Tumor Necrosis Factor Receptor-associated Factor 6 Is an Intranuclear Transcriptional Coactivator in Osteoclasts*

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Tumor necrosis factor receptor-associated factor 6 (TRAF6) associates with the cytoplasmic domain of receptor activator of NF-κB (RANK) and is an essential component of the signaling complex mediating osteoclastogenesis. However, the osteoclastic activity of TRAF6 is blunted by its association with four and half LIM domain 2 (FHL2), which functions as an adaptor protein in the cytoplasm and transcriptional regulator in the nucleus. We find that TRAF6 also localizes in the nuclei of osteoclasts but not their bone marrow macrophage precursors and that osteoclast intranuclear abundance is specifically increased by RANK ligand (RANKL). TRAF6 nuclear localization requires FHL2 and is diminished in fhl2−/− osteoclasts. Suggesting transcriptional activity, TRAF6 interacts with the transcription factor RUNX1 in the osteoclast nucleus. FHL2 also associates with RUNX1 but does so only in the presence of TRAF6. Importantly, TRAF6 recognizes FHL2 and RUNX1 in osteoclast nuclei, and the three molecules form a DNA-binding complex that recognizes and transactivates the RUNX1 response element in the fhl2 promoter. Finally, TRAF6 and its proximal activator, RANKL, polyubiquitinate FHL2, prompting its proteasomal degradation. These observations suggest a feedback mechanism whereby TRAF6 negatively regulates osteoclast formation by intracytoplasmic sequestration of FHL2 to blunt RANK activation and as a component of a transcription complex promoting FHL2 expression.

Osteoclasts are the exclusive resorptive cells of bone, and their derivation from monocyte/macrophage precursors occurs upon interaction of receptor activator of NK-κB ligand (RANKL)³ with its receptor, RANK (1). Occupied RANK initiates a series of signals that prompt the osteoclast phenotype and activate the mature osteoclast, principally by promoting organization of its cytoskeleton (2).

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The abbreviations used are: RANKL, receptor activator of NF-κB ligand; BMM, bone marrow macrophage; FHL2, four and half LIM domain 2; HA, hemagglutinin; IL, interleukin; mAb, monoclonal antibody; M-CSF, macrophage colony-stimulating factor; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; WT, wild type.

RANK is a member of the tumor necrosis factor (TNF) receptor superfamily, and as such, its cytoplasmic domain binds a number of TNF receptor-associated factors (TRAFs). In this regard, TRAF6 is perhaps the most important RANK-associated molecule regulating osteoclast formation and function. Accordingly, traf6−/− mice are severely osteopetrotic (3, 4). Although their ultimate consequences are often transcriptional, the osteoclast-regulating signals directly mediated by TRAF6, identified to date, occur in the cytoplasm. These signals include activation of NF-κB by a process of ubiquitination in which TRAF6 functions as an E3 ligase (5).

Four and half LIM domain 2 (FHL2) is a LIM-domain-only protein, not present in bone marrow macrophages (BMMs) but induced by RANKL, which binds TRAF6, thereby inhibiting its interaction with RANK (6). In consequence, FHL2 blunts osteoclast formation and function. Committed osteoclast precursors lacking the protein exhibit enhanced NF-κB activation, specifically in response to RANKL, and FHL2 overexpression exerts the opposite effect. Consequently, fhl2−/− BMMs undergo accelerated generation of osteoclasts and stimulation of the polykaryon’s resorptive capacity, in vitro. Moreover, mice lacking FHL2 exhibit enhanced bone destruction in response to inflammatory arthritis. These observations suggest that FHL2 inhibits osteoclast formation and function by restricting TRAF6-RANK interaction.

However, FHL2 is also a transcriptional corepressor and coactivator. In other cells, the protein shuttles between the cytoplasm and nucleus where it functions in a promoter-specific manner (7). The fact that TRAF6 associates with FHL2 raised the possibility that it also translocates to the nucleus and participates in transcriptional events. We find that TRAF6 localizes in the osteoclast nucleus where its abundance is enhanced by FHL2. Moreover, nuclear TRAF6 and FHL2 associate with the transcription factor, RUNX1. This TRAF6:FHL2:RUNX1 complex recognizes and transactivates the fhl2 promoter and therefore represents a DNA-binding moiety with the capacity to promote fhl2 expression and serve as a negative feedback mechanism counteracting the proosteoclastic effects of RANK signaling.

