Published in final edited form as:
J Cell Biochem. 2012 April ; 113(4): 1274–1281. doi:10.1002/jcb.23460.

NIP45 Negatively Regulates RANK Ligand Induced Osteoclast Differentiation

Shanmugarajan Srinivasan[1], **Courtney J. Haycraft**[2], **Sakamuri V. Reddy**[1], and **William L. Ries**[2]

[1]Charles P. Darby Children's Research Institute, Medical University of South Carolina, Charleston, SC

[2]College of Dental Medicine, Medical University of South Carolina, Charleston, SC

Abstract

Receptor activator of NF-κB ligand (RANKL)-RANK receptor signaling to induce NFATc1 transcription factor is critical for osteoclast differentiation and bone resorption. RANK adaptor proteins, tumor necrosis factor receptor-associated factors (TRAFs) play an essential role in RANKL signaling. Evidence indicates that NIP45 (NFAT interacting protein) binds with TRAFs and NFATc2. We therefore hypothesized that NIP45 regulates RANKL induced osteoclast differentiation. In this study, we demonstrate that RANKL treatment down regulates NIP45 expression in mouse bone marrow derived preosteoclast cells. Lentiviral (pGIPZ) mediated shRNA knock-down of NIP45 expression in RANKL stimulated preosteoclast cells resulted in increased levels of NFATc1, NFATc2 and TRAF6 but not TRAF2 expression compared to control shRNA transduced cells. Also, NIP45 suppression elevated p-IκB-α levels and NF-κB-luciferase reporter activity. Confocal microscopy demonstrated NIP45 colocalized with TRAF6 in the cytosol of osteoclast progenitor cells. In contrast, RANKL stimulation induced NIP45 nuclear translocation and colocalization with NFATc2 in these cells. Coimmuneprecipitation assasy demonstrated NIP45 binding with NFATc2 but not NFATc1. We further show that shRNA knock-down of NIP45 expression in preosteoclast cells significantly increased RANKL induced osteoclast differentiation and bone resorption activity. Taken together, our results indicate that RANKL signaling down regulates NIP45 expression and that NIP45 is a negative regulator of osteoclast differentiation.

Keywords

Osteoclast; RANK ligand; NIP45; NFAT

Introduction

Osteoclast is the bone resorbing cell. Receptor activator of NF-κB ligand (RANKL) is a member of the tumor necrosis factor (TNF) family that is produced by osteoblasts in the

*Address for Correspondence: Srinivasan Shanmugarajan, Ph.D, Charles P. Darby Children's Research Institute, 173 Ashley Avenue, Charleston, SC 29425; Tel. Ph. 843-792-9755; Fax: 843-792-7927; srini@musc.edu.

The authors declare no conflict of interest with this work.

bone microenvironment and the RANK receptor is expressed on committed osteoclast precursors. RANKL interaction with RANK results in recruitment of TNF receptor-associated factor (TRAF) family of adaptor proteins (Lomaga et al., 1999) and activates NF-κB, c-Jun N-terminal kinase (JNK) activity in osteoclast precursor cells, which then fuse to form multinucleated osteoclasts (Boyle et al., 2003). Further, RANKL signaling induces nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), the master regulator of osteoclast differentiation (Takayanagi, 2007b). The NFAT (nuclear factor of activated T cells) family comprises five members including NFATc1 (NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), NFATc4 (NFAT3) and NFAT5 which are activated by specific phosphatases through calcium/calmodulin signaling (Day et al., 2005). RANKL costimulatory signaling with immunereceptor tyrosine-based activation motif (ITAM) bearing adapter molecules such as FcRγ and DAP12 results in activation of calcium signaling and autoinduction of NFATc1 which is critical for osteoclast differentiation (Negishi-Koga and Takayanagi, 2009). Further over-expression of NFATc2 has been shown to promote osteoclast differentiation in the absence of RANKL stimulation (Ikeda et al., 2004). NFATc1 has also been shown to induce dendritic cell specific transmembrane protein (DC-STAMP) which plays an essential role in fusion of preosteoclast cells to form multinucleated osteoclasts (Kim et al., 2008). Also, NFATc1 has been shown to modulate calcitonin receptor, mouse tartrate-resistant acid phosphatase (TRAP) and human beta-3 integrin expression during osteoclast differentiation (Crotti et al., 2006). In the immune cells NFATs hetero-dimerize with a number of other nuclear proteins that serve to modify their transcriptional activity. NFATs associate with AP-1 and IRF-4 transcription factors to regulate gene expression (Rao et al., 1997). NIP45 (NFAT interacting protein of 45 kDa) has been shown to interact with TRAFs and NFATc2 to modulate gene transcription (Bryce et al., 2006). NFAT driven IL-4 gene transcription is potentiated by NIP45 (Hodge et al., 1996). It has been demonstrated that arginine methylation at the amino terminus of NIP45 modulates interaction with NFAT to augment cytokine production in T cells. NIP45 recruits the arginine methyltransferase and histone modifier protein arginine methyltransferase 1 (PRMT1) to the NFAT complex to facilitate gene transcription (Mowen et al., 2004). However a functional role of NIP45 during osteoclast differentiation remains unknown. In this study, we show that RANKL down regulates NIP45 expression which negatively regulates osteoclast differentiation.

Materials and Methods

Reagents and antibodies

Cell culture and DNA transfection reagents were purchased from Invitrogen, Inc (Carlsbad, CA). mRANKL and mM-CSF were obtained from R&D systems, Inc (Minneapolis, MN). Goat-anti-NIP45 antibody, rabbit anti-NFATc1, NFATc2, TRAF2, TRAF6 antibodies and peroxidase-conjugated anti-rabbit and anti-goat secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-rabbit antibody conjugated Alexa 488 and donkey anti-goat antibody conjugated Alexa 568 are obtained from Invitrogen Inc. (Carlsbad, CA). pNF-κB-Luc cis-reporter plasmids were obtained from Stratagene (La Jolla, CA). Super-signal enhanced chemiluminescence (ECL) reagent was obtained from Amersham Bioscience (Piscataway, NJ), and nitrocellulose membranes were purchased

from Millipore (Bedford, MA). A luciferase reporter assay system was obtained from Promega (Madison, WI).

