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Homer2 and Homer3 modulate RANKL-induced NFATc1 signaling in osteoclastogenesis and bone metabolism

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Abstract

The receptor activator of nuclear factor-kappa B ligand (RANKL) induces osteoclastogenesis by induction of Ca²⁺ oscillation, calcineurin activation and translocation into the nucleus of nuclear factor of activated T cells type c1 (NFATc1). Homer proteins are scaffold proteins. They regulate Ca²⁺ signaling by modulating the activity of multiple Ca²⁺ signaling proteins. Homers 2 and 3, but not Homer1, also independently affect the interaction between NFATc1 and calcineurin. However, to date, whether and how the Homers are involved in osteoclastogenesis remains unknown. In the present study, we investigated Homer2 and Homer3 roles in Ca²⁺ signaling and NFATc1 function during osteoclast differentiation. Deletion of Homer2/Homer3 (Homer2/3) markedly decreased the bone density of the tibia, resulting in bone erosion. RANKL-induced osteoclast differentiation is greatly facilitated in Homer2/3 DKO bone marrow-derived monocytes/macrophages (BMMs) due to increased NFATc1 expression and nuclear translocation. However, these findings did not alter RANKL-induced Ca²⁺ oscillations. Of note, RANKL treatment inhibited Homer proteins interaction with NFATc1, but it was restored by cyclosporine A treatment to inhibit calcineurin. Finally, RANKL treatment of Homer2/3 DKO BMMs significantly increased the formation of multinucleated cells. These findings suggest a novel potent mode of bone homeostasis regulation through osteoclasts differentiation. Specifically, we found that Homer2 and Homer3 regulate NFATc1 function through its interaction with calcineurin to regulate RANKL-induced osteoclastogenesis and bone metabolism.

Key Words

- ▶ RANKL
- ▶ osteoclast differentiation
- ▶ scaffold protein
- ▶ osteoporosis
- ▶ NFATc1

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Introduction

Bone is a dynamic organ. It is constantly renewing through a bone remodeling process, mediated by osteoblastic bone formation and osteoclastic bone resorption. In numerous bone diseases, an imbalance in this process

can be found. Examples are autoimmune arthritis, osteoporosis, periodontitis, osteopetrosis and bone tumors (Takayanagi 2007, Zaidi 2007). Multinucleated mature osteoclasts originate from bone marrow-derived

monocytes/macrophages (BMMs) through two essential factors. One is receptor activator of nuclear factor- κ B ligand (RANKL) secreted by osteoblasts. The other is macrophage-colony-stimulating factor (M-CSF) (Takayanagi 2007). RANK receptor's activation by RANKL leads to oscillations in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). The latter is linked to the activation of Gq. In turn, Gq generates inositol 1,4,5-triphosphate (IP_3) to activate the IP_3 receptors (IP_3Rs), Ca^{2+} release from the ER and activation of Ca^{2+} influx from the extracellular space by the Orai channels and transient receptor potential (TRP) channels. Ultimately, as previously reported, this generates an increase in intracellular reactive oxygen species (Yang *et al.* 2009, 2013, Kim *et al.* 2010, Son *et al.* 2012, Park *et al.* 2013). Subsequently, the RANKL-mediated $[\text{Ca}^{2+}]_i$ oscillations increase NFATc1's nuclear translocation, multinucleated cells' formation and bone resorption (Takayanagi *et al.* 2002, Yang *et al.* 2009, Kim *et al.* 2010).

Members of the NFAT family of transcription factors play a central role in immune responses and in the development of cardiac and skeletal muscles, bone and the nervous system. In these tissues, the NFAT family members are expressed in most cells. The NFAT family consists of five members (NFATc1-5). NFATc1 through NFATc4 are regulated by Ca^{2+} signaling. Specifically, Ca^{2+} activates calcineurin by calmodulin. This dephosphorylates NFAT to expose its nuclear import signal (Olson & Williams 2000, Crabtree & Olson 2002, Hogan *et al.* 2003, Takayanagi 2007).

Ca^{2+} is a universal intracellular messenger that is involved in various cellular functions and processes, such as cell proliferation, differentiation and apoptosis (Bootman *et al.* 2001, Berridge *et al.* 2003). Disruption of Ca^{2+} signaling is one mechanism by which RANKL function, NFATc1 activation and the associated bone metabolism can be altered. An additional less explored mechanism NFAT function's regulation is through their interaction with adapter proteins. As for example SH3 domain-binding protein 2 (3BP2), NFAT-interacting protein (NIP45) and most prominently the Homer proteins (Takayanagi *et al.* 2002, Foucault *et al.* 2005, Huang *et al.* 2008, GuezGuez *et al.* 2010, Shanmugarajan *et al.* 2012). The Homer family of scaffolding proteins consists of Homer1, Homer2 and Homer3 and several splice variants. The short Homer1a's gene coding was identified as an immediate early gene transcriptionally upregulated by synaptic activity in neuronal cells

(Brakeman *et al.* 1997, Xiao *et al.* 1998, Bottai *et al.* 2002). Homer1a predominantly consists of the N-terminal Ena/VASP homology 1 (EVH) protein-binding domain. The remaining Homer proteins (i.e., Homer1b/c, Homer2 and Homer3, referred to as 'long Homers') are composed of an EVH domain and a C-terminus. The latter includes a coiled-coil multimerization domain and leucine zipper (Tu *et al.* 1998, Worley *et al.* 2007). Of note, the EVH domain binds to several G protein coupled-receptors (GPCRs). Specifically, these are as follows: canonical TRP (TRPC) channels, IP_3Rs , ryanodine receptors (