunoblotting with anti-FLAG. *B*, Ubc13-C87A blocks TRAF6-dependent NF- κ B activation. HEK293 cells were co-transfected with empty vector or with increasing amounts of FLAG-Ubc13-C87A in the absence or presence of TRAF6 together with an NF- κ B-luciferase reporter and processed essentially as described in *A*. Expression of the transfected TRAF6 and Ubc13-C87A constructs was determined by immunoblotting with anti-FLAG. *C*, TRAF6, but not TRAF6-C70A, induces spontaneous osteoclast differentiation. RAW cells (*left panels*) or mouse BMM (*right panels*) were infected with the indicated retrovirus as described under "Experimental Procedures." Images were then captured with a 10 \times -objective lens for phase (bright field) and fluorescence (GFP), after which some of the samples were fixed and stained for TRAP. *D*, osteoclasts derived from TRAF6 infection resorb bone and form actin rings. For pit formation, infected RAW cells were seeded in 24-well plates containing dentine slices (ALPCO) and after 1 week, the dentine slices were processed for pit formation. Cells from TRAF6 infection were plated on glass coverslips, fixed, and stained with rhodamine-phalloidin and Hoechst. *E*, TRAF6, but not TRAF6-C70A, induces nuclear expression of NFATc1. Cells from *C* were harvested, and cytoplasmic and nuclear fractions were prepared. Protein from the nuclear extracts (NE) was immunoblotted with anti-NFATc1, and then the membrane was stripped and reprobed with anti-tubulin. Cytoplasmic extracts (CE) were immunoblotted with anti-FLAG, and then the membrane was stripped and reprobed with anti-actin.

and MAPK machinery, and, when TRAF6 is overexpressed in an osteoclast progenitor, the intact RING domain is required for osteoclast differentiation and function.

Identification of Lys-124 as the Key Ubiquitin Lysine Acceptor Site on TRAF6—The TRAF6-C70A mutation abolished the ligase activity of TRAF6 and consequently its auto-ubiquitination and signaling capacity. To address the role of the auto-ubiquitination of TRAF6 and its ligase activity in signaling events, it is essential to map the Ub lysine acceptor site(s) of TRAF6 and characterize the functional effects of eliminating the site(s). Because the RING domain of TRAF6 mediates its interaction with the E2 complex Ubc13/Uev1A (22), we hypothesized that Lys residues near the RING domain of TRAF6 may be in close proximity to the catalytic Cys-87 of

Ubc13 (23) and may serve as likely candidates for auto-ubiquitination.

Alignment of a region of TRAF6 from its RING domain to the first zinc finger domain from six different species revealed seven highly conserved Lys residues (Fig. 3A). To determine whether these Lys residues reside on the surface and whether they can be mutated without altering the conformation of TRAF6; we constructed a three-dimensional molecular model of this region of TRAF6. A portion of the Rag1 dimerization domain (24), which contains a RING and a zinc finger domain, aligned well with TRAF6 with only one single residue gap and was used as a structural template. The TRAF6 model clearly showed that all seven Lys residues are on the surface of the structure and exposed to solvent. Therefore, mutations of these