EXPERIMENTAL PROCEDURES

Plasmids and Reagent—RUNX1 cDNA was a generous gift from Dr. G. David Roodman (University of Pittsburgh School of Medicine). Recombinant murine macrophage colony-stimulating factor (M-CSF) was from R&D Systems (Minneapolis, MN). Murine glutathione S-transferase-RANKL was expressed as described (8). Anti-hemagglutinin (HA) antibody was obtained from Covance Research Products (Princeton, NJ). Anti-TRAFA6
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antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human FHL2 promoter and anti-FHL2 antibody were provided generously by Dr. Roland Schule (Frauenklinik und Zentrum für Klinische Forschung, Klinikum der Universität Freiburg, Freiburg, Germany). Anti-FLAG antibody was obtained from Sigma-Aldrich. Anti-RUNX1 antibody was provided generously by Dr. Roland Schule (Universität Freiburg, Freiburg, Germany). Anti-FLAG antibody was obtained from Santa Cruz Biotechnology, Inc. Anti-TRAF6, and polyclonal anti-TRAF6 (Santa Cruz Biotechnology, Inc.).

Primary Cells and Cell Lines—Macrophages/osteoclast precursors and osteoclasts were generated from bone marrow precursors as described (9). COS-7 and 293T cells were obtained from ATCC. 293T cells were utilized for coimmunoprecipitation experiments and COS-7 cells for those involving nuclear protein assessment.

Immunoprecipitation and Western Blotting—FLAG-tagged full-length and truncated TRAF6, FLAG-tagged fhl2, and HA-tagged RUNX1 cDNAs were subcloned into the mammalian expression vector pcDNA3 (Invitrogen). 293T cells were transfected with expression constructs using FuGENE 6 as the transfection reagent according to the manufacturer’s instructions (Roche Applied Science). Cells were lysed 48 h after transfection, and lysates were immunoprecipitated and immunoblotted with anti-FLAG or anti-HA antiserum. Anti-TRAF6, anti-FHL2, and anti-RUNX1 antibodies were used to detect endogenous protein-protein interaction and for electrophoretic mobility shift assay (EMSA).

Preparation of Nuclear-enriched Protein Fractions—Cells were washed and centrifuged in phosphate-buffered saline (PBS). The pellet was resuspended in 200 μl of hypotonic buffer (10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiobitol, Roche protease inhibitor mixture 1:25, 1 mM NaF, 1 mM Na3VO4). After the addition of 1% Nonidet P-40 (10 μl), lysates were vortexed for 30 s and centrifuged for 5 min at 3,000 rpm. Supernatant was reserved as cytosol. The pellet was washed once in hypotonic buffer and resuspended in nuclear extraction buffer (20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM/ml aprotinin, 10 mM NaF, and 1 mM dithiobitol). Then the nuclear extracts were shaken vigorously at 4 °C for 30 min and centrifuged for 20 min at 14,000 rpm. The supernatant represented nuclear-enriched fraction as determined by nucleophosmin content.

Immunofluorescence—Intracellular TRAF6 and FHL2 were labeled with fluorescent markers as described (6). In brief, cells were fixed with 3% paraformaldehyde in PBS for 20 min. Free aldehyde groups were quenched with 50 mM NH4Cl in PBS for 10 min. Nonspecific binding was blocked by incubation in PBS containing 0.2% bovine serum albumin and 0.05% saponin (PBSBS) for 15 min. The cells were then incubated with primary antibodies in PBSBS for 1 h. Primary antibody binding was visualized using fluorescent dye-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBSBS for 45 min. Immunofluorescence analysis was performed using a Nikon fluorescence microscope equipped with a CCD camera. Primary antibodies used include monoclonal anti-FHL2, and polyclonal anti-TRAF6 (Santa Cruz Biotechnology, Inc.).

