Ubiquitin E3 Ligase Itch Negatively Regulates Osteoclast Formation by Promoting Deubiquitination of Tumor Necrosis Factor (TNF) Receptor-associated Factor 6

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Background: TRAF6 activity is crucial for osteoclastogenesis, which is decreased by deubiquitination. The role of Itch in this process is studied.

Results: Itch−/− osteoclast precursors formed more osteoclasts, had prolonged RANKL-induced NF-κB activation, and had delayed TRAF6 deubiquitination. Itch bound to the deubiquitinating enzyme cylindromatosis.

Conclusion: Itch inhibits osteoclastogenesis by binding to cylindromatosis and prompting TRAF6 deubiquitination.

Significance: Itch is a new inhibitor of osteoclastogenesis.

Itch is a ubiquitin E3 ligase that regulates protein stability. Itch−/− mice develop an autoimmune disease phenotype characterized by itchy skin and multiorgan inflammation. The role of Itch in the regulation of osteoclast function has not been examined. We report that Itch−/− bone marrow and spleen cells formed more osteoclasts than cells from WT littermates in response to receptor activator of NF-κB ligand (RANKL) and was associated with increased expression of the osteoclastogenic transcription factors c-fos and Nfatc1. Overexpression of Itch in Itch−/− cells rescued increased osteoclastogenesis. RANKL increased Itch expression, which can be blocked by a NF-κB inhibitor. The murine Itch promoter contains NF-κB binding sites. Overexpression of NF-κB p65 increased Itch expression, and RANKL promoted the binding of p65 onto the NF-κB binding sites in the Itch promoter. Itch−/− osteoclast precursors had prolonged RANKL-induced NF-κB activation and delayed TNF receptor-associated factor 6 (TRAF6) deubiquitination. In WT osteoclast precursors, Itch bound to TRAF6 and the deubiquitinating enzyme cylindromatosis. Adult Itch−/− mice had normal bone volume, but they had significantly increased LPS-induced osteoclastogenesis and bone resorption. Thus, Itch is a new RANKL target gene that is induced during osteoclastogenesis. Itch interacts with the deubiquitinating enzyme and is required for deubiquitination of TRAF6, thus limiting RANKL-induced osteoclast formation.

Autoimmune diseases and systemic inflammation can perturb normal bone homeostasis and cause bone loss, which is typically due to increased osteoclast formation and bone resorption (1). The mechanisms underlying the increased osteoclast functions in these pathological conditions are complex and diverse. Osteoimmunology studies have demonstrated that the co-stimulatory signaling that regulates immune cell functions often also plays a role in osteoclastogenesis (2). Thus, the molecular mechanisms that mediate the disturbed immune cell functions in autoimmune diseases could also be operational in osteoclasts and contribute to the increased bone loss seen in patients with these conditions.

Itch is a ubiquitin E3 ligase (3). Itch−/− mice on a C57BL/6J background develop a progressive autoimmune disease (4). Patients with Itch mutations have autoimmune inflammatory cell infiltration in various tissues (5). Recent molecular studies demonstrate that Itch limits TNF-induced NF-κB activation by facilitating A20-mediated ubiquitination and degradation of the adaptor protein receptor-interacting protein in the TNF receptor complex in T cells (6) and macrophages (7). Itch is also required for negative regulation of TNF- and lipopolysaccharide (LPS)-mediated TNF receptor-associated factor 6 (TRAF6) ubiquitination in macrophages (8). Itch depletion results in persistent activation of NF-κB in cells, thereby causing inflammation. However, the role of Itch in bone cell regulation has not been investigated.

TRAF6 is an essential signaling component of RANKL/RANK signaling in osteoclasts and osteoclast precursors (OCPs). The activity of TRAF6 is regulated by ubiquitination. Ubiquitination is a post-translational modification of target proteins. The functional consequences of this modification are either target protein degradation through the proteasome or

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3 The abbreviations used are: TRAF, TNF receptor-associated factor; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; OCP, osteoclast precursor; DUB, deubiquitinating enzyme; CYLD, cylindromatosis; PE, phycoerythrin; M-CSF, macrophage colony-stimulating factor; TRAP, tartrate-resistant acid phosphatase; IP, immunoprecipitation.
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activation of the target protein via conformational changes depending on the ubiquitin linkage. Generally, the ubiquitin molecules are linked through the lysine residue at position 48 or 63 of ubiquitin (known as Lys48 and Lys63 polyubiquitination, respectively) (9, 10). The different types of polyubiquitin chains have different effects on the substrate: a protein with Lys48 polyubiquitination is mainly recognized by the 26 S proteasome and undergoes degradation, whereas a protein with Lys63 polyubiquitination usually becomes activated to mediate downstream signaling events such as kinase activation, alteration of intracellular locations, and DNA repair (11). Protein ubiquitination is carried out in part by ubiquitin E3 ligases, which play an important role in the pathogenesis of autoimmune diseases and inflammation (1, 2).

Biochemical experiments demonstrate that TRAF6 functions as a ubiquitin ligase. After cells are stimulated by cytokines such as RANKL, IL-1, and LPS, TRAF6 promotes Lys63 polyubiquitination on target proteins in NF-κB signaling and on itself (9). This Lys63-linked polyubiquitination on TRAF6 can be reversed by deubiquitinating enzymes (DUB) such as cylindromatosis (CYLD) and A20 (12, 13). Osteoclast precursors from Cyld−/− mice are hyper-responsive to RANKL-induced osteoclasts differentiation. CYLD targets TRAF6 for deubiquitination and inhibits the downstream signaling events as part of its mechanism to regulate osteoclast formation. A20−/− mice develop spontaneous inflammation because A20 is required for the termination of IL-1R/Toll-like receptor 4 signaling in immune cells by promoting TRAF6 deubiquitination (13). These studies highlight the importance of deubiquitination as one of the molecular mechanisms to regulate TRAF6 function, which has not been well studied in osteoclast biology.