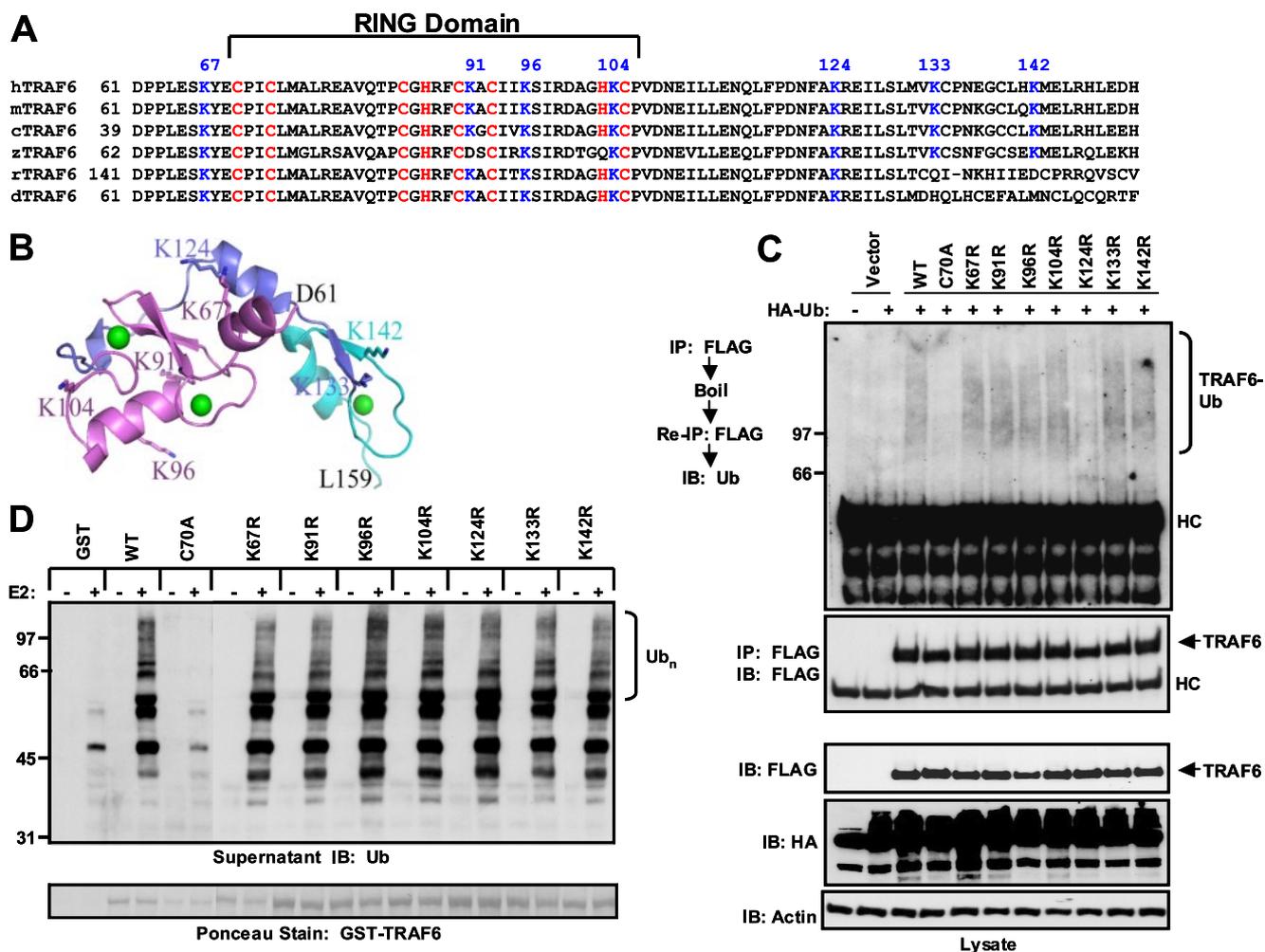


FIGURE 3. Identification of the auto-ubiquitination site(s) in TRAF6. *A*, schematic diagram of the TRAF6 RING domain. Shown is an alignment of the RING domain and the region before the first zinc finger of TRAF6 from six different species (*h*, human; *m*, mouse; *c*, chicken; *z*, zebra fish; *r*, rat; and *d*, dog). Conserved Lys residues are colored in blue, and the Cys and His residues that constitute the RING domain are colored in red. Residue numbers are for mouse TRAF6. *B*, a molecular model of TRAF6 from the RING domain to the first zinc finger domain (residues Asp-61 through Leu-159), modeled onto the Rag1 dimerization domain. Magenta, RING domain; blue, residues between the RING and the first zinc finger; cyan, the first zinc finger. Side chains of the seven conserved Lys residues are shown and labeled. *C*, Lys-124 is the predominant site of TRAF6 auto-ubiquitination. HEK293 cells were transfected with vector (Vector), FLAG-TRAF6 (WT), or the indicated TRAF6 mutants in the presence of HA-Ub (0.2 μ g). Cells were lysed (Buffer A) and immunoprecipitated with anti-FLAG in Buffer A followed by boiling in 1% SDS and a second immunoprecipitation with anti-FLAG. The bound proteins were subjected to SDS-PAGE and immunoblotted with anti-HA. The membrane was stripped and reprobed with anti-FLAG. Cell lysates were immunoblotted with the indicated antibody (bottom). *D*, lysine mutants of TRAF6 retain ubiquitin ligase activity. The indicated GST fusion proteins were subjected to *in vitro* ubiquitination assay as described in Fig. 1C, except the supernatant of the reaction was subjected to SDS-PAGE and immunoblotted with anti-Ub (top). The membrane was stained with Ponceau S (bottom).

residues should not induce any significant structural perturbation (Fig. 3B).

To identify the auto-ubiquitination sites in TRAF6, we systematically replaced each of these Lys residues to an Arg residue, which maintains the positive charge but does not serve as an acceptor site for Ub modification. The FLAG-tagged TRAF6 constructs were transfected into HEK293 cells along with HA-Ub, immunopurified by anti-FLAG antibody, boiled in the presence of SDS, and again immunoprecipitated with anti-FLAG followed by SDS-PAGE and immunoblotting with anti-HA. Of the seven Lys mutants examined, K124R consistently appeared to be less ubiquitinated than the other single point mutants (Fig. 3C). Although auto-ubiquitination of the TRAF6-K124R mutant was impaired, all of the mutants except for TRAF6-C70A were active as E3s in supporting free polyubiquitin chain formation *in vitro* (Fig. 3D), which suggests that these mutations did not alter the conformation of the RING

structure. Therefore, because Lys-124 is absolutely conserved, exposed on the TRAF6 surface, and has the most significant auto-ubiquitination defect when mutated, it is likely that Lys-124 is the predominant Ub acceptor site for TRAF6-mediated auto-ubiquitination.