EMSA—DNA oligonucleotides were labeled with biotin at the 5’-ends during synthesis, annealed, and purified as described (10). 3 μg of nuclear extract was mixed with EMSA binding buffer, 5 mM MgCl2, 25% glycerol, 50 ng/μl poly(dI-dC), 0.05% Nonidet P-40, 0.2 mM AML-1 binding site to a final volume of 20 μl and kept at room temperature for 20 min. The mixture was then subjected to 6% polyacrylamide gel electrophoresis. Anti-FLAG and anti-HA antibodies were added with the mixture to detect the components of the protein complex. Biotin complex signals were developed with the Pierce kit following the manufacturer’s instruction. Oligonucleotides used were: 5’-biotin-ATTCTTTAGGTCACTGTGGTGGTTA-TTTCTGTAAGAAG-3’ and 5’-biotin-CTTCTTTCGCAATA-TGAACCAACACAGATGACATATAAAGAT-3’.

Luciferase Assay—293T cells, cultured in 24-well plates were transfected with FHL2 promoter luciferase plasmid, pTK-Renilla luciferase plasmid, pcDNA3-FLAG-Traf6, pcDNA3-HA-FHL2, and pcDNA3-AML-1. Total plasmid amount was balanced with pcDNA3 as needed. 48 h after transfection, cells were lysed in 100 μl of lysis buffer (Promega, Madison, WI), and firefly and Renilla luciferase activity was determined using substrates from Promega and a luminometer.

RESULTS

TRAF6 Localizes in the Osteoclast Nucleus—TRAF6 resides in the cytoplasm where it associates with a number of transmembrane receptors. However, it also binds FHL2, which locates in both the cytoplasm and nucleus, suggesting that TRAF6 may also enjoy a nuclear distribution (6). To determine whether TRAF6 localizes in nuclei of osteoclasts and/or their precursors, we cytokine-starved naïve BMMs and those induced to undergo osteoclastogenesis by exposure to M-CSF and RANKL for 5 days. 3 h later, the cells were maintained in absence of cytokine or exposed to optimal concentrations of interleukin (IL)-1 (10 ng/ml) or RANKL (100 ng/ml) for 30 min (8, 11). Nuclear proteins were extracted and immunoblotted for TRAF6 (Fig. 1A). Although TRAF6 is undetectable in the nuclei of BMMs, regardless of cytokine treatment, it is constitutively present in those of osteoclasts. Furthermore, short exposure to RANKL, but not IL-1, enhances intranuclear TRAF6 in the mature bone-resorptive cells.

FHL2 Enhances TRAF6 Nuclear Translocation—despite its RANKL-stimulated intranuclear translocation, TRAF6 does not contain an apparent nuclear localization sequence. Furthermore, although TRAF6 is expressed in BMMs and osteoclasts, it is resident in the nucleus only in the mature resorptive polykaryon (Fig. 1A). These findings suggest that TRAF6 utilizes an adaptor protein expressed in osteoclasts, but not BMMs, to transit into the nucleus. Given that FHL2, which interacts with TRAF6, is present in osteoclast cytosol and nucleus and is not expressed by BMMs, we reasoned FHL2 may also enjoy a nuclear distribution (6). To determine whether TRAF6 localizes in nuclei of osteoclasts and/or their precursors, we cytokine-starved naïve BMMs and those induced to undergo osteoclastogenesis by exposure to M-CSF and RANKL for 5 days. 3 h later, the cells were maintained in absence of cytokine or exposed to optimal concentrations of interleukin (IL)-1 (10 ng/ml) or RANKL (100 ng/ml) for 30 min (8, 11). Nuclear proteins were extracted and immunoblotted for TRAF6 (Fig. 1A). Although TRAF6 is undetectable in the nuclei of BMMs, regardless of cytokine treatment, it is constitutively present in those of osteoclasts. Furthermore, short exposure to RANKL, but not IL-1, enhances intranuclear TRAF6 in the mature bone-resorptive cells.

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and second lanes), but a substantial amount translocates to the nucleus in its presence (Fig. 1B, third and fourth lanes). As expected, FHL2 is located in both compartments. To determine whether FHL2 shuttles TRAF6 into the osteoclast nucleus, we treated wild-type (WT) or FHL2<sup>-/-</sup>/fhl2<sup>-/-</sup>BMMs with M-CSF and RANKL for 5 days. Immunoblot analysis revealed that nuclear TRAF6 is decreased in osteoclasts lacking FHL2 (Fig. 1C). Once again, nuclear FHL2 is present in WT cells. Finally, we immunostained osteoclasts in culture with anti-TRAF6 and anti-FHL2 antibodies and observed both moieties colocalizing in the nucleus (Fig. 1D).