In the present study, we examined the effects of Itch deficiency on osteoclastogenesis, NF-κB activation, and TRAF6 deubiquitination. We found that Itch−/− mice have increased osteoclast numbers and are more responsive to LPS-stimulated osteoclast formation and bone resorption. Osteoclast precursors from Itch−/− mice have prolonged RANKL-induced NF-κB activation and significantly delayed TRAF6 deubiquitination upon RANKL withdrawal. Itch is a negative regulator of osteoclastogenesis by promoting TRAF6 deubiquitination. Itch-regulated TRAF6 deubiquitination may represent a new mechanism for persistent NF-κB activation in immune cells to account for autoimmune phenotypes of Itch−/− mice.

EXPERIMENTAL PROCEDURES

Animal Studies—Itch−/− mice were generated previously on a C57BL/6 background (4) and were genotyped by PCR analysis. All animal procedures were conducted using procedures approved by the University of Rochester Committee for Animal Resources. For in vivo bone resorption studies, mice (n = 4/group) were given LPS (Sigma) injections (100 μg in 25 μl of PBS) or 25 μl of PBS into the loose subcutaneous tissue overlying the calvaria using a Hamilton syringe (Hamilton Co., Reno, NV) as described previously (14). Injections were performed daily for 3 days. Animals were sacrificed 1 day after the last injection. Calvarial bones were harvested for histology.

Plasmids, Antibodies, and Retrovirus—HA-CYLD and FLAG-Itch expression plasmids were provided by Dr. Shao-Cong Sun (The University of Texas M. D. Anderson Cancer Center) and Dr. Derek Abbott (Case Western Reserve University), respectively. Antibodies to FLAG, HA, and β-actin were purchased from Sigma; antibodies to ubiquitin, CYLD, TRAF6, and NF-κB p65 were from Santa Cruz Biotechnology Inc.; antibody to Itch was from BD Biosciences; and antibodies to IκBα, phospho-IκBα, JNK, phospho-JNK, ERK, and phospho-ERK were from Cell Signaling Technology. FITC-anti-CyD5, PE-Cy7-anti-c-Kit, PE-anti-CD11b, and allophycocyanin-anticytokeratin Fms used in fluorescence-activated cell sorting (FACS) were from eBioscience. To generate an Itch retroviral expression vector, the Itch coding region was amplified by PCR from the FLAG-Itch plasmid and cloned into the pMX-GFP retroviral vector at the BamHI and NotI sites to generate the pMX-Itch-GFP expression vector. The pMX-GFP vector was used as a control for infection efficiency. These retroviral vectors were transiently transfected into the Plat-E retroviral packaging cell line, and viral supernatant was collected 48 h later as we described previously (15).

Osteoclastogenesis and Viral Infection—Bone marrow cells or spleenocytes were cultured with conditioned medium (1:50 dilution) from an M-CSF-producing cell line (16) for 3 days in α-modified essential medium with 10% fetal calf serum (Hyclone Laboratories, Logan, UT) to enrich for OCPs. For osteoclastogenesis, OCPs were cultured with M-CSF-conditioned medium and RANKL (10 ng/ml) or LPS (100 ng/ml) for 2–3 or 6–7 days. For viral infection, OCPs were infected with retroviral supernatants from Itch−, NF-κB p65−, or GFP virus-infected Plat-E packaging cells in the presence of M-CSF and Polybrene (8 μg/ml) for 2 days. Cells were then cultured with M-CSF and RANKL to form osteoclasts. After multinucleated cells were observed under a microscope, the cells were fixed and stained for TRAP activity. TRAP+ cells containing ≥3 nuclei were counted as described (17, 18).

Deubiquitination Assay—OCPs were treated with RANKL plus N-ethylmaleimide for 48 h to block deubiquitination. Whole cell lysates were incubated with UbiQapture-Q matrix (Biomol) to pull down all ubiquitinated proteins according to the manufacturer’s instructions. Briefly, cell lysates containing 200 μg of protein were incubated with washed UbiQapture-Q matrix by gentle agitation at 4 °C overnight. After washing three times, captured proteins were eluted with 2× SDS-PAGE loading buffer and analyzed by Western blotting using anti-TRAF6 antibody as described previously (19, 20).

Immunoprecipitation and Western Blotting—Proteins from cell lysates were quantitated using a kit from Bio-Rad. Proteins were subjected to immunoprecipitation as described in the technical bulletin from Sigma. Briefly, 300 μg of proteins in 1 ml of cell lysis buffer was mixed with 1 μg of antibody, incubated for 1 h at 4 °C, and then incubated with prewashed EZview Red Protein G Affinity Gel beads from Sigma for 1 h at 4 °C. The bound antigens were eluted from the beads by boiling samples for 5 min. Eluted samples were fractionated by SDS-PAGE and transferred to PVDF membranes. For NF-κB and ERK signaling analysis, OCPs were starved without serum for 8 h and then treated with PBS or RANKL for various time periods. Cells were harvested and lysed with lysis buffer containing 1 mM Na3VO4, 1 mM PMSF, and 1 mM NaF. After protein quantification, 50 μg...
of protein was fractionated by SDS-PAGE and transferred to PVDF membranes. Immunoblotting was carried out as described previously (20).

Chromatin Immunoprecipitation (ChIP)—A ChIP assay was performed with the MAGnify Chromatin Immunoprecipitation System (Invitrogen) according to the manufacturer’s instructions. OCPs were treated with RANKL or PBS for 48 h. Cells were fixed with 1% formaldehyde for 15 min and sonicated on ice 8 times with a 20-s on and 20-s off cycle at high power using the Bioruptor UCD-200 sonicator. Antibody against p65 (Santa Cruz Biotechnology Inc.) or control rabbit IgG (Invitrogen) were used in the immunoprecipitation step. The amounts of each specific DNA fragment in immunoprecipitates were determined by quantitative PCR. The following primers were used: NF-κB binding site 1 (5′-3′): forward, 5′-GCAGAAATGTCCCAAAGA-3′; reverse, 5′-TGGAAAGCCGAGAACG-3′; NF-κB binding site 2 (5′-3′): forward, 5′-TATGGGATTATTAGGCTGG TG-3′; reverse, 5′-GAGCAGGGTCTACAAGT-3′.