TRAF6-K124R Is Impaired in Activation of NF- κ B—If auto-ubiquitination of TRAF6 is required in mediating its downstream signaling, mutation of the Ub acceptor site Lys in TRAF6 should prevent its signaling ability. When expressed in HEK293 cells with an NF- κ B luciferase reporter plasmid, only the K124R mutant exhibited a significant loss of NF- κ B luciferase reporter activity (Fig. 4A), which supports a critical role of Lys-124 in TRAF6-dependent signaling. Because TRAF6 has been proposed to activate the IKK pathway through the interaction with TAB2-TAK1, we next examined the role of Lys-124 of TRAF6 in activating the TAK1 and IKK complexes. To address this question, we generated HEK293 cells stably

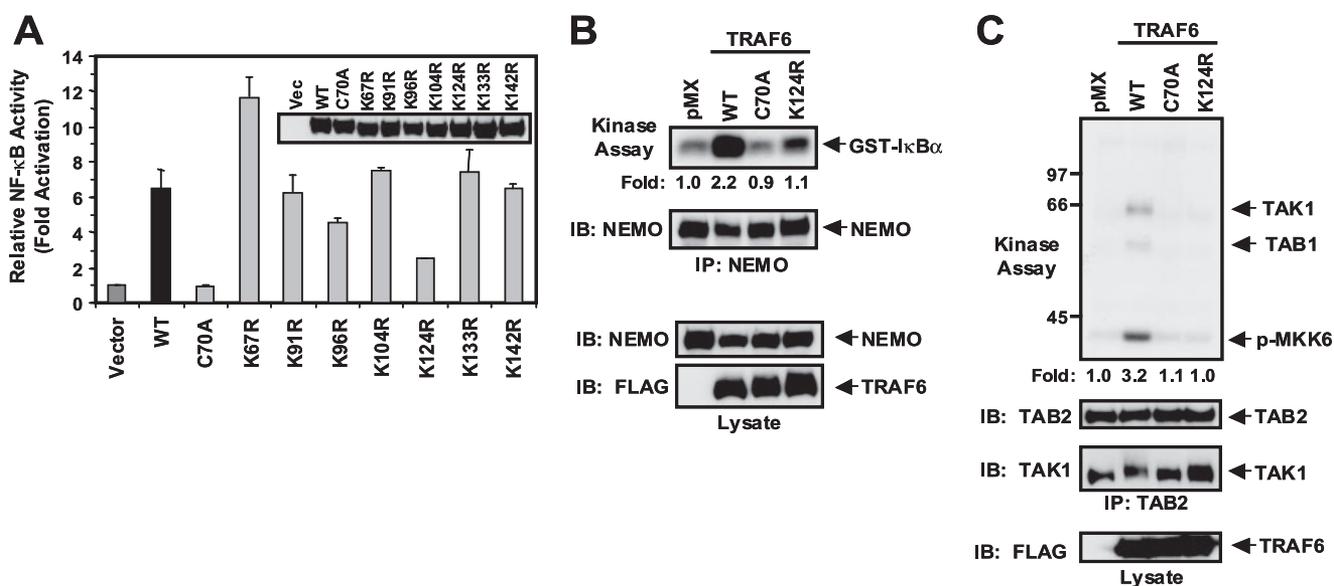


FIGURE 4. TRAF6-K124R is required for its signaling capacity. *A*, activation of an NF- κ B luciferase reporter by TRAF6 and its mutants. HEK293 cells were transfected with vector, FLAG-TRAF6 (WT), or the indicated TRAF6 mutants together with an NF- κ B-luciferase reporter. Thirty-six hours after transfection, cells were harvested, and then luciferase activity was measured. Expression of TRAF6 and its mutants was determined by immunoblotting with anti-FLAG (*inset*). *B*, Lys-124 of TRAF6 is required for IKK activity. Lysates from the indicated HEK293 stable cell lines were immunoprecipitated with anti-NEMO in Buffer A followed by an *in vitro* kinase assay with GST-I κ B α . The membrane was probed with anti-NEMO. Cell lysates were immunoblotted with the indicated antibodies. *C*, Lys-124 of TRAF6 is required for TAB2-TAK1 kinase activity. Lysates from the indicated HEK293 stable cell lines were immunoprecipitated with anti-TAB2 in Buffer A followed by an *in vitro* kinase assay with catalytically inactive MKK6. The membrane was probed with anti-TAB2 and then stripped and reprobed with anti-TAK1. Expression of the variants of TRAF6 was determined by immunoblotting with anti-FLAG.

expressing empty vector (pMX), TRAF6, TRAF6-K124R, and TRAF6-C70A by retroviral transduction. Only HEK293 cells stably expressing TRAF6, but not TRAF6-C70A or TRAF6-K124R, were able to activate the IKK complex (Fig. 4*B*). Furthermore, we immunoprecipitated endogenous TAB2 and assayed the presence of TAK1 kinase activity in phosphorylating catalytically inactive His-tagged MKK6. Although TAK1 co-precipitated with TAB2 in all of the cell lines, only cells expressing TRAF6, but not TRAF6-C70A or TRAF6-K124R, had TAK1 kinase activity that could phosphorylate MKK6 (Fig. 4*C*). Two phosphorylated proteins (TAK1 and TAB1) were identified in the TAB2 immunoprecipitate from the TRAF6-expressing cells as judged by size and Western blotting. Consistent with the NF- κ B reporter assay, TRAF6 auto-ubiquitination appears to be the upstream ubiquitination event that controls the activation of TAK1 and IKK complexes.