TRAF6 Interacts with RUNX1—FHL2 binds TRAF6 and inhibits its association with RANK (6). FHL2 is also induced as BMMs assume the osteoclast phenotype, suggesting its regulated expression may represent a negative feedback of RANKL-mediated osteoclast differentiation and function (6).

RUNX1 is a transcription factor implicated in the osteolysis attending multiple myeloma (12). Importantly, the fhl2 promoter contains three RUNX1 recognition sites (Fig. 2A). Because TRAF6 translocates to the nucleus but contains no DNA binding domain, we postulated that it interacts with transcription factors, such as RUNX1, to regulate fhl2 expression. Hence, 293T cells were transiently transfected with combinations of traf6-FLAG and RUNX1 to determine whether they associate intracellularly. The transfected lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted for RUNX1. As shown in Fig. 2B, RUNX1 and traf6 coimmunoprecipitate.

To determine whether the RUNX1-TRAF6 complex recognizes the FHL2 promoter, we performed EMSA. COS-7 cells were transfected with traf6-FLAG and RUNX1-HA. Nuclear protein was extracted and incubated with biotin-labeled fhl2 promoter and antibodies at room temperature for 30 min. Nuclear protein-DNA complexes were analyzed by native gel electrophoresis.

FIGURE 1. FHL2 promotes TRAF6 nuclear localization. A, naïve BMMs or those differentiated into osteoclasts by 5 days exposure to M-CSF and RANKL were starved for 3 h and maintained cytokine-free or stimulated with IL-1 (10 ng/ml) or RANKL (100 ng/ml) for 30 min. Nuclear protein was extracted and TRAF6 immunoblotted. Nucleophosmin serves as nuclear marker and loading control. B, traf6-FLAG with or without fhl2-HA was transfected into COS-7 cells. Cytoplasm- and nuclear-enriched fractions were separated. TRAF6 and FHL2 were immunoprecipitated with anti-FLAG and anti-HA mAbs, respectively. Nucleophosmin serves as marker of nuclear enrichment. C, WT and fhl2<sup>-/-</sup>(KO) osteoclasts were generated by treating BMMs with RANKL and M-CSF for 5 days. Nuclear protein was extracted and its TRAF6 content determined by immunoblotting. Nucleophosmin serves as nuclear marker and loading control. D, osteoclasts generated from WT BMMs were fixed and subjected to fluorescence microscopy using an anti-TRAF6 rabbit polyclonal antibody and FITC-labeled secondary antibody (green reaction product) or anti-FHL2 mAb and TRITC-labeled secondary antibody (red reaction product). FHL2-TRAF6 colocalization was documented by simultaneous exposure yielding the yellow reaction product. Arrows indicate nuclei.

FIGURE 2. TRAF6 associates with RUNX1. A, fhl2 promoter detailing RUNX1 recognition sites. B, traf6-FLAG and RUNX1 plasmids were transiently transfected into 293T cells. Nuclear protein was extracted and immunoprecipitated (IP) with anti-FLAG and immunoblotted (IB) with anti-RUNX1 mAbs. TCL represents total cell lysate. C, traf6-FLAG and RUNX1-HA plasmids were transfected into 293T cells. Nuclear enriched protein was extracted 48 h later and mixed with biotin-labeled fhl2 promoter RUNX1-binding element and antibodies at room temperature for 30 min. Polycrylamide gel electrophoresis was performed, and biotin complex signals were developed. D, nuclear-enriched protein (NEP), extracted from osteoclasts generated from BMMs by M-CSF and RANKL, was immunoprecipitated with anti-TRAF6 mAb and the immunoprecipitate immunoblotted for RUNX1 and TRAF6.
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TRAF6 enhances RUNX1-mediated fhl2 promoter activity. A, combinations of traf6-FLAG, fhl2-FLAG, and RUNX1-HA were transfected into 293T cells. 48 h later lysates were immunoprecipitated (IP) with anti-HA (RUNX1) mAb. The immunoprecipitates were immunoblotted (IB) with anti-FLAG mAb. FHL2 and TRAF6 were distinguished on the basis of molecular weight. B, nuclear-enriched osteoclast protein using biotin-labeled fhl2 promoter RUNX1-binding DNA elements as probe. D, human fhl2 promoter-driven luciferase reporter plasmid was transiently transfected into 293T cells with combinations of RUNX1, fhl2, and fhl2 plasmids as indicated. Luciferase activity was measured 48 h later. Data are presented as mean ± S.D.