NF-κB Activation—The DNA binding capacity of NF-κB (p65 subunit) was measured in the nuclear extracts of OCPs using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo, Rockford, IL) and TransAMe NF-κB kits (Active Motif, Carlsbad, CA) according to the manufacturers’ instructions. Briefly, the assay is based on a 96-well plate to which an oligonucleotide containing the NF-κB consensus binding site (5′-GGGACCTTCC-3′) has been immobilized. The activated NF-κB contained in nuclear extracts specifically binds to this nucleotide. By using an antibody that is directed against an epitope on p65 that is accessible only when NF-κB is bound to its target DNA, the NF-κB bound to the oligonucleotide is detected. Addition of a secondary antibody conjugated to horseradish peroxidase provides a sensitive colorimetric read-out that is quantified by densitometry.

FACS—Cells were harvested, and red blood cells were lysed. Cells were stained with FITC-anti-CD45, PE-Cy7-anti-c-Kit, PE-anti-CD11b, and allophycocyanin-anti-c-Fms for 30 min and subjected to FACS analysis according to the manufacturer’s instructions (21). Results were analyzed by FlowJo7 software.

Quantitative Real Time RT-PCR—Total RNA was extracted from cell cultures using TRizol reagent (Invitrogen). cDNAs were synthesized using the iSCRIPT cDNA Synthesis kit (Bio-Rad). Quantitative real time RT-PCR amplifications were performed in the iCycler (Bio-Rad) real time PCR machine using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions. Sequence-specific primers of c-fos, Nfatc1, and β-actin used for quantitative real time PCR amplification were described previously (15). The following sequence-specific primers of Itch were used: forward, 5′-TCACCTTGCGCATAGGTCTCT-3′; reverse, 5′-TGTGGCCAGACACTGAGTTA-3′. The primer sequences for c-fos and Nfatc1 were described previously (15). To determine the number of copies of target DNA in the samples, purified PCR fragments of known concentration were serially diluted and served as external standards for each experiment. Data were normalized to Gapdh levels.

Micro-computed Tomography, Histology, and Histomorphometry of Bone Sections—Femurs obtained from 3-month-old mice were dissected free of soft tissue, fixed overnight in 70% ethanol, and scanned at high resolution (10.5 μm) on a VivaCT40 micro-computed tomography scanner (Scanco Medical, Bassersdorf, Switzerland) using an integration time of 300 ms, energy of 55 peak kilovoltage, and intensity of 145 μA. The three-dimensional images were generated using a constant threshold of 275 for all samples.

The calvariae and limbs were removed from mice after sacrifice, fixed in 10% buffered formalin, decalcified in 10% EDTA, and embedded in paraffin. Sections (4 μm thick) were then stained with H&E and for TRAP activity. Histomorphometric analysis of osteoclast numbers (expressed as the number of osteoclasts per millimeter of bone surface) and of eroded surfaces (expressed as a percentage of the eroded surface of total bone surface) was performed in calvarial sections using an Osteometrics image analysis software system (Osteometrics, Atlanta, GA) (22).

Statistics Analysis—Data are presented as mean ± S.D., and all experiments were repeated at least twice with similar results. Statistical analyses were performed with Stat View statistical software (SAS, Cary, NC). Differences between the two groups were compared using unpaired Student’s t test, and more than two groups were compared using one-way analysis of variance followed by a Bonferroni/Dunnett test. p values less than 0.05 were considered to be statistically significant.

RESULTS

Itch−/− Mice Have Increased Osteoclast Formation in Vitro—To examine whether Itch regulates osteoclastogenesis, we cultured bone marrow cells or spleen cells from Itch−/− mice and their WT littermates with M-CSF and RANKL in osteoclastogenic assays. Cells from bone marrow of Itch−/− mice formed significantly more osteoclasts than WT cells at all cell densities tested (Fig. 1A). Increased osteoclast formation was also observed when Itch−/− splenocytes were used (number of osteoclasts/well, 360 ± 24 versus 20 ± 12 in WT cells). The expression levels of osteoclast transcription factors c-fos and Nfatc1 were increased in Itch−/− osteoclasts (Fig. 1B). Osteoclasts are derived from myeloid precursors. Several studies (23, 24) including reports from our group (25, 26) demonstrate that OCPs can be identified by a combination of cell surface markers such as CD45+/c-Kit+/c-Fms+ cells. Furthermore, bone marrow OCPs include both CD45+/c-Kit+/c-Fms+/CD11b+ and CD45+/c-Kit+/c-Fms+/CD11b− cells, whereas peripheral OCPs only contain CD45+/c-Kit+/c-Fms-/CD11b+ cells (18, 24, 25). To determine whether the increased osteoclast formation in the absence of Itch is due to increased OCPs in Itch−/− mice, we examined the frequency of OCPs in bone marrow and spleen cells (peripheral OCPs) by FACS analysis. A similar percentage of OCPs was detected in bone marrow and spleen cells from Itch−/− mice and WT littermates (Fig. 1C).

We then examined whether overexpression of Itch can rescue the increased osteoclastogenesis phenotype of Itch−/− cells using a retroviral delivery approach. Overexpression of Itch in WT OCPs reduced RANKL-induced osteoclast formation (number of osteoclasts/well, 27 ± 12 versus 40 ± 22 in GFP

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controls). Overexpression of Itch in Itch−/− OCPs prevented increased osteoclast formation (number of osteoclasts/well, 138 ± 18 versus 259 ± 32 in GFP-infected cells) (Fig. 2A). Itch expression after retroviral infection in WT and Itch−/− cells was confirmed by Western blotting (Fig. 2B).