TRAF6-K124R Is Impaired in Inducing Osteoclast Differentiation—To address whether Lys-124 of TRAF6 is important for its osteoclastogenic potential, we assayed the ability of TRAF6 and its mutants to spontaneously form osteoclasts as described earlier. For this set of experiments, we focused on the single-site mutants K124R, K133R, and K142R. RAW cells infected with TRAF6-K124R showed significantly reduced numbers of TRAP-positive osteoclasts as compared with TRAF6, although a similar number of GFP-positive cells was achieved (Fig. 5, *A–C*). In contrast, RAW cells infected with TRAF6-K133R or -K142R had comparable numbers of TRAP-positive osteoclasts to that of TRAF6. Moreover, equivalent expression of TRAF6, TRAF6-K133R, and TRAF6-K142R induced similar high levels of nuclear NFATc1, whereas pMX and TRAF6-K124R induced much lower levels of nuclear NFATc1 (Fig. 5*D*). Collectively, these results suggest that Lys-

124 of TRAF6 is a predominant Ub acceptor site that is important for its ability to activate the NF- κ B pathway, which is an essential step in the differentiation and activation of mature osteoclasts.

TRAF6-K124R Is Deficient in Auto-ubiquitination, NEMO Ubiquitination, IKK Activation, and Osteoclast Differentiation in TRAF6-deficient Cells—Similar to our earlier observations in HEK293 cells, the TRAF6-K124R mutant retained some signaling capacity in RAW cells, albeit at a much reduced level, suggesting that TRAF6-K124R may exert its effects through endogenous TRAF6. To avoid this possibility, we expressed TRAF6 and its mutants in MEFs and spleen-derived monocytes from TRAF6-deficient mice. First, we examined the extent of ubiquitination of TRAF6, TRAF6-C70A, TRAF6-K124R, and TRAF6-K133R after transient infection into TRAF6-deficient MEFs. The anti-FLAG immunoprecipitates indicated that only TRAF6 and TRAF6-K133R were ubiquitinated, whereas ubiquitination of TRAF6-C70A and TRAF6-K124R was significantly impaired (Fig. 6*A*). Furthermore, because transient ectopic expression of TRAF6 is sufficient to engage signaling components leading to the activation of IKK, we examined the extent of IKK activity in these cells. Only expression of TRAF6, TRAF6-K133R, and TRAF6-K142R, but not TRAF6-C70A and TRAF6-K124R, were able to activate IKK leading to the phosphorylation of I κ B α (Fig. 6*B*). From these observations, we propose that site-specific auto-ubiquitination of TRAF6 plays an important role in IKK activation.

We next asked whether site-specific TRAF6 auto-ubiquitination is required for NEMO ubiquitination. This is important, because, although NEMO is known to be ubiquitinated in response to many stimuli (25–28), thus far it is not clear what the E3 is for this ubiquitination and whether