FIGURE 3. TRAF6 enhances RUNX1-mediated fhl2 promoter activity. A, combinations of traf6-FLAG, fhl2-FLAG, and RUNX1-HA were transfected into 293T cells. 48 h later lysates were immunoprecipitated (IP) with anti-HA (RUNX1) mAb. The immunoprecipitates were immunoblotted (IB) with anti-FLAG mAb. FHL2 and TRAF6 were distinguished on the basis of molecular weight. B, nuclear-enriched osteoclast protein using biotin-labeled fhl2 promoter RUNX1-binding DNA elements as probe. D, human fhl2 promoter-driven luciferase reporter plasmid was transiently transfected into 293T cells with combinations of RUNX1, fhl2, and fhl2 plasmids as indicated. Luciferase activity was measured 48 h later. Data are presented as mean ± S.D.

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TRAF6 Enhances RUNX1-mediated fhl2 Promoter Activity—Having established TRAF6 is a nuclear protein and interacts with RUNX1 and FHL2, we asked whether the three form an intracellular complex. Combinations of traf6-FLAG, fhl2-FLAG, and RUNX1-HA were transfected into 293T cells. Cell lysates were immunoprecipitated with anti-HA mAb, and the product was immunoblotted with anti-FLAG mAb (Fig. 3A). FHL2-FLAG and TRAF6-FLAG were distinguished by molecular weight. RUNX1 and FHL2, alone, do not interact but do so in the presence of TRAF6. Thus, the three proteins form an intracellular entity when overexpressed in 293T cells. To determine whether the same obtains in native osteoclast nuclei, a nucleophosmin-rich fraction was immunoprecipitated with anti-TRAF6 antibody and immunoblotted for RUNX1 and FHL2 (Fig. 3B). In keeping with immunofluorescent localization (Fig. 1D), TRAF6 coimmunoprecipitates with FHL2 and once again, with RUNX1.

The fact that each protein resides in the osteoclast nucleus suggests the TRAF6-FHL2-RUNX1 composite may have transcriptional properties. Hence, we performed EMSA on osteoclast nuclear proteins, probing with biotin-labeled fhl2 promoter RUNX1 response element (Fig. 3C). Again, osteoclast nuclear proteins are present which bind the probe (lane 1), and the protein-DNA complex is unaltered by anti-IgG antibody (lane 2). In contrast, anti-RUNX1 (lane 3), anti-TRAF6 (lane 4), and anti-FHL2 (lane 5) mAbs each reduce signal intensity, indicating this DNA-binding entity contains all three proteins. To assess the functional implications of this complex, we transiently transfected 293T cells with combinations of RUNX1, fhl2, and traf6, as well as a luciferase reporter, driven by the FHL2 promoter. RUNX1 alone or with either FHL2 or TRAF6 moderately increases reporter activity. However, all three proteins, in toto, enhance transcription 7-fold (Fig. 3D).

FHL2 Is Ubiquitinated by TRAF6—TRAF6 is an E3 ubiquitin ligase (5), raising the possibility that it alters FHL2 function by polyubiquitination. To address this issue, we transfected 293T cells with fhl2-HA, with or without traf6-FLAG. FHL2 was immunoprecipitated with an anti-HA mAb, and the immunoprecipitate was stained for ubiquitin content. Consistent with the E3 ligase activity of TRAF6, FHL2 polyubiquitination is increased as the two proteins associate (Fig. 4A).

TRAF6 typically functions as an E3 ligase by facilitating assembly of LysK63-linked polyubiquitin chains (13). To determine whether this is so in the context of TRAF6-mediated FHL2 polyubiquitination, we transfected 293T cells with traf6-FLAG and fhl2-FLAG and fhl2-HA as well as WT or inactive K63R ubiquitin. Because of evidence that TRAF6 may also promote LysK48-linked polyubiquitination, we also examined the effects of the K48R mutant (14). K29R ubiquitin served as control. The cells were treated with the proteasome inhibitor, MG132, and FHL2 was immunoprecipitated from the lysate. The immunoprecipitates were blotted with anti-FHL2, anti-TRAF6, and anti-ubiquitin mAbs. FHL2 and TRAF6 coreplicate regardless of the cotransfected ubiquitin construct (Fig. 4B). In keeping with its E3 ligase activity in other cells, the capacity of TRAF6 to polyubiquitinate FHL2 is reduced in the presence of ubiquitin K63R. Ubiquitin K48R moderately dampens FHL2 polyubiquitination whereas ubiquitin K29R is as effective as the WT construct. Thus, TRAF6 principally ubiquitinates FHL2 in a LysK63-dependent manner, but also promotes LysK48 ubiquitin chain linkage.