RANKL Up-regulates Itch Expression via NF-κB—To investigate the regulation of Itch during osteoclast differentiation, we treated WT OCPs with RANKL for 4–72 h. RANKL significantly increased Itch mRNA expression starting at 4 h and peaking at 24 h (Fig. 2C), and this was confirmed by Western blot (Fig. 2D). RANKL did not affect TRAF6 mRNA expression and slightly increased TRAF6 protein levels at later time points.

NF-κB is one of the downstream transcription factors of RANKL in osteoclasts and regulates the transcription of a number of target genes. To examine whether NF-κB mediates RANKL-induced Itch expression, we first treated WT OCPs with the NF-κB inhibitor pyrrolidine dithiocarbamate in the presence or absence of RANKL. Pyrrolidine dithiocarbamate itself did not affect Itch expression, but it remarkably reduced RANKL-induced Itch expression (Fig. 2E). We then overexpressed NF-κB p65 in WT OCPs and found that p65 increased Itch expression (Fig. 2F). Finally, we searched NF-κB binding sites within −4000 bp of the murine Itch promoter (NCBI RefSeq accession number NC_000068) using TFSEARCH software (version 1.3) and identified two putative NF-κB binding sites at the −3473/−3465 and −3183/−3175 location. We performed ChIP assays using anti NF-κB p65 antibody to pull down protein-chromatin complexes and two primer sets around these NF-κB binding sites. Results showed p65 binding to both NF-κB binding sites that was enhanced by RANKL treatment (Fig. 2G).

Itch Negatively Regulates RANKL-induced Activation of NF-κB Signaling—Itch has been reported to be a negative regulator of the NF-κB pathway in T cells by prolonging TNF-α-induced NF-κB activation (6). NF-κB is essential for osteoclast formation (27). We examined the effect of Itch deficiency on RANKL-induced activation of NF-κB signaling in osteoclasts. OCPs from Itch−/− or WT mice were treated with RANKL (10 ng/ml) for different times, and the expression of IκBα and phosphorylated IκBα, a commonly used readout for activation of canonical NF-κB signaling, was examined. RANKL increased IκBα phosphorylation in WT cells at 15 min, and then it subsequently declined. However, RANKL-induced IκBα phosphorylation persisted for 240 min in Itch−/− cells (Fig. 3A). We observed increased JNK phosphorylation in WT cells at 15 min, and then it subsequently declined, whereas RANKL-induced
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JNK phosphorylation persisted for 240 min. However, the change of ERK phosphorylation was similar. RANKL increased ERK phosphorylation at 15 min, and it decreased gradually from 30 to 240 min. However, the basal level of ERK phosphorylation in Itch-/- cells was higher than that in WT cells (Fig. 3A). The NF-κB activation was also examined using an ELISA-based assay in which the DNA binding capacity of NF-κB p65 subunit was assessed. In WT cells, RANKL-triggered NF-κB activation peaked at 15 min and declined thereafter, whereas in Itch-/- cells, RANKL-triggered NF-κB activation persisted for 1 h (Fig. 2B). RANKL/RANK interaction recruits several TRAF adaptor proteins including TRAF2, -3, -5, and -6 to mediate downstream signaling. TRAF6 is a unique adapter protein for RANKL signaling in osteoclasts, and Itch induces TRAF6 ubiquitination, thereby transducing positive signaling from the initial receptor/ligand interaction to downstream signaling proteins (28, 29). We examined the ubiquitination of TRAF6. WT cells showed an increased ubiquitination of TRAF6 at 15 min of RANKL treatment, whereas Itch-/- cells showed a constitutive ubiquitination from 15 min to 1 h of RANKL treatment (Fig. 3C).

Itch Binds to TRAF6 and Limits TRAF6 Deubiquitination—To examine whether Itch affects RANKL-induced TRAF6 signaling, we first determined whether Itch interacts with TRAF6 in WT osteoclasts in the presence or absence of RANKL. Cells were treated with RANKL for 2 days, and whole cell lysates were immunoprecipitated with anti-TRAF6 antibody or IgG control and blotted with anti-Itch antibody. In the absence of RANKL, we observed small quantities of Itch associating with TRAF6 (Fig. 4A). RANKL greatly increased the interaction between Itch and TRAF6 (Fig. 4A). RANKL has been reported to induce TRAF6 ubiquitination and activate downstream signals (28, 29), which are limited via deubiquitination (30). To investigate whether Itch is involved in the regulation of TRAF6 ubiquitination and deubiquitination, we treated WT and Itch-/- osteoclasts with RANKL to promote TRAF6 ubiquitination and subsequently...
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removed RANKL to facilitate its deubiquitination using a published protocol (12). RANKL induced TRAF6 ubiquitination in WT and _Itch^-/-_ OCPs to the same degree after 2 days of treatment (Fig. 4B). However, ubiquitinated TRAF6 disappeared after RANKL withdrawal in WT cells, but it persisted in _Itch^-/-_ cells, suggesting dysregulation of TRAF6 deubiquitination in the absence of Itch (Fig. 4C).