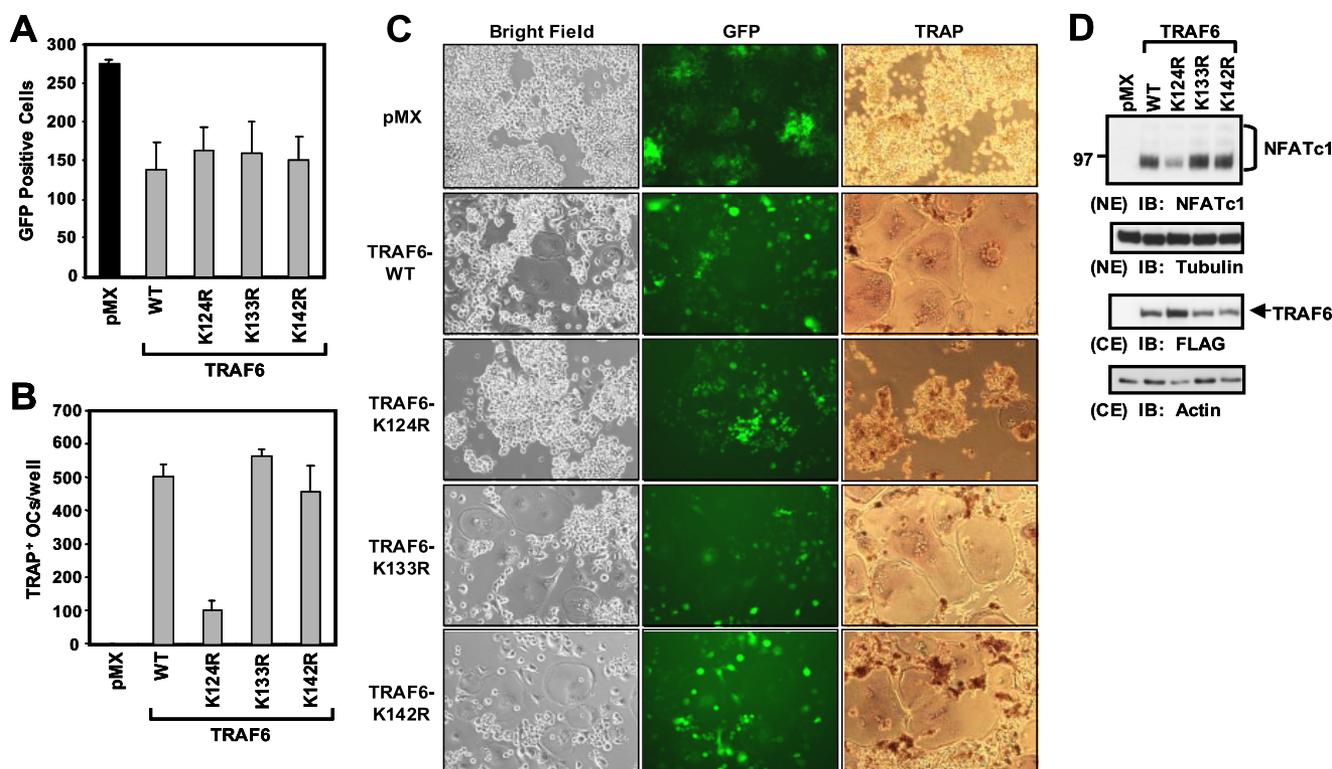


FIGURE 5. Effect of TRAF6 mutants on spontaneous osteoclast differentiation. A–C, Lys-124 of TRAF6 is required for osteoclast differentiation. RAW cells were infected with the indicated retrovirus and processed as described in Fig. 2C. Prior to seeding in 24-well plates, the total number of GFP-positive cells was counted to ensure equivalent infection (A). The total number of TRAP⁺, multinucleated osteoclasts was counted from wells plated in quadruplicate and shown as the average with standard deviation (B). Shown are representative images captured with a 10 \times objective lens (C). Similar results were obtained in at least three independent experiments. D, TRAF6-Lys-124 is required for efficient induction of nuclear NFATc1 expression. Cells from B were harvested, and cytoplasmic and nuclear fractions were prepared and processed as described in Fig. 2E.

TRAF6 plays a role in this process. The extent of endogenous NEMO ubiquitination was much greater in the TRAF6-expressing cells than in cells expressing either TRAF6-K124R or TRAF6-C70A (Fig. 6C). These data suggest that auto-ubiquitination of TRAF6 is necessary for the ubiquitination of NEMO and that NEMO may be a substrate of the TRAF6 ubiquitin ligase. To determine whether NEMO is indeed a direct target of the TRAF6 Ub ligase activity, we performed an *in vitro* ubiquitination assay with bacterial expressed TRAF6 and NEMO. Inclusion of TRAF6 and Ubc13/Uev1A, but not TRAF6-C70A or GST, supported the ubiquitination of NEMO (Fig. 6D). These results clearly provide evidence that TRAF6 is the E3 Ub ligase that facilitates the Lys-63-linked ubiquitination of NEMO.

Furthermore, we infected spleen-derived monocytes from TRAF6-deficient mice and demonstrated that neither TRAF6-C70A nor TRAF6-K124R was able to induce spontaneous osteoclast differentiation, because no TRAP-positive multinucleated osteoclasts were observed (Fig. 6E). Taken together, these results demonstrate that Lys-124 of TRAF6 plays a dominant role in TRAF6 auto-ubiquitination and activation of signal transduction pathways to induce efficient osteoclast differentiation. These data are consistent with our hypothesis that site-specific auto-ubiquitination of TRAF6 is a prerequisite for the activation of IKK.

IL-1-dependent Ubiquitination of TRAF6 at Lys-124 Is Required for IKK Activation—Stimulation of cells with IL-1 causes TRAF6 ubiquitination (9); however, the consequence of

this ubiquitination event has not been investigated. To determine whether site-specific ubiquitination of TRAF6 is required for IL-1-induced IKK activation, we established cell lines from TRAF6-deficient MEFs expressing, respectively, the same level of FLAG-tagged TRAF6, TRAF6-C70A, and TRAF6-K124R. Following stimulation with IL-1 for various times and immunoprecipitation with anti-FLAG revealed ubiquitination of TRAF6, but not with TRAF6-C70A or TRAF6-K124R (Fig. 7A). Furthermore, IL-1 stimulation of IKK activity and detection of phospho-I κ B α was significantly impaired in cells expressing either TRAF6-C70A or TRAF6-K124R as compared with TRAF6 (Fig. 7B), which confirms an essential requirement of the TRAF6 Ub ligase activity and its site-specific auto-ubiquitination for IL-1 signaling. These data suggest that Lys-124 of TRAF6 is the primary ubiquitination site for ligand-stimulated TRAF6 auto-ubiquitination.

DISCUSSION

TRAF6 is one of the key adaptor molecules in the signal transduction of members of the TNF receptor superfamily and the IL-1 receptor/Toll-like receptor superfamily. To date, TRAF6 is the only member of the TRAF family that plays a critical role in osteoclastogenesis; its recruitment to the cytoplasmic domain of RANK initiates a signaling cascade that is crucial for the maturation of monocyte precursors to fully differentiated osteoclasts. However, the molecular events associated with TRAF6 in this process are not well understood. In this study, we clearly show that the RING domain of TRAF6 and the dimeric E2