Lentiviral constructs and transduction

GIPZ lentiviral shRNAmir (control non-silencing and mouse NIP45 shRNA) vectors (Clone ID. V2LMM_82925 and gene access no. BC113761) and NIP45 cDNA expression construct in the pCR-BluntI-TOPO vector were obtained from Open Biosystems (Clone IDs 40054445). NIP45 cDNA insert was excised using BamH1 and XhoI restriction enzymes and sub-cloned into the lentiviral pLEX vector. Lentiviral packaging was done in TLA-HEK293T cells in accordance with the Trans-Lentiviral packaging system (Open Biosystems, Huntsville, AL). After 12 hr of transfection, the packaging cocktail was aspirated and replaced with complete medium. After 72 hr, lentiviral containing supernatants were collected, centrifuged at 3000 rpm for 20 min and stored at −80 °C for further use. Titers of all lentiviral particles were determined by transducing TLA-HEK293 cells with serial dilutions of lentiviral supernatants for 48 hr and GFP positive cells were scored using a fluorescent microscope. Mouse bone marrow derived non-adherent cells were transduced with lentiviral stock at a multiplicity of infection (MOI) of 5 and 10 (i.e., TLA-HEK293 transducing units per target cell) diluted in 2 ml DMEM containing 10 μg/ml polybrene (Millipore, MA). After 6 hr, an equal amount of DMEM with 20% FBS was added to each well. The following day, the transfection cocktail was removed and replaced with complete medium containing puromycin (2 μg/ml) to select for NIP45 shRNA or cDNA expressing cells and total cell lysates were subjected to Western blot analysis for NIP45 expression as described.

Western blot analysis

Mouse bone marrow derived non-adherent cells transduced with control and NIP45 shRNA were cultured with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 hr. Total cell lysates were analyzed by Western blot for TRAF2, TRAF6, p-IκBα, NFATc1, NFATc2 and NIP45 expression. Band intensities were quantified by densitometric analysis using the NIH Image J program. β-actin expression levels were also analyzed to normalize the protein samples.

Co-Immunoprecipitation assay

Mouse bone marrow derived non-adherent cells transduced with control non-specific siRNA or NIP45, NFATc1, NFATc2 siRNA and cultured for 48 hr. Total cell lysates were subjected to a coimmunoprecipitation assay (Shanmugarajan et al., 2010). Briefly, 250 μg of protein was incubated with protein G agarose for 2 hr and centrifuged to pre-clear non-specific binding. The supernatant was incubated with anti-NIP45 antibody or control IgG overnight at 4 °C on an orbital shaker. The immune complexes were incubated with 100 μL protein G agarose beads for 2 hr at 4 °C and the immune complexes were collected by centrifugation. The pellet was boiled for 5 min in reducing sample buffer and subjected to SDS-PAGE. The gels were processed for Western blot analysis to confirm the presence of NFATc1 and NFATc2 in the immune complex.

pNF-κB-Luc reporter gene assay

RAW 264.7 cells were transduced with control non-silencing vector (EV) or NIP45 shRNA vector as described above. Cells were transfected with pGL2 basic or pNF-κB-Luc reporter plasmids and cultured in the presence of RANKL (100 ng/ml) for 48 hr. Cell monolayer was washed twice with PBS and incubated at room temperature for 15 min with 0.3 ml cell lysis buffer. The cell lysates collected were spun briefly in a microfuge to pellet the debris. Then, a 20 μl aliquot of each sample was mixed with 100 μl of the luciferase assay reagent. Light emission was measured for 10 sec of integrated time using a Sirius Luminometer (Promega, Madison, WI). Transfection efficiency was normalized by co-transfection with 0.2 μg of pRSV β-gal plasmid and measuring β-galactosidase activity in the cells. LacZ cytochemical activity staining (Invitrogen Inc, San Diego, CA) indicated a DNA transfection efficiency (>80%) in RAW 264.7 cells.

Osteoclast culture and bone resorption assay

Mouse bone marrow derived non-adherent cells (1.3×10^6/ml) were transduced with control non-silencing vector (EV) or NIP45 shRNA lentiviral vector and cultured in 96-well plates for 5 days in the presence of RANKL (100 ng/ml) and M-CSF (10 ng/ml). Half the culture media was changed every alternate day and replaced with fresh media and cytokines. At the end of the culture period, cells were fixed in 2% glutaraldehyde in PBS for 20 min and stained for tartrate-resistant acid phosphatase (TRAP) activity. TRAP positive multinucleated cells containing three or more nuclei were scored microscopically. Bone resorption activity was assayed by culturing osteoclasts for 10 days on dentine slices. At the end of the culture period, TRAP positive osteoclasts were scored and cells were removed from the dentine disc using 1 M NaOH followed by staining with 0.1% toluidine blue. The bone resorption area (mm^2) per osteoclast was quantified using computerized image analysis (Adobe Photoshop and Scion MicroImaging version 4.2) and percentage of the resorbed area was calculated relative to the total dentine disc area (Shanmugarajan et al., 2008).