**Itch Increases the Interaction of Deubiquitinating Enzyme, Cylindromatosis, and TRAF6**—Deubiquitination is carried out by deubiquitinating enzymes including CYLD (31). In osteoclasts, CYLD promotes TRAF6 deubiquitination and negatively regulates osteoclast formation (12). _Itch_ does not have deubiquitinating activity (32). To determine whether _Itch_ promotes binding of CYLD to TRAF6 to explain the reduced TRAF6 deubiquitination in _Itch_-deficient cells, we overexpressed FLAG-Itch and HA-CYLD in 293T cells and performed Western blotting and immunoprecipitation (IP) assays in whole cell lysates using anti-HA antibody for CYLD and anti-FLAG to detect _Itch_. A weak _Itch_ band was detected in the IP complex by the IgG control, whereas a much stronger _Itch_ band was observed in the IP complex by anti-HA (CYLD) (Fig. 5A). To determine whether _Itch_ binds to CYLD in osteoclasts, WT OCPs were treated with PBS or RANKL and subjected to immunoprecipitation using an anti-CYLD antibody. In the absence of RANKL, a weak _Itch_ band was detected that was enhanced by RANKL treatment (Fig. 5B). To test whether _Itch_ could facilitate CYLD binding to TRAF6, we immunoprecipitated TRAF6 with anti-TRAF6 antibody in OCPs from WT and _Itch^-/-_ mice in the presence and absence of RANKL for 48 h. RANKL increased the binding of CYLD to TRAF6 in WT OCPs, but this binding was attenuated in _Itch^-/-_ cells. RANKL-increased expression of CYLD was not changed in _Itch^-/-_ cells (Fig. 5C).

**_Itch^-/-_ Mice Have Increased Osteoclast Formation in Response to LPS Stimulation**—Data from the above _in vitro_ studies suggest that _Itch^-/-_ mice may have an osteoporotic phenotype due to increased osteoclast formation. However, when we examined bones from 3- and 6-month-old _Itch^-/-_ mice using micro-computed tomography and histology, we found no difference in bone volumes or other bone mass parameters between _Itch^-/-_ mice and WT littermates (see Fig. 6A for data from 3-month-old mice; data from 6-month-old mice are not shown). Biomechanical testing did not detect any differences in bone strength between _Itch^-/-_ mice and WT littermates (data not shown). TRAP-stained femoral sections showed that _Itch^-/-_ mice have 30% higher osteoclast numbers than WT littermates (Fig. 6B). To examine whether _Itch_ mediates increased osteoclast formation under pathological conditions such as periodontal disease, we injected LPS into the loose subcutaneous tissues overlying the calvaria of WT and _Itch^-/-_ mice. No significant difference was observed between osteoclast numbers in WT and _Itch^-/-_ mice in response to PBS. However, LPS injection induced significantly more osteoclasts and a greater eroded surface in _Itch^-/-_ mice than in WT mice (Fig. 6C). The osteoclast formation with LPS (100 ng/ml) in the absence of _Itch_ expression _in vitro_ was also examined. The number of TRAP^+_ osteoclasts was increased by more than 6-fold in cells from _Itch^-/-_ mice compared with WT cells (Fig. 6D).

**DISCUSSION**

In the present study, we demonstrated that the ubiquitin E3 ligase _Itch_ inhibits RANKL-induced osteoclast formation. Using osteoclast precursors from _Itch^-/-_ mice, we identified two molecular mechanisms mediating the inhibitory effect of _Itch_ on osteoclastogenesis. _Itch_ limits TRAF6 deubiquitination by binding the deubiquitinating enzyme CYLD. In the absence of _Itch_, RANKL-induced NF-κB activation is prolonged. _Itch_ transcription is directly regulated by RANKL during osteoclast differentiation via NF-κB. Furthermore, LPS-induced osteoclastic bone resorption is significantly increased in _Itch^-/-_ mice, indicating that _Itch_ negatively regulates osteoclast formation _in vivo_ under pathological conditions such as periodontal disease and bacterial infection-induced bone loss.

Similar to ubiquitination, deubiquitination is another important post-translational mechanism to regulate protein stability and thereby cell function. Protein deubiquitination is carried...
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FIGURE 4. Itch−/− osteoclast precursors have prolonged TRAF6 ubiquitination. OCPs from Itch−/− mice and WT littermates were used. A, cells were treated with RANKL for 2 days, and whole cell lysates (WCL) were subjected to IP with anti-TRAF6 antibody and blotted with anti-Itch antibody. B, cells were treated with RANKL for 2 days, and then whole cell lysates were incubated with UbiQapture-Q matrix to pull down ubiquitinated proteins. Ubiquitinated (Ub) proteins were blotted with anti-TRAF6 antibody. C, cells were treated with RANKL for 2 days and then cultured for an additional 2 days without RANKL. Whole cell lysates were incubated with UbiQapture-Q matrix to pull down ubiquitinated proteins. Ubiquitinated proteins were blotted with anti-TRAF6 antibody.

FIGURE 5. Itch Interacts with the deubiquitinating enzyme CYLD. A, 293T cells were co-transfected with FLAG-Itch and HA-CYLD expression vectors. Whole cell lysates (WCL) were subjected to IP with anti-HA antibody and blotted with anti-FLAG antibody. B, OCPs were treated with RANKL, and whole cell lysates were subjected to IP with anti-CYLD antibody and blotted with anti-Itch antibody. C, OCPs from Itch−/− mice and WT littermates were treated with RANKL, and whole cell lysates were subjected to IP with anti-TRAF6 antibody and blotted with anti-CYLD antibody. IB, immunoblot.

Deubiquitination of the Lys63-linked ubiquitination of the adaptor protein receptor-interacting protein and switching receptor-interacting protein ubiquitination toward Lys48 linkage, leading to its proteasomal degradation (6). A20 also deubiquitinates TRAF6 in liver cells (34) and terminates Toll-like receptor-mediated responses (13). A20 mice have premature death due to severe inflammation and cachexia (35). Silencing A20 increases TRAF6 protein and NF-κB activity in osteoclasts in the presence of LPS (36). Whether this is due to alteration of TRAF6 deubiquitination is unknown. Given the known interaction of Itch and A20 in other cell types, it is likely that Itch may facilitate the inhibitory effect of A20 on osteoclast formation.