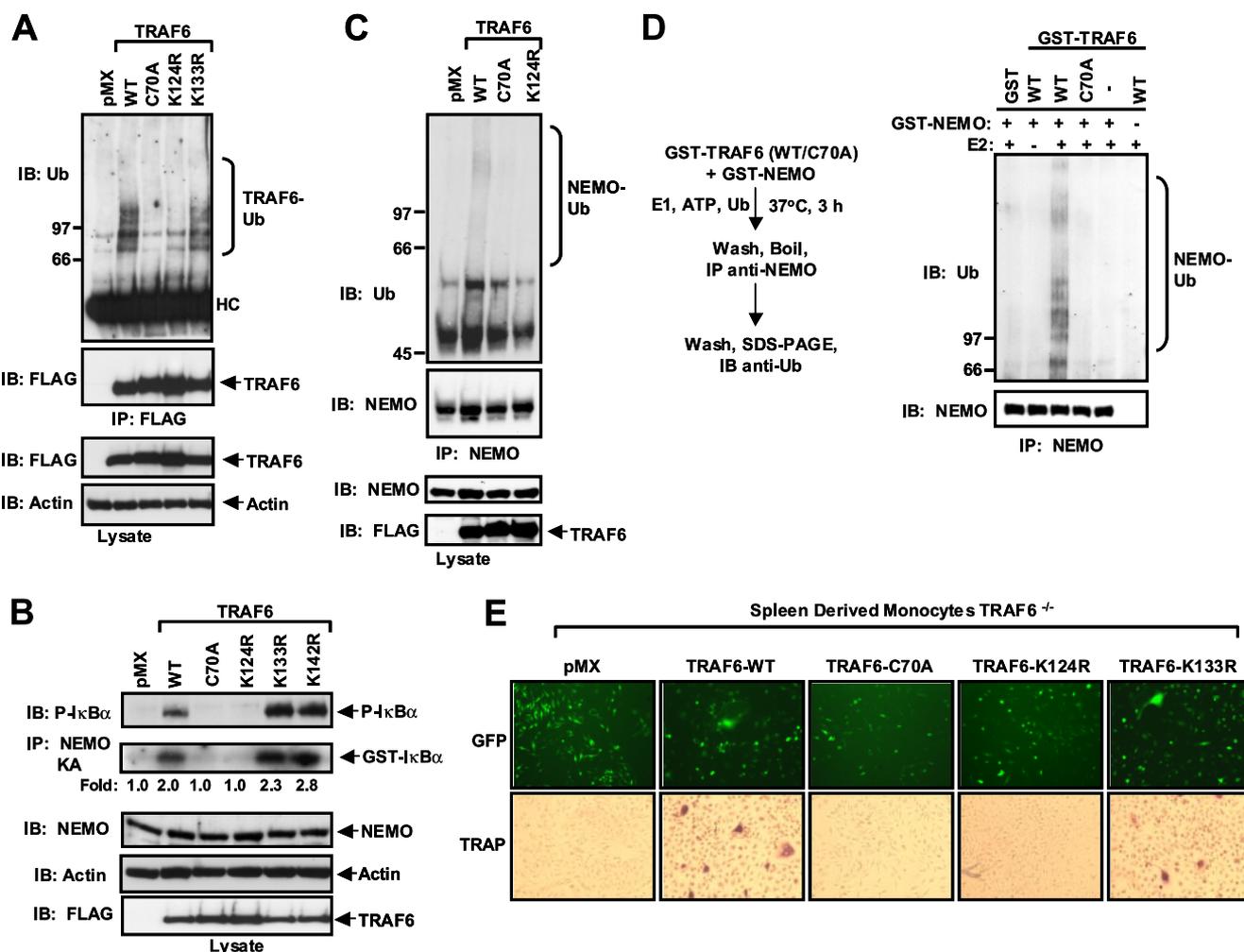


FIGURE 6. Lys-124 of TRAF6 is required for its auto-ubiquitination and signaling in TRAF6-deficient cells. *A*, TRAF6-K124R is not ubiquitinated in TRAF6-deficient MEFs. TRAF6^{-/-} MEFs, which express vector (pMX) or TRAF6 and the indicated TRAF6 mutants were serum-starved overnight. Cell lysates prepared from the indicated cell lines were immunoprecipitated with anti-FLAG in Buffer C and then immunoblotted with anti-Ub. The membrane was stripped and reprobed with anti-FLAG. Cell lysates were immunoblotted with indicated antibodies. *B*, Lys-124 of TRAF6 is required to activate the IKK complex. Cell lysates from the indicated cell lines were immunoblotted with anti-p-IκBα, and then the membranes were stripped and reprobed with anti-actin and anti-FLAG. Lysates were also immunoprecipitated with anti-NEMO antibody followed by an *in vitro* kinase assay with GST-IκBα essentially as described in Fig. 4B. Cell lysates were immunoblotted with the indicated antibodies. *C*, NEMO ubiquitination is impaired in TRAF6-K124R-expressing cells. Cell lysates from the indicated cell lines were immunoprecipitated with anti-NEMO in Buffer A, followed by boiling in 1% SDS and immunoprecipitated again with anti-NEMO. The eluted proteins were subjected to SDS-PAGE and immunoblotted with anti-Ub. The membrane was stripped and reprobed with anti-NEMO. Cell lysates were immunoblotted with the indicated antibodies. *D*, TRAF6 directly ubiquitinates NEMO. Bacterial expressed and purified GST, GST-TRAF6, and GST-NEMO bound to glutathione-agarose beads were processed for *in vitro* ubiquitination assays as described (*left*) and NEMO immunoprecipitates were immunoblotted with anti-Ub. The membrane was stripped and reprobed with anti-NEMO. *E*, effect of TRAF6 mutants on spontaneous osteoclast differentiation in TRAF6-deficient monocytes. Spleen-derived monocytes from a TRAF6-deficient mouse were infected with the indicated retrovirus and processed as described in Fig. 2C.