LysK63-linked polyubiquitination typically activates events such as protein-protein recognition and cytoplasm to nuclear translocation (15). Because these phenomena are both components of the TRAF6-FHL2-RUNX1 signaling pathway we asked if they are mediated by FHL2 polyubiquitination. First, we transfected 293T cells with combinations of fhl2-FLAG, RUNX1-HA, traf6-FLAG, and increasing concentrations of ubiquitin-His. As seen in Fig. 4C, the FHL2-RUNX1 association is not altered by the presence or absence of ubiquitin.
To determine whether the TRAF6 ubiquitination of FHL2 mediates nuclear localization, COS-7 cells were transfected with traf6-FLAG, fhl2-FLAG, and ubiquitin-HA. TRAF6-FLAG and FHL2-FLAG were immunoblotted in cytosolic and nuclear-enriched fractions, the latter characterized by an abundance of nucleophosmin. Again, FHL2 is necessary for TRAF6 nuclear localization, but the process is unaffected by the presence of nucleophosmin. Hence, the E3 ligase activity of TRAF6 regulates neither its inclusion in a transcription complex nor its nuclear translocation.

Lys63-linked polyubiquitination does not promote proteasomal degradation of target proteins. However, we and others find that TRAF6 also induces Lys48-linked ubiquitination (14). Moreover, RANKL, which activates TRAF6, induces proteasome-mediated degradation of proteins such as IkB. We therefore asked whether RANKL prompts FHL2 ubiquitination and degradation. To this end, we transduced BMMs with fhl2-HA, cytokine-starved the cells, and exposed them to RANKL, with time, in the presence or absence of the proteasome inhibitor MG132 (Fig. 4E). HA immunoprecipitates were immunoblotted for ubiquitin. Although monoubiquitinated FHL2 is unaltered by MG132, osteoclast precursors express increasing amounts of the polyubiquitinated species with RANKL exposure, only when proteasome activity is arrested. Thus, RANKL-stimulated TRAF6 activity is associated with ubiquitination of FHL2 and its proteasomal degradation.

DISCUSSION

The magnitude of osteoclast activity profoundly affects skeletal balance because virtually all osteoporotic diseases reflect its enhancement relative to bone formation. Thus, precise regulation of osteoclast formation and function is required to maintain normal bone mass. The discovery of RANKL provides the most important clues as to how osteoclasts are generated and activated. Less is known, however, as to how RANKL-stimulated osteoclastogenesis is negatively controlled.

The RANK intracellular domain recognizes a variety of TRAFs, but only TRAF6 is central to the osteoclastogenic process (16). Other receptors, such as CD40, TLR4, and those recognizing IL-1 and IL-17, also bind TRAF6, but unlike RANK,
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directly is not established. Thus, its intranuclear function probably mirrors its cytoplasmic role, namely as a recruiter of proteins that, in this circumstance, directly or indirectly modulate transcription. For example, FHL2 interacts with transcription factors, such as β-catenin (19) and the androgen and estrogen receptors (20, 21). FHL2 also recruits CBP/p300 to target gene (22).

We established that FHL2 sequesters TRAF6 in osteoclasts and in so doing inhibits its association with RANK, thereby diminishing osteoclast formation and function (6). We also noted that FHL2 is not expressed in osteoclast precursors but appears with their exposure to RANKL and M-CSF. Taken together, these data raised the possibility that FHL2 synthesis may represent a negative feedback mechanism for accelerated bone resorption. Buttressing this posture is the fact that FHL2 is expressed by osteoclasts, in vivo, only in states of pathological bone resorption such as inflammatory osteolysis, where presence of the adaptor protein has a protective effect on periarticular osteolysis (6).