Our study indicates that, apart from the interaction with A20, Itch binds to CYLD, the first DUB shown to deubiquitinate TRAF6 in osteoclasts (12). This Itch/CYLD interaction promotes CYLD-mediated TRAF6 deubiquitination in osteoclasts. p62 protein enhances CYLD binding to TRAF6, thereby facilitating its deubiquitination (12). It is not known whether Itch interacts with CYLD-p62-TRAF6. However, the findings that Itch−/− mice develop autoimmune disease and have enhanced osteoclast formation in response to LPS suggest that Itch may play an indispensable role in limiting inflammation and osteoclastogenesis in vivo.

We found that adult Itch−/− mice have normal bone volumes, similar to p62−/− mice (37). Thus, basal osteoclastogenesis is not affected by the loss of p62 or Itch. Interestingly, both Itch−/− and Cyld−/− mice develop multiorgan inflammation (6, 38), but they have different bone phenotypes. Cyld−/− mice have osteoporosis at 8 weeks of age (12), whereas 3–6-month-old Itch−/− mice have normal bone volume. This difference in bone volume may be due to different effects of Itch and CYLD on osteoblasts. Osteoblast differentiation is similar in WT and Cyld−/− mice (12). We found that Itch−/− mice have increased osteoblast numbers and bone formation rate. The bone marrow stromal cells from Itch−/− mice have increased osteoblast differentiation but a normal percentage of mesenchymal stem cells. At the molecular level, Itch promotes degradation of JunB protein, a transcription factor in osteoblasts (39). Increased osteoblast differentiation of Itch−/− cells may also be related to the known function of Itch-mediated degradation of bone mor-

out by DUBs, a group of proteases carrying DUB domains. Genomic and functional scans of the human genome found 95 DUBs including A20 and CYLD. Itch does not contain a DUB domain, suggesting that Itch itself does not have DUB activity (32). However, Itch interacts with A20 (6) and CYLD (this study), indicating that it may affect protein deubiquitination through these DUBs. A recent bioinformatics study indicates that a given DUB interacts with multiple adaptor proteins (33), highlighting the complexity of the deubiquitination process in regulating cellular functions.

In T cells and mouse embryonic fibroblasts, Itch negatively regulates TNF-induced NF-κB activation by facilitating A20 
phogenetic protein (40) and TGFβ signaling proteins (41). Consequently, increased osteoblastic bone formation compensates for increased osteoclastic bone resorption in Itch+/− mice. What is the potential biological relevance of Itch in bone? First, modification of Itch levels specifically in osteoclasts or osteoblasts may be useful to treat bone diseases such as osteoporosis. Second, local Itch inhibition may be helpful for the bone repair process because it creates a high bone turnover microenvironment by increasing the function of both osteoclasts and osteoblasts. Finally, Itch−/− mice may provide a new model for investigating the involvement of the deubiquitination process in bone cell regulation. Furthermore, because the Itch−/− mice have autoimmune disease and multiorgan inflammation, cellspecific depletion of Itch using the Cre/LoxP system will be required to delineate the functions of Itch in osteoclasts and osteoblasts in vivo.

RANKL increases Itch expression in OCPs within 24 h, consistent with a direct effect on transcription. Interestingly, other proteins that play a role in controlling TRAF6 deubiquitination including CYLD (12), A20 (36), and p62 (42) are also up-regulated by RANKL in osteoclasts. The A20 promoter contains functional NF-κB binding sites (43). We found NF-κB binding sites at the Itch promoter. Similarly, the Cyld and p62 promoters also contain NF-κB binding sites (−3812/−3804 bp in Cyld and −2882/−2874 and −2416/−2408 bp in p62). These findings suggest that RANKL may up-regulate the expression of genes that encode DUBs and adaptor proteins through NF-κB to terminate NF-κB activation. Thus, deubiquitination of

FIGURE 6. Increased LPS-induced osteoclastogenesis in Itch−/− mice. A, femoral bones from 3-month-old Itch−/− mice and WT littermates were subjected to micro-computed tomography analysis. The bone volumes and other parameters were measured. Values are mean ± S.D. (error bars) of four mice per group. B, TRAP staining for femoral bones from 3-month-old Itch−/− mice and WT littermates. The number of osteoclasts along the trabecular bone surface was measured. Values are mean ± S.D. (error bars) of four mice per group. C, LPS or PBS was injected over the calvarial bones of Itch−/− mice and WT littermates. Calvarial bones were harvested for histological analysis. Paraffin-embedded sections were stained for TRAP activity. The number of TRAP-positive osteoclasts and eroded surface were measured. The values are mean ± S.D. (error bars) of four mice per group. *, p < 0.05 versus LPS-injected WT mice. D, 10⁵ bone marrow cells/well in a 96-well plate were cultured with LPS and M-CSF for 10 days. TRAP-positive cells were counted. Values are mean ± S.D. (error bars) of 4 wells.

Oc, osteoclast; BV, bone volume; TV, total volume; B.S., bone surface; B.Pm., bone perimeter; E.S., eroded surface; OCs, osteoclasts.
Ubiquitination activates the osteoclastogenic genes and genes encoding DUB components such as ubiquitin. Itch binds to TRAF6 and CYLD, leading to TRAF6 deubiquitination and resulting in termination of RANKL-induced TRAF6/CYLD signaling to influence inflammatory signaling pathways. Cytoplasm.

TRAF6 is a negative feedback mechanism to limit RANKL-mediated osteoclast formation (Fig. 7).

Briefly, Itch−/− mice have increased osteoclast formation due to continuous activation of NF-κB signaling. Itch promotes TRAF6 deubiquitination by recruiting CYLD to the TRAF6 signal transduction complex. Itch expression is up-regulated by RANKL, which is involved in NF-κB signaling. Thus, Itch is a new RANKL target gene that is induced during osteoclastogenesis and functions as a deubiquitinating adaptor molecule to limit RANKL-induced osteoclast formation via the deubiquitinating enzyme CYLD.