enzyme Ubc13/Uev1A are critical for Lys-63-linked TRAF6 auto-ubiquitination and the ability of TRAF6 to activate IKK leading to the activation of NF-κB. In contrast to most studies of Ub-dependent TRAF6 signaling that only examined the activation of IKK, we further addressed this molecular mechanism on TRAF6 for its capacity to regulate osteoclast differentiation and maturation. Similar to previous reports (29, 30), we observed that retroviral infection of RAW cells or mouse BMMs with TRAF6 caused RANKL-independent osteoclast differentiation. However, we further show that a RING domain mutant of TRAF6 could not induce expression of NFATc1 nor induce the differentiation of monocytes to mature functional osteoclasts. Collectively, our results demonstrate the biological relevance of the RING domain of TRAF6 and, hence, its auto-

ubiquitination for its ability to induce NFATc1 and terminal differentiation of osteoclasts.

Without the knowledge of specific ubiquitination sites, the precise role of TRAF6 auto-ubiquitination in its ability to activate downstream signaling events cannot be rigorously addressed. Therefore, we took on the task of identifying the Ub acceptor Lys residues in TRAF6 that are responsible for its auto-ubiquitination. Because the RING domain of TRAF6 interacts with its E2 and the ubiquitination site(s) should be sufficiently close to the E2 for ubiquitination to occur, we mutated the seven Lys residues within and surrounding the RING domain of TRAF6. By using a molecular model of the TRAF6 RING structure, we predicted that all of the mutated lysine residues are solvent-exposed, and their mutation to Arg