Confocal microscopy analysis

Confocal microscopy was used to localize NIP45 and TRAF6 expression in the preosteoclast cells. Briefly, mouse bone marrow derived non-adherent cells (1×10^6/ml) were cultured with M-CSF (10 ng/ml), RANKL (100 ng/ml) for 24 hr to form pre-osteoclasts in an 8-well lab-tec chamber and RAW 264.7 cells were fixed with methanol, permeabilized with 0.1% Triton X-100 followed by blocking with 2% normal human serum for 1 hr at room temperature. Subsequently, cells were washed with PBS and incubated with anti-NIP-45, NFATc2 or anti-TRAF6 antibody. Cells were washed three times and incubated with Alexa 568 or Alexa 488 conjugated secondary antibodies. The nuclei were stained with DRAQ5 (Axxora Platform, CA) for 10 min and confocal image analysis of the cells were performed with Zeiss 510 META Laser Scanning Confocal Microscope.

Statistical analysis

Results are presented as the mean ± SD for three independent experiments and were compared by Student's *t* test or one-way ANOVA. Values were considered significantly different for $^*p < 0.05$.

Results

RANKL down regulates NIP45 expression in preosteoclasts

RANKL induces nuclear factor of activated T cells cytoplasmic 1 (NFATc1) critical for osteoclast differentiation (Takayanagi, 2007a). However, the role of NFAT family interacting proteins in osteoclast differentiation is unknown. Therefore, we examined RANKL regulation of NIP45 expression in preosteoclast cells. Mouse bone marrow derived non-adherent mononuclear cells were stimulated with RANKL (100 ng/ml) for a variable period (0–72 hr). Western blot analysis of total cell lysates obtained demonstrated a significant decrease in NIP45 expression in a time dependent manner. Densitometric quantification indicated a 3.5-fold decrease in NIP45 expression at a 24 hr period of RANKL stimulation (Fig. 1A). We further examined the RANKL dose dependent inhibition of NIP45 expression in mouse bone marrow derived preosteoclast cells. Western blot analysis of total cell lysates obtained from cells stimulated with RANKL at different concentration (0–200 ng/ml) for 12 hr period demonstrated a 5.2-fold decrease in NIP45 expression (Fig. 1B). Relative levels of NIP45 expression was normalized with respect to β-actin expression in these cells. These results suggest that RANKL negatively regulates NIP45 expression during osteoclast differentiation.

NIP45 modulates RANKL-RANK signaling

The RANKL-RANK signal transduction pathway is critical for OCL differentiation, activation, and survival (Reddy, 2004). To further understand the role of NIP45 in RANKL-RANK signal transduction, we used GIPZ shRNA lentiviral vectors to knockdown NIP45 expression in mouse bone marrow derived non-adherent cells as described in the methods. We determined a multiplicity of lentiviral infection (MOI) of 10 can down regulate 48% of NIP45 mRNA expression (data not shown) and therefore used this concentration for further experiments. RANKL signaling recruits TRAF adaptor proteins to RANK during osteoclast differentiation (Boyle WJ, 2003). We therefore examined RANKL stimulation of TRAF2 and TRAF6 expression in NIP45 shRNA transduced cells. Total cell lysates obtained from the control and NIP45 shRNA transduced cells stimulated with or without RANKL (100 ng/ml) for a 48 hr period were subjected to Western blot analysis. As shown in Fig. 2A, shRNA knock-down of NIP45 expression results in a 3.5-fold increase in TRAF6; however no change occurred in the level of RANK, TRAF2 expression in RANKL stimulated preosteoclast cells compared to control non-silencing shRNA transduced cells. We further examined the status of RANKL induced IκB activation in NIP45 shRNA transduced mouse bone marrow derived preosteoclast cells. Total cell lysates obtained from the control and NIP45 shRNA transduced cells stimulated with RANKL for a variable period (0–60 min) were subjected to Western blot analysis for phospho-IκB (p-IκB) expression. As shown in Fig. 2B, NIP45 shRNA transduced cells demonstrated a 3.0 and 4.8-fold increase in p-IκB expression with and without RANKL stimulation (0–60 min) compared to control cells respectively. We further examined NIP45 regulation of RANKL stimulated NF-κB transcriptional activity in RAW 264.7 cells. To obtain high transfection efficiency, control non-silencing or NIP45 shRNA transduced RAW 264.7 cells were transiently transfected with a control pGL2 Basic vector or pNF-κB-Luc cis-reporter plasmid and stimulated with RANKL (100 ng/ml) for a 48 hr period. Total cell lysates obtained from these cells were

analyzed for luciferase activity as described. As shown in Fig. 2C, RANKL stimulation increased (4.3-fold) NF-κB reporter activity with NIP45 shRNA compared to control shRNA transduced cells. These results suggest that inhibition of NIP45 enhances NF-κB activation in preosteoclast cells. We further examined the RANKL induction of NFATc1 and NFATc2 expression in control and NIP45 shRNA transduced mouse bone marrow derived non-adherent cells. Western blot analysis of total cell lysates obtained from NIP45 shRNA transduced cells stimulated with and without RANKL (0–48 hr) demonstrated 3.7 and 1.5-fold increase in NFATc1 expression compared to control cells respectively. Further, RANKL stimulation of NIP45 transduced cells showed an increase (2.4-fold) in NFATc2 expression (Fig. 2D). Taken together, NIP45 negatively regulates RANKL signaling during osteoclast differentiation.

Colocalization of NIP45 with TRAF6 and NFATc2 in preosteoclast cells

Evidence indicates that NIP45 interacts with TRAFs and NFATc2 (Hodge et al., 1996; Lieberson et al., 2001). We therefore examined NIP45 localization in preosteoclast cells stimulated with and without RANKL by confocal microscopy. We identified NIP45 cytosolic colocalization with TRAF6 in homogeneous population of RAW 264.7 cells without RANKL stimulation (Fig. 3A). In contrast, RANKL stimulation induced partial nuclear translocation of NIP45 and colocalization with NFATc2 in RANKL stimulated mouse bone marrow derived primary preosteoclast cells (Fig. 3B). Further, cells transfected with non-specific control, NFATc1 or NFATc2 siRNA were treated with RANKL for 48 hr. Total cell lysates obtained were subjected to co-immune precipitation assay demonstrated NIP45 interaction with NFATc2 but not with NFATc1 (Fig. 3C). These results suggest NIP45 interaction with TRAF6 and NFATc2 regulate RANKL signaling during osteoclast differentiation.