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REFERENCES

1. Fang, D., Elly, C., Gao, B., Fang, N., Altman, Y., Joazeiro, C., Hunter, T., Copeland, N., Jenkins, N., and Liu, Y. C. (2002) Dysregulation of T lymphocyte function in itchy mice: a role for Itch in TH2 differentiation. Nat. Immunol. 3, 281–287

2. Vinuesa, C. G., Cook, M. C., Angelucci, C., Athanassopoulos, V., Rui, L., Hill, K. M., Yu, D., Domaschenz, H., Whittle, B., Lambe, T., Roberts, I. S., Copley, R. R., Bell, I. L., Cornell, R. J., and Goodnow, C. C. (2005) A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmune inactivity. Nature 435, 452–458

3. Melino, G., Gallagher, E., Asqelan, R. I., Knight, R., Peterson, A., Rossi, M., Scialpi, F., Malatesta, M., Zocchi, L., Browne, G., Ciechanover, A., and Bernassola, F. (2008) Itch: a HECT-type E3 ligase regulating immunity, skin and cancer. Cell Death Differ. 15, 1103–1112

4. Perry, W. L., Hustad, C. M., Swing, D. A., O’Sullivan, T. N., Jenkins, N. A., and Copeland, N. G. (1998) The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. Nat. Genet. 18, 143–146

5. Lohr, N. J., Molleston, J. P., Strauss, K. A., Torres-Martinez, W., Sherman, E. A., Squires, R. H., Rider, N. L., Chikwava, K. R., Cummings, C. W., Morton, D. H., and Puffenberger, E. G. (2010) Human ITCH E3 ubiquitin ligase deficiency causes syndromic multisystem autoimmune disease. Am. J. Hum. Genet. 86, 447–453

6. Shembade, N., Harhaj, N. S., Parvatiyar, K., Copeland, N. G., Jenkins, N. A., Matesic, L. E., and Harhaj, E. W. (2008) The E3 ligase Itch negatively regulates inflammatory signaling pathways by controlling the function of the ubiquitin-editing enzyme A20. Nat. Immunol. 9, 254–262

7. Tao, M., Scacheri, P. C., Marinis, J. M., Harhaj, E. W., Matesic, L. E., and Abbott, D. W. (2009) ITCH K63-ubiquitinates the NOD2 binding protein, RIP2, to influence inflammatory signaling pathways. Cell. Biol. 19, 1255–1263

8. Shembade, N., Parvatiyar, K., Harhaj, N. S., and Harhaj, E. W. (2009) The ubiquitin-editing enzyme A20 requires RNF11 to downregulate NF-κB signalling. EMBO J. 28, 513–522

9. Chen, Z. J. (2005) Ubiquitin signalling in the NF-κB pathway. Nat. Cell Biol. 7, 759–765

10. Skaug, B., Jiang, X., and Chen, Z. J. (2009) The role of ubiquitin in NF-κB regulatory pathways. Annu. Rev. Biochem. 78, 769–796

11. Ballaert, A., Heyninck, K., Janssens, S., and Beyaert, R. (2006) Ubiquitin: tool and target for intracellular NF-κB inhibitors. Trends Immunol. 27, 533–540

12. Jia, W., Chang, M., Paul, E. M., Babu, G., Lee, A. J., Reiley, W., Wright, A., Zhang, M., You, J., and Sun, S. C. (2008) Deubiquitinating enzyme CYLD negatively regulates RANK signaling and osteoclastogenesis in mice. J. Clin. Investig. 118, 1858–1866

13. Boone, D. L., Turer, E. E., Lee, E. G., Ahmad, R. C., Wheeler, M. T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hittosumatsu, O., McNally, E., Pickard, C., and Ma, A. (2004) The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat. Immunol. 5, 1052–1060

14. Chiaram, C. Y., Kyritsis, G., Graves, D. T., and Amar, S. (1999) Interleukin-1 and tumor necrosis factor activities partially account for calvarial bone resorption induced by local injection of lipopolysaccharide. Infect. Immun. 67, 4231–4236

15. Yamashita, T., Yao, Z., Li, F., Zhang, Q., Badell, I. R., Schwarz, E. M., Takeshita, S., Wagner, E. F., Noda, M., Matsu, K., Xing, L., and Boyle, B. F. (2007) NF-κB p50 and p52 regulate receptor activator of NF-κB ligand (RANKL) and tumor necrosis factor-induced osteoclast precursor differentiation by activating c-Fos and NFATc1. J. Biol. Chem. 282, 18245–18253

16. Takeshita, S., Kaji, K., and Kudo, A. (2000) Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J. Bone Miner. Res. 15, 1477–1488

17. Hughes, D. E., Dai, A., Tiffee, J. C., Li, H. H., Mundy, G. R., and Boyce, B. F. (1996) Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-β. Nat. Med. 2, 1132–1136

18. Yao, Z., Li, P., Zhang, Q., Schwarz, E. M., Peng, P., Arbini, A., Boyle, B. F., and Xing, L. (2006) Tumor necrosis factor-α increases circulating osteoclast precursor numbers by promoting their proliferation and differentiation in the bone marrow through up-regulation of c-Fms expression. J. Biol. Chem. 281, 11846–11855

19. Guo, R., Yamashita, M., Zhang, Q., Zhou, Q., Chen, D., Reynolds, D. G., Awad, H. A., Yanosso, L., Zhao, L., Schwarz, E. M., Zhang, Y. E., Boyle, B. F., and Xing, L. (2008) Ubiquitin ligase Smurf1 mediates tumor necrosis factor-α induced systemic bone loss by promoting proinflammatory activation of bone morphogenetic signaling proteins. J. Biol. Chem. 283, 23084–23092

20. Zhang, H. W., Ding, J., Jin, J. L., Guo, J., Liu, J. N., Karaplis, A., Goltzman, D., and Miao, D. (2010) Defects in mesenchymal stem cell self-renewal and cell fate determination lead to an osteopenic phenotype in Bmi-1 null mice. J. Bone Miner. Res. 25, 640–652