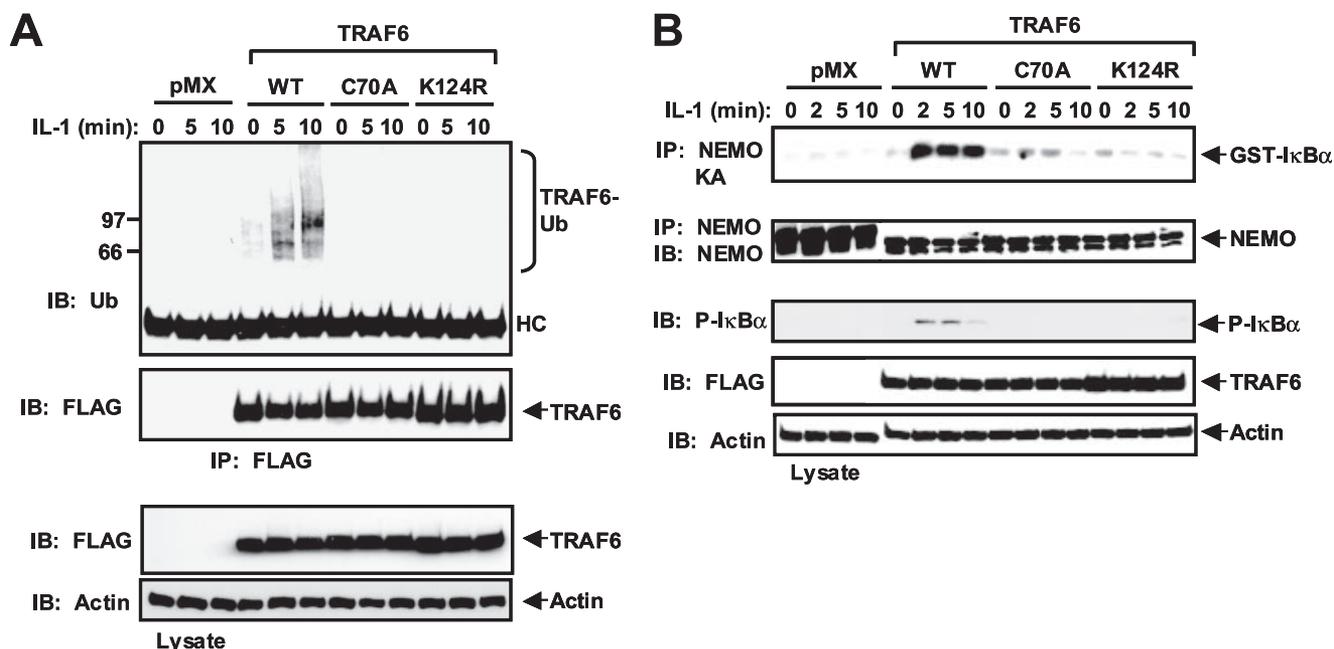


FIGURE 7. Lys-124 of TRAF6 is required for IL-1-dependent ubiquitination and IKK activation. *A*, IL-1-dependent ubiquitination of TRAF6 at Lys-124. Stable TRAF6^{-/-} MEFs, which express vector (pMX) or TRAF6, and the indicated TRAF6 mutants were serum-starved overnight and then treated with IL-1 (0.1 ng/ml) for the indicated times. Cell lysates were prepared in Buffer A and immunoprecipitated with anti-FLAG followed by boiling in 1% SDS and immunoprecipitated again with anti-FLAG. The eluted proteins were subjected to SDS-PAGE and immunoblotted with anti-Ub. The membrane was stripped and reprobed with anti-FLAG. Cell lysates were immunoblotted with the indicated antibodies. *B*, IL-1-dependent IKK activation is impaired in TRAF6-K124R-expressing cells. Stable TRAF6^{-/-} MEFs, which express vector (pMX) or TRAF6, and the indicated TRAF6 mutants were serum-starved overnight and then treated with IL-1 (0.1 ng/ml) for the indicated times. Lysates from the indicated stable cell lines were immunoprecipitated with anti-NEMO in Buffer A followed by an *in vitro* kinase assay with GST-IκBα. The membrane was probed with anti-NEMO. Cell lysates were immunoblotted with the indicated antibodies.

would not alter the RING structure. Consistent with our prediction, each of the TRAF6 Lys mutants retained their ability to synthesize free poly-Ub chains. Through mutagenesis, we demonstrated that Lys-124 is the major Ub acceptor site in TRAF6, which is required for its auto-ubiquitination. Importantly, the transient expression of the K124R mutant results in both signaling and biological defects: decreased TRAF6 auto-ubiquitination, loss of NEMO ubiquitination, lack of TAK1 and IKK activation, deficiency in NFATc1 induction, and impairment in mature osteoclast formation. Significantly, IL-1-dependent TRAF6 ubiquitination and IKK activation were abolished in stable TRAF6-deficient MEFs reconstituted with either the RING domain or the K124R mutant of TRAF6. Thus, by identifying a specific Lys-63-linked ubiquitination site on TRAF6 and determining the importance of this site, we have established a link between site-specific TRAF6 auto-ubiquitination and its ability to activate the IKK complex.

The identification of a single Lys residue for TRAF6 auto-ubiquitination points to a model of poly-, rather than multi-ubiquitination in NF-κB signaling, a question raised in a recent review (31), and reveals the potential specificity in this pathway of Lys-63-linked ubiquitination. In contrast, Lys-48-linked ubiquitination is often not so site-specific. This is perhaps consistent with the different roles of Lys-48- versus Lys-63-linked ubiquitination. The former is simply a "signal" for proteasomal destruction, which does not need to be site-specific. In contrast, Lys-63-linked ubiquitination may be important for assembly of signaling complexes, which might require the poly-Ub chains to be at a specific site of the target. Because Lys-63-linked ubiquitination uses the heterodimeric E2 enzyme Ubc13/Uev1A,

whereas K48-linked ubiquitination uses single component E2 enzymes, it is tempting to speculate that this difference may play a role in the apparent specificity.

Strikingly, the lack of TRAF6 auto-ubiquitination also resulted in the loss of NEMO ubiquitination, suggesting that TRAF6 may also be the E3 for NEMO. Up to now, the E3 for NEMO has been obscure. In the TNF pathway, cIAP1 was shown to be the E3 for NEMO ubiquitination (26), whereas the E3 for NOD2- or Bcl10-induced NEMO ubiquitination has not been identified (25, 28). In this regard, because TRAF6 and TRAF2 appear to be the common elements in these pathways, it is possible that TRAFs are the unifying E3s for NEMO ubiquitination (32, 33). Indeed, we have clearly demonstrated that TRAF6 serves as an E3 for NEMO ubiquitination using recombinant proteins, which is further supported by the lack of NEMO ubiquitination in TRAF6-deficient MEFs reconstituted with either a TRAF6 RING or K124R mutant. The failure of NEMO ubiquitination with these two TRAF6 mutants may reflect the loss of recruitment of NEMO to the TRAF6 complex, because neither of these TRAF6 mutants are ubiquitinated. Consistent with this hypothesis, recent data have emerged implicating proteins with binding specificity toward Lys-63-linked poly-Ub chains. Two such molecules TAB2 (or TAB3) through its C-terminal zinc finger domain and NEMO through a novel Ub-binding domain have been shown to preferentially bind Lys-63-linked poly-Ub chains (10, 34, 35).⁴ These data may explain the necessity of Lys-63-linked auto-ubiquitination

⁴ B. G. Darnay, unpublished observations.

of TRAF6 to facilitate the recruitment and activation of the IKK complex. Nevertheless, the functional mechanism of ubiquitinated NEMO in assisting the activation of IKK remains to be investigated. One way to resolve these questions would be to identify the Ub acceptor sites on NEMO and assess the functional effects of eliminating them, which is currently under investigation.

In summary, the data presented here may provide molecular insight into the non-traditional role of Ub in TRAF6 signaling. In particular, we have shown that site-specific auto-ubiquitination of TRAF6 via the Lys-63 linkage is crucial for the activation of downstream kinases and transcription factors and further define a role of TRAF6 auto-ubiquitination in regulating osteoclast differentiation. Site-specific TRAF6 auto-ubiquitination appears to be a prerequisite that initiates a cascade of further downstream ubiquitination events, including the ubiquitination of components in the TAK1 and IKK complexes. Further studies are required to determine the specific role of each of the ubiquitination events in TRAF6-mediated IKK activation.

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