Inhibition of NIP45 increases osteoclast differentiation and bone resorption activity

We next determined the role of NIP45 in RANKL induced osteoclast differentiation/bone resorption activity. Mouse bone marrow derived non-adherent cells were transduced with control or NIP45 shRNA lentiviral vectors and cultured with M-CSF and RANKL for 5 days. TRAP positive multinucleated cells (MNC) formed in these cultures were scored. RANKL stimulation of NIP45 shRNA transduced cells showed a significant increase in the number of TRAP positive multi-nucleated osteoclasts formed compared to control shRNA transduced cells. Quantification of these results indicated that shRNA inhibition of NIP45 resulted in a 43% increase in osteoclast formation compared to control shRNA transduced cells (Fig. 4A). We also tested the effect of NIP45 shRNA on the bone resorption capacity of osteoclasts formed in mouse bone marrow cultures as described. Osteoclasts formed in NIP45 shRNA transduced cells demonstrated a significant increase in resorption area per osteoclast on dentine slices compared to control shRNA (Fig. 4B). These results suggest that NIP45 negatively regulates osteoclast differentiation and bone resorption activity.

Discussion

Osteoclast formation and bone resorption activity are regulated by complex signaling mechanisms. The RANKL-RANK signaling pathway activates transcription factors such as

NF-κB and activator protein 1 (AP-1) which induces NFATc1 transcription factor expression critical for osteoclastogenesis (Takayanagi, 2007a). Our results show that RANKL down regulates NIP45 expression in preosteoclast cells suggesting a functional role for NIP45 in osteoclast differentiation/bone resorption activity. Suppression of NIP45 significantly increased NFATc1, NFATc2 and TRAF6 expression which implicate NIP45 as a negative regulator of RANK signaling in preosteoclast cells. NIP45 colocalized with TRAF6 and NIP45 shRNA increase basal level expression of p-IκB and NFATc1 without RANKL stimulation. However, we observe no change in RANK expression in NIP45 shRNA transduced preosteoclast cells which suggest that NIP45 regulates preosteoclast commitment to osteoclast lineage. Previously, it has been shown that RANKL stimulation elevates interferon-β which down regulates c-Fos expression which is essential for osteoclast differentiation (Takayanagi et al., 2002). Similarly, IFN-γ inhibits osteoclastogenesis through degradation of TRAF6 (Takayanagi et al., 2000). Thus, both extracellular and intracellular signaling events can regulate RANK signaling and osteoclast differentiation. Ubiquitination of the RANK adaptor protein, TRAF6 plays an important role in activation of NF-κB during osteoclast differentiation (Lamothe et al., 2007). p62 is a scaffold protein which interacts with the atypical PKCs and TRAF6 to modulate RANK signaling. The deubiquitinating enzyme, CYLD has been shown to interact with p62 UBA domain to inhibit TRAF6 ubiquitination and thereby negatively regulates RANK signaling and osteoclastogenesis (Jin et al., 2008). It has been shown that TRAF1 association with NIP45 in the cytoplasm prevents nuclear translocation and regulates gene transcription in T cells (Bryce et al., 2006). Furthermore, TRAF2 interaction with NIP45 has been implicated in IL-4 regulation in vivo (Lieberson et al., 2001). We show that NIP45 is colocalized with TRAF6, however it is unlikely that NIP45 affects TRAF6 ubiquitination. RANK signaling through TRAF6 results in activation of NF-κB (Takayanagi, 2007a). Elevated levels of p-IκB-α and NF-κB-luciferase reporter activity observed in NIP45 knock-down RANKL stimulated preosteoclast cells further indicate that RANKL regulation of NIP45 expression modulates NF-κB activation. RANKL significantly decreased NIP45 expression and promoted nuclear translocation and colocalization with NFATc2 in preosteoclast cells. Previously, it has been demonstrated that NFATc2 induce NFATc1 expression and thereby indirectly promotes osteoclastogenesis (Takayanagi, 2007b). An increased level of NFATc2 expression observed in NIP45 suppressed cells favors NFATc1 expression. NIP45 has been shown to be a cofactor in NFATc2/c-Maf driven IL-4 promoter activity and endogenous IL-4 expression (Hodge et al., 1996). Therefore, NIP45 interaction with NFATc2 may regulate gene expression during osteoclast differentiation. NIP45 knockdown significantly increased RANKL induced osteoclast formation/bone resorption activity further suggest that NIP45 is a negative regulator of osteoclast formation. However, NIP45 over-expression in osteoclast progenitor cells did not modulate osteoclast formation (data not shown) which indicated that it has no feedback control on RANKL signaling and that constitutive levels of NIP45 expression are sufficient to suppress osteoclastogenesis. Further, cytokines such as IL-6, TNF-α and PTHrP treatment did not alter NIP45 expression in osteoclast progenitor cells (data not shown). NFAT transcription factors also play an important role in gene transcription in other cell types such as chondrocytes and immune cells. NFATc2 has been shown to be a repressor of cartilage cell growth and differentiation (Ranger et al., 2000). Therefore, NIP45 interaction and down-regulation of NFATc2 expression can modulate

chondrogenesis in the bone microenvironment. Recently, targeted deletion of NIP45 in T-helper cells in mice resulted in a significant decrease in serum levels of cytokines such as IL-3, IL-4, IL-5 and IL-13; however no change occurred in interferon-γ levels (Fathman et al., 2010). IL4 has been shown to inhibit osteoclastogenesis (Abu-Amer, 2001). It is possible that NIP45 regulated cytokine expression may also play a role in RANKL induced osteoclast formation. In summary, RANKL signaling down regulates NIP45 expression and that NIP45 is a negative regulator of osteoclast differentiation.