21. Darzykowski, Z., Roederer, M., and Tanke, H. J. (eds) (2004) Cytometry, 4th Ed., Vol. 75, Academic Press, New York

22. Xing, L., Carlson, L., Story, B., Tai, Z., Peng, P., Siebenlist, U., and Boyle, B. F. (2003) Expression of either NF-κB p50 or p52 in osteoclast precursors is required for IL-1-induced bone resorption. J. Bone Miner. Res. 18, 260–269
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23. Arai, F., Miyamoto, T., Ohneda, O., Inada, T., Sudo, T., Brasel, K., Miyata, T., Anderson, D. M., and Suda, T. (1999) Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor κB (RANK) receptors. J. Exp. Med. 190, 1741–1754

24. Jacone-Galarza, C. E., Lee, S. K., Lorenzo, J. A., and Aguila, H. L. (2013) Identification, characterization, and isolation of a common progenitor for osteoclasts, macrophages, and dendritic cells from murine bone marrow and periphery. J. Bone Miner. Res. 28, 1203–1213

25. Li, P., Schwarz, E. M., O’Keefe, R. J., Ma, L., Looney, R. J., Ritchlin, C. T., Boyce, B. F., and Xing, L. (2010) Functions of nuclear factor κB in bone. Annu. N.Y. Acad. Sci. 1192, 367–375

26. Yao, Z., Xing, L., Qin, C., Schwarz, E. M., and Boyce, B. F. (2008) Osteoclast precursor interaction with bone matrix induces osteoclast formation directly by an interleukin-1–mediated autocrine mechanism. J. Biol. Chem. 283, 9917–9924

27. Boyce, B. F., Yao, Z., and Xing, L. (2010) Functions of nuclear factor κB in bone. J. Biol. Chem. 285, 37641–37649

28. Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Lamothe, B., Webster, W. K., Gopinathan, A., Besse, A., Campos, A. D., Sowa, M. E., Bennett, E. J., Gygi, S. P., and Harper, J. W. (2009) Defining the signaling cross-talk between RANKL and IFN-γ. Nature 408, 600–605

29. Wilkinson, K. D. (2000) Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. Semin. Cell Dev. Biol. 11, 141–148

30. Katz, E. J., Isasa, M., and Crosas, B. (2010) A new map to understand deubiquitination. Biochem. Soc. Trans. 38, 21–28

31. Sowa, M. E., Bennett, E. J., Gygi, S. P., and Harper, J. W. (2009) Defining the human deubiquitinating enzyme interaction landscape. Cell 138, 389–403

32. Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) A genomic and functional inventory of deubiquitinating enzymes. Cell 123, 773–786

33. Raysor, W. W., Jin, W., Lee, A. J., Wright, A., Wu, X., Tewalt, E. F., Leonard, J. O., Norbury, C. C., Fitzpatrick, L., Zhang, M., and Sun, S. C. (2007) Deubiquitinating enzyme CYLD negatively regulates the ubiquitin-dependent kinase Taki1 and prevents abnormal T cell responses. J. Exp. Med. 204, 1475–1485

34. Jensen, L. E., and Whitehead, A. S. (2003) Ubiquitin activated tumor necrosis factor receptor associated factor-6 (TRAF6) is recycled via deubiquitination. FEBS Lett. 553, 190–194

35. Lee, E. G., Boone, D. L., Chai, S., Libby, S. L., Chien, M., Lodolce, J. P., and Ma, A. (2000) Failure to regulate TNF-induced NF-κB and cell death responses in A2O-deficient mice. Science 289, 2350–2354

36. Mabilleau, G., Chappard, D., and Sabokbar, A. (2011) Role of the A20–TRAF6 axis in lipopolysaccharide-mediated osteoclastogenesis. J. Biol. Chem. 286, 3242–3249

37. Durán, A., Serrano, M., Leitges, M., Flores, J. M., Picard, S., Brown, J. P., Moscat, J., and Diaz-Meco, M. T. (2004) The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. Dev. Cell 6, 303–309

38. Reiley, W. W., Jin, W., Lee, A. J., Wright, A., Wu, X., Tewalt, E. F., Leonard, T. O., Norbury, C. C., Fitzpatrick, L., Zhang, M., and Sun, S. C. (2007) Deubiquitinating enzyme CYLD negatively regulates the ubiquitin-dependent kinase Taki1 and prevents abnormal T cell responses. J. Exp. Med. 204, 1475–1485

39. Hengwei, Z., and Lianping, X. (2013) Ubiquitin E3 ligase Itch negatively regulates osteoblast differentiation from mesenchymal progenitor cells. Stem Cells, in press

40. Durrington, H. J., Upton, P. D., Hoer, S., Boname, J., Dunmore, B. J., Yang, J., Crilley, T. K., Butler, L. M., Blackbourn, D. J., Nash, G. B., Lehner, P. J., and Morrell, N. W. (2010) Identification of a lysosomal pathway regulating degradation of the bone morphogenetic protein receptor type II. J. Biol. Chem. 285, 37641–37649

41. Bai, Y., Yang, C., Hu, K., Elly, C., and Liu, Y. C. (2004) Itch E3 ligase Itch negatively regulates osteoblast differentiation from mesenchymal progenitor cells. Stem Cells, in press

42. Kurihara, N., Hiruma, Y., Zhou, H., Subler, M. A., Dempster, D. W., Singer, F. R., Reddy, S. V., Gruber, H. E., Windle, J. J., and Roodman, G. D. (2007) Mutation of the sequestosome 1 (p62) gene increases osteoclastogenesis but does not induce Paget disease. J. Clin. Investig. 117, 133–142

43. Laherty, C. D., Perkins, N. D., and Dixit, V. M. (1993) Human T cell leukemia virus type 1 Tax and phorbol 12-myristate 13-acetate induce expression of the A20 zinc finger protein by distinct mechanisms involving nuclear factor κB. J. Biol. Chem. 268, 5032–5039