Because it is bound by FHL2, which is abundant in the nucleus, we asked whether the same is true regarding TRAF6. We noted that although TRAF6 is constitutively present in osteoclast nuclei and further accumulates under the influence of RANKL, none is detectable in the nuclei of BMMs despite its abundant expression in these cells. FHL2 being absent in BMMs, but induced with exposure to RANKL, suggested that FHL2, may, at least in part, mediate TRAF6 nuclear localization. In fact, meaningful TRAF6 nuclear translocation requires coexpression of FHL2 in 293T cells and is substantially reduced in FHL2-deficient osteoclast nuclei.

FHL2 and TRAF6 each lack an apparent nuclear localization motif. However, the relatively modest molecular mass (32 kDa) of FHL2 would theoretically permit it to transit nuclear pores, although such would not be the case for TRAF6 (57 kDa). Thus, its association with FHL2 may optimize the nuclear localization of TRAF6. However, the presence of residual TRAF6 in fhl2−/− osteoclast nuclei suggests that the event may also be mediated by a shuttling protein in addition to FHL2.

TRAF6 contains a RING domain structurally homologous to E3 ubiquitin ligases, and it polyubiquitinates itself and a number of target proteins via Lys63-linked polyubiquitin chains that promote molecular signaling rather than protein degradation (5, 13, 23). We find that TRAF6 also stimulates FHL2 ubiquitination, predominantly in a Lys63-dependent manner. However, TRAF6 mimics its effect on the IL-17 receptor (14) by promoting a modest amount FHL2 polyubiquitination via Lys48, a ubiquitin residue prompting proteasomal degradation (24, 25).

Having established that TRAF6 ubiquitinates FHL2, we asked whether this event regulates nuclear translocation. Our first exercise was to determine whether FHL2 residence in the nucleus depends upon TRAF6 as an E3 ligase. Our rationale for these experiments was the fact that TRAF6-mediated ubiquitination regulates nuclear translocation of the p75 neutrophil receptor interactor, NIFR (15), and the nerve growth factor receptor, TrkA (26). However, FHL2 transports TRAF6 into the nucleus regardless of ubiquitination.

The nuclear localization and association of TRAF6 and FHL2 in osteoclasts raise the possibility that they may participate in gene transcription. Neither protein, however, contains a DNA binding consensus, indicating that if they impact gene expression it would be as coregulators associated with a primary transcription factor. Thus, our challenge was to discover a candidate gene regulated by TRAF6/FHL2 and identify relevant response elements in its promoter. Our rationale for selecting the fhl2 gene, itself, was the fact that it is induced with TRAF6 activation, by RANKL, and its product impacts osteoclast formation and function (6). Thus, we examined the fhl2 promoter and discovered two RUNX1 recognition sequences.

RUNX1 is a Runt-related protein essential for hematopoietic development (27). It is also among the most targeted transcription factors associated with various forms of leukemia. RUNX1 interacts with many coregulators and transcription factors, including PU.1, thereby inducing expression of the M-CSF receptor, c-Fms (28, 29). Both PU.1 and c-Fms are essential for osteoclastogenesis. RUNX1 also associates with molecules recognized by FHL2, such as CBP/p300 (22). We find that FHL2 interacts with RUNX1 but only in the context of TRAF6 wherein the three proteins form a DNA-binding complex that transactivates the fhl2 promoter. The fact that RUNX1 fails to associate with FHL2 in the absence of TRAF6 suggested that polyubiquitinated FHL2 may recruit additional proteins, such as RUNX1 (5). We find, however, that like TRAF6 nuclear localization, RUNX1-FHL2 association is independent of ubiquitin.

These and our previous data (6) suggest a model whereby TRAF-6 and FHL2 autoregulate osteoclastogenesis. Upon RANKL stimulation, TRAF6 activates an array of osteoclastogenic molecules, including NF-κB and NFAT-c1 (30). At the same time, TRAF6 is sequestered by FHL2, which inhibits its binding to RANK, thus attenuating osteoclast formation. FHL2 and TRAF6 translocate to the nucleus where they both associate with RUNX1 to form a transcription complex that transactivates the fhl2 gene. Increased FHL2 expression serves to further dampen RANKL-induced osteoclast formation and function. Alternatively, the antiosteoclastogenic properties of FHL2 are blunted by RANKL-induced ubiquitination and proteasomal degradation. Thus, development of FHL2 mimetics may represent a strategy for preventing pathological bone resorption such as that attending inflammatory arthritis.

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