Acknowledgments

This work was supported by a grant C06RR015455 from the Extramural Research Facilities Program of the National Center for Research Resources.

References

Abu-Amer Y. IL-4 abrogates osteoclastogenesis through STAT6-dependent inhibition of NF-kappaB. J Clin Invest. 2001; 107(11):1375–1385. [PubMed: 11390419]

Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003; 423(6937): 337–342. [PubMed: 12748652]

Bryce PJ, Oyoshi MK, Kawamoto S, Oettgen HC, Tsitsikov EN. TRAF1 regulates Th2 differentiation, allergic inflammation and nuclear localization of the Th2 transcription factor, NIP45. Int Immunol. 2006; 18(1):101–111. [PubMed: 16352630]

Crabtree GR, Olson EN. NFAT signaling: choreographing the social lives of cells. Cell. 2002; 109(Suppl):S67–79. [PubMed: 11983154]

Crotti TN, Flannery M, Walsh NC, Fleming JD, Goldring SR, McHugh KP. NFATc1 regulation of the human beta3 integrin promoter in osteoclast differentiation. Gene. 2006; 372:92–102. [PubMed: 16513293]

Day CJ, Kim MS, Lopez CM, Nicholson GC, Morrison NA. NFAT expression in human osteoclasts. J Cell Biochem. 2005; 95(1):17–23. [PubMed: 15759284]

Fathman JW, Gurish MF, Hemmers S, Bonham K, Friend DS, Grusby MJ, Glimcher LH, Mowen KA. NIP45 controls the magnitude of the type 2 T helper cell response. Proc Natl Acad Sci U S A. 2010; 107(8):3663–3668. [PubMed: 20133688]

Hodge MR, Chun HJ, Rengarajan J, Alt A, Lieberson R, Glimcher LH. NF-AT-Driven interleukin-4 transcription potentiated by NIP45. Science. 1996; 274(5294):1903–1905. [PubMed: 8943202]

Ikeda F, Nishimura R, Matsubara T, Tanaka S, Inoue J, Reddy SV, Hata K, Yamashita K, Hiraga T, Watanabe T, Kukita T, Yoshioka K, Rao A, Yoneda T. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. J Clin Invest. 2004; 114(4):475–484. [PubMed: 15314684]

Jin W, Chang M, Paul EM, Babu G, Lee AJ, Reiley W, Wright A, Zhang M, You J, Sun SC. Deubiquitinating enzyme CYLD negatively regulates RANK signaling and osteoclastogenesis in mice. J Clin Invest. 2008; 118(5):1858–1866. [PubMed: 18382763]

Kim K, Lee SH, Ha Kim J, Choi Y, Kim N. NFATc1 induces osteoclast fusion via up-regulation of Atp6v0d2 and the dendritic cell-specific transmembrane protein (DC-STAMP). Mol Endocrinol. 2008; 22(1):176–185. [PubMed: 17885208]

Lamothe B, Webster WK, Gopinathan A, Besse A, Campos AD, Darnay BG. TRAF6 ubiquitin ligase is essential for RANKL signaling and osteoclast differentiation. Biochem Biophys Res Commun. 2007; 359(4):1044–1049. [PubMed: 17572386]

Lieberson R, Mowen KA, McBride KD, Leautaud V, Zhang X, Suh WK, Wu L, Glimcher LH. Tumor necrosis factor receptor-associated factor (TRAF)2 represses the T helper cell type 2 response through interaction with NFAT-interacting protein (NIP45). J Exp Med. 2001; 194(1):89–98. [PubMed: 11435475]

Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, Morony S, Capparelli C, Van G, Kaufman S, van der Heiden A, Itie A, Wakeham A, Khoo W, Sasaki T, Cao Z, Penninger JM,

Paige CJ, Lacey DL, Dunstan CR, Boyle WJ, Goeddel DV, Mak TW. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev. 1999; 13(8): 1015–1024. [PubMed: 10215628]

Mowen KA, Schurter BT, Fathman JW, David M, Glimcher LH. Arginine methylation of NIP45 modulates cytokine gene expression in effector T lymphocytes. Mol Cell. 2004; 15(4):559–571. [PubMed: 15327772]

Negishi-Koga T, Takayanagi H. Ca2+-NFATc1 signaling is an essential axis of osteoclast differentiation. Immunol Rev. 2009; 231(1):241–256. [PubMed: 19754901]

Ranger AM, Gerstenfeld LC, Wang J, Kon T, Bae H, Gravallese EM, Glimcher MJ, Glimcher LH. The nuclear factor of activated T cells (NFAT) transcription factor NFATp (NFATc2) is a repressor of chondrogenesis. J Exp Med. 2000; 191(1):9–22. [PubMed: 10620601]

Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol. 1997; 15:707–747. [PubMed: 9143705]

Reddy SV. Regulatory mechanisms operative in osteoclasts. Crit Rev Eukaryot Gene Expr. 2004; 14(4):255–270. [PubMed: 15663356]

Shanmugarajan S, Youssef RF, Pati P, Ries WL, Rao DS, Reddy SV. Osteoclast inhibitory peptide-1 (OIP-1) inhibits measles virus nucleocapsid protein stimulated osteoclast formation/activity. J Cell Biochem. 2008; 104(4):1500–1508. [PubMed: 18348201]

Shanmugarajan S, Beeson CC, Reddy SV. Osteoclast inhibitory peptide-1 binding to the Fc gammaRIIB inhibits osteoclast differentiation. Endocrinology. 2010; 151(9):4389–99. [PubMed: 20610564]

Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol. 2007a; 7(4):292–304. [PubMed: 17380158]

Takayanagi H. The role of NFAT in osteoclast formation. Ann N Y Acad Sci. 2007b; 1116:227–237. [PubMed: 18083930]

Takayanagi H, Kim S, Matsuo K, Suzuki H, Suzuki T, Sato K, Yokochi T, Oda H, Nakamura K, Ida N, Wagner EF, Taniguchi T. RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. Nature. 2002; 416(6882):744–749. [PubMed: 11961557]

Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, Nakamura K, Taniguchi T. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. Nature. 2000; 408(6812):600–605. [PubMed: 11117749]

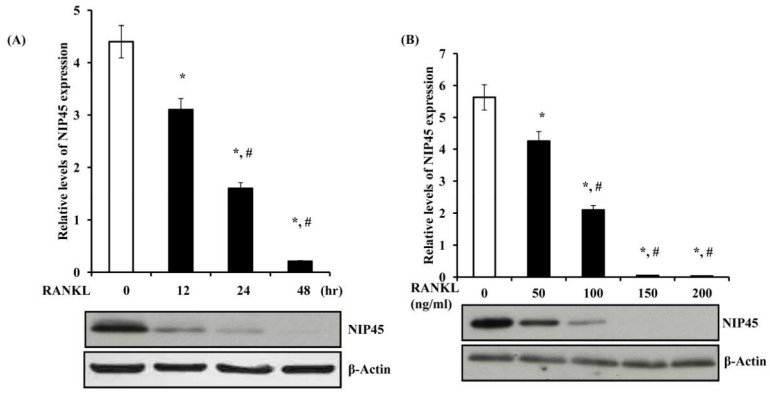
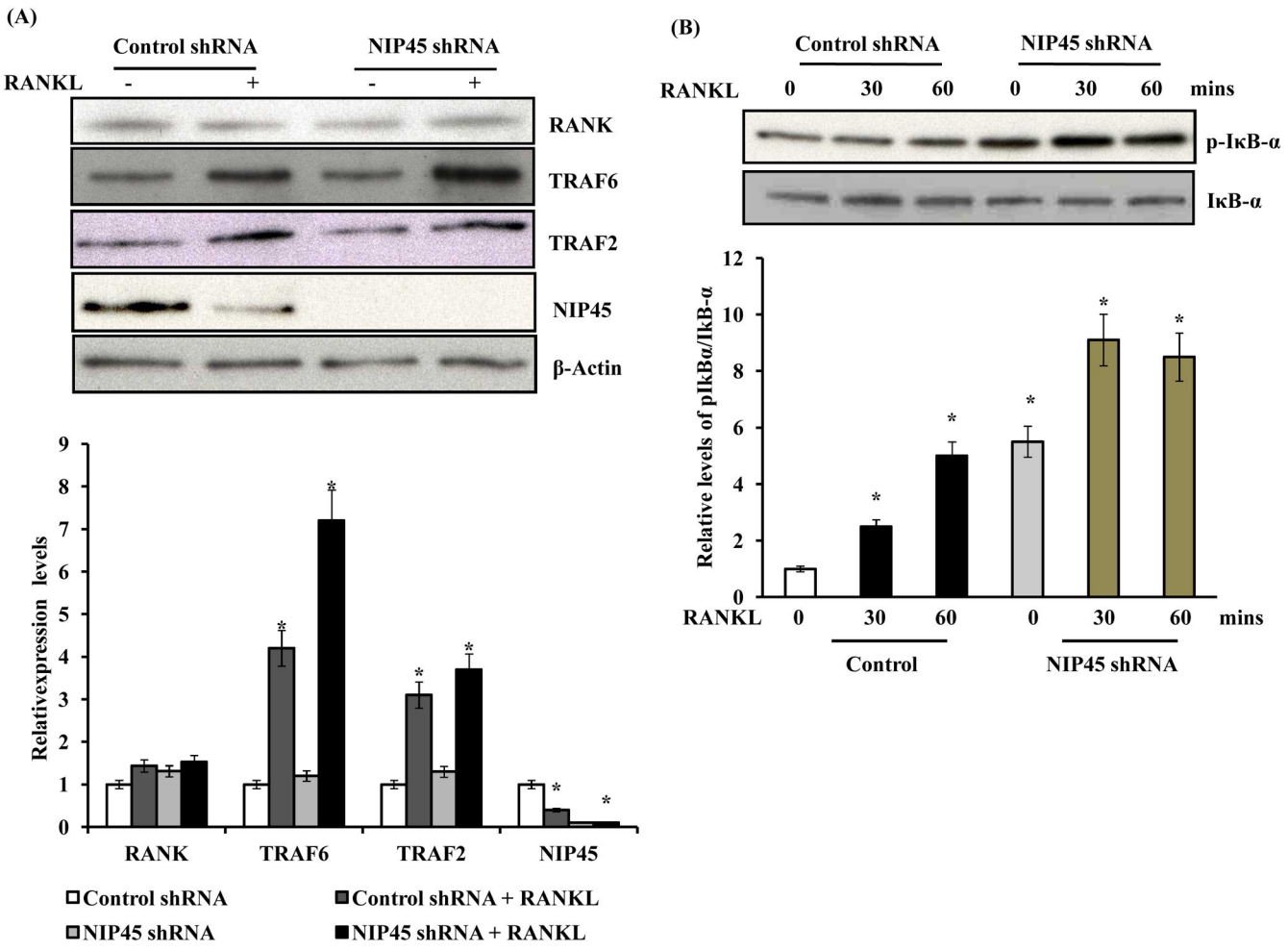


Figure 1. RANKL down regulates NIP45 expression in preosteoclast cells

Mouse bone marrow derived non-adherent cells were cultured with M-CSF (10 ng/mL) for 48 hr. (A) Cells were stimulated with RANKL (100 ng/ml) for variable time points as indicated. (B) Cells were stimulated with RANKL at different concentrations (0–200 ng/ml) for 12 hr. Total cell lysates obtained were subjected to Western blot analysis for NIP45 expression. β-Actin expression levels were also analyzed to normalize the protein loading onto the gels in all the samples. Band intensities were quantified by densitometric analysis using the NIH Image J program. Data represent three independent experiments *$p < 0.05$ versus control and $^{\#}P < 0.05$ versus RANKL treated group.



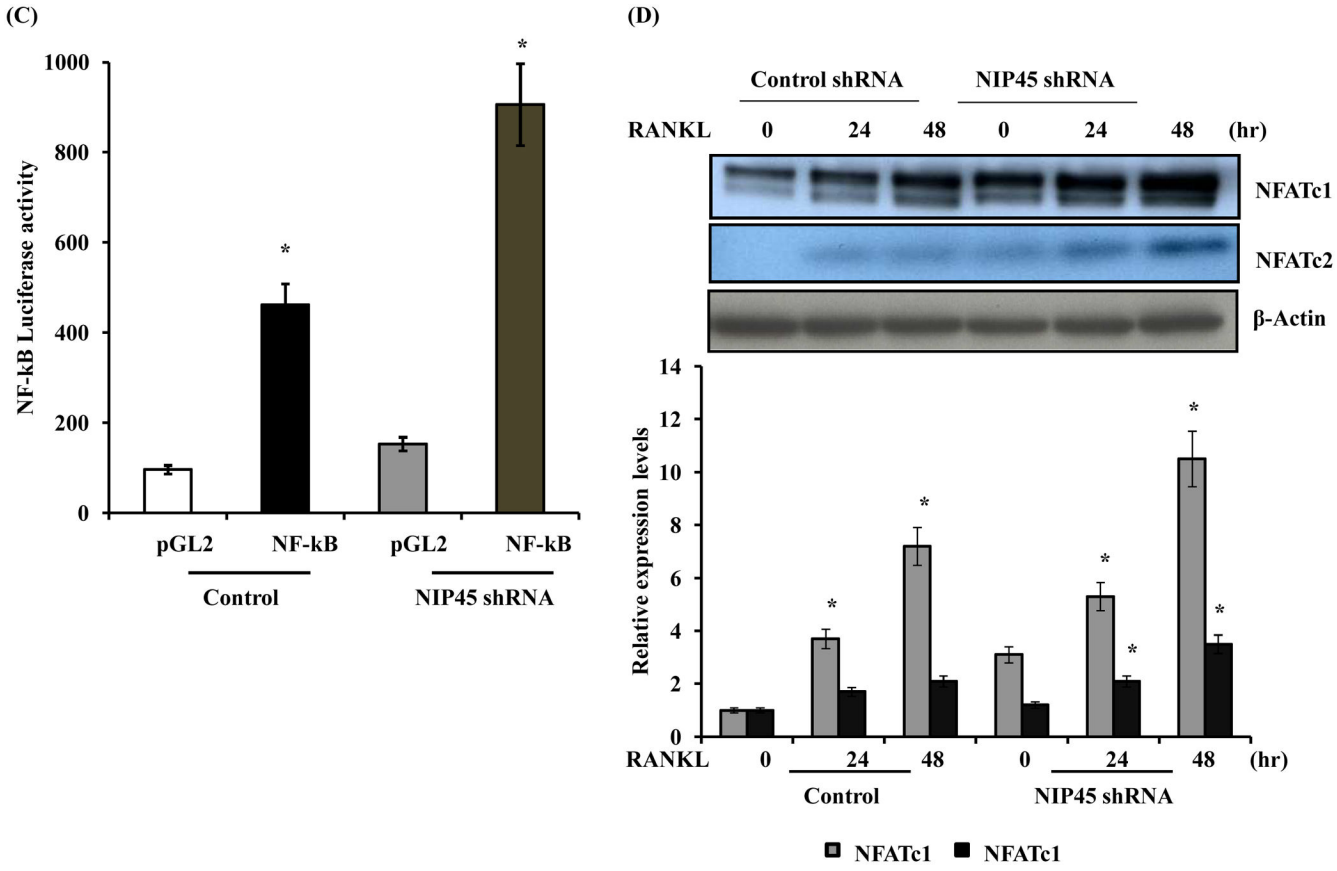


Figure 2. shRNA knock down of NIP45 expression modulates RANKL-RANK signaling in preosteoclast cells

(A) Mouse bone marrow derived non-adherent cells were transduced with control or NIP45 shRNA lentiviral vector and cultured with M-CSF (10 ng/ml) in the presence or absence of RANKL (100 ng/mL) for 48 hr and total cell lysates prepared were subjected to Western blot analysis for TRAF2, TRAF6 and NIP45 expression. (B) Cells transduced with control or NIP45 shRNA lentiviral vector were cultured with M-CSF and RANKL for indicated time point (0–60 mins). Total cell lysates were subjected to Western blot analysis for IκB-α and p-IκB-α expression. (C) RAW 264.7 cells were transduced with control vector (EV) or NIP45 shRNA vector. After 24 hr, cells were transfected with control pGL2 basic vector or pNF-κB-Luc reporter plasmid and stimulated with RANKL (100 ng/ml) for 48 hr. Total cell lysates prepared were assayed for luciferase activity. The transfection efficiency was normalized by β-galactosidase activity co-expressed in these cells. The results represent three independent experiments and data shown as mean ± SD, (*$p<0.05$). (D) Mouse bone marrow derived non-adherent cells transduced with control or NIP45 shRNA lentiviral vector and cultured with M-CSF (10 ng/ml) in the presence or absence of RANKL (100 ng/ml) for indicated time point (0–48 hr). Total cell lysates were subjected to Western blot analysis for NFATc1 and NFATc2 expression. Band intensities were quantified densitometrically using the NIH Image J program and relative expression levels were normalized for *β*-actin or IκB-α expression expression. The data represent three independent experiments ($n = 6$ mice; $p < 0.05$)

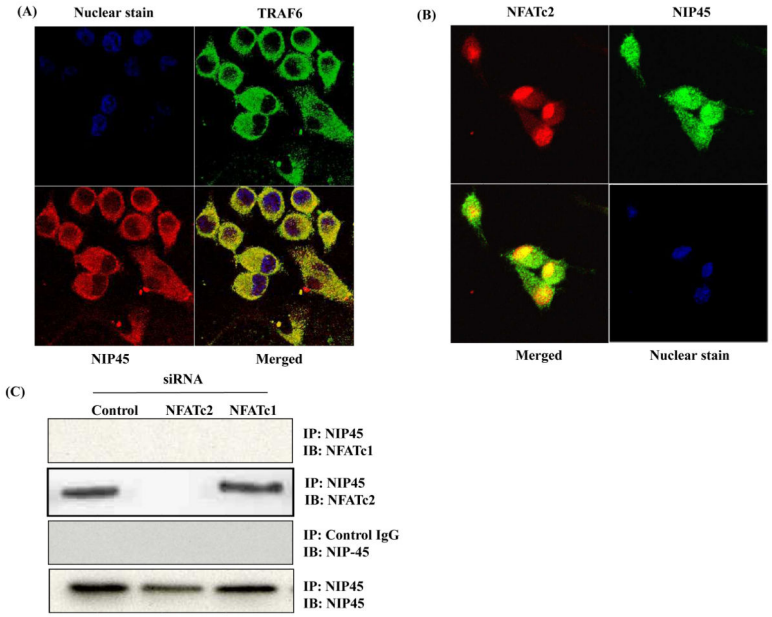


Figure 3. Confocal microscopy analysis of NIP45 colocalization with TRAF6 and NFATc2 in preosteoclasts

(A) RAW 264.7 cells subjected to confocal microscopy analysis using anti-TRAF6 and anti-NIP45 antibodies. The merged image demonstrated cytosolic co-localization of TRAF6 with NIP45. (B) Mouse bone marrow derived non-adherent cells treated with RANKL and M-CSF for 24 hr and analyzed by confocal microscopy using anti-NFATc2 and anti-NIP45 antibodies. Merged image demonstrated nuclear co-localization of NFATc2 and NIP45. The nuclei were stained with DRAQ5. Magnification 60x. (C) NIP45 interacts with NFATc2 but not with NFATc1 in preosteoclast cells. Bone marrow derived non-adherent cells were transfected with non-specific control siRNA, NFATc1 and NFATc2 siRNA (10 nM) by TransIT-TKO transfection reagent (Mirus), after 48 hr total cell lysates were subjected to immunoprecipitation assay with control IgG, goat anti-NIP45 antibody and subjected to Western blot analysis using anti-NFATc1 and anti-NFATc2 antibody.

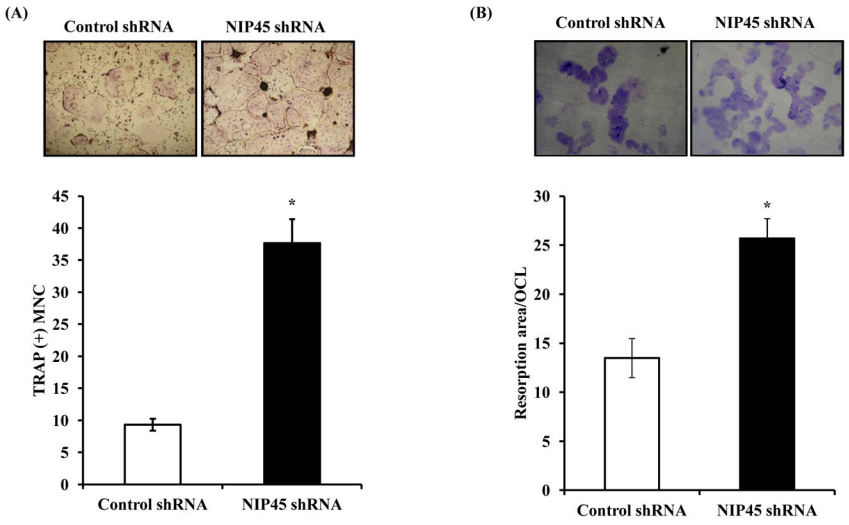


Figure 4. shRNA knock-down of NIP45 expression increases osteoclast formation/bone resorption activity in mouse bone marrow cultures

(A) Mouse bone marrow non-adherent cells were transduced with control or NIP45 shRNA lentiviral vector and cultured for osteoclast formation in the presence of M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 5 days. At the end of the culture period, cells were fixed and stained for TRAP activity. (B) TRAP (+) multinucleated cells (MNC) formed were scored under a light microscope. The results represent quadruplicate cultures of three independent experiments and data shown as mean±SD, (*$p<0.05$). Magnification, 20x. (C) NIP45 knock-down increases osteoclast bone resorption activity. Mouse bone marrow derived non-adherent cells (1×10^6/ml) transduced with control shRNA or NIP45 shRNA lentiviral vector were cultured on dentine slices for 10 days in the presence of M-CSF (10 ng/mL) and RANKL (100 ng/ml). At the end of the culture period, cells were removed and stained with 0.1% toludine blue. (D) The mineralized surface area (mm^2) resorbed per osteoclast was quantified as described in the methods. The results represent quadruplicate cultures of three independent experiments and data shown as mean ± SD, (*$p<0.05$). Magnification, 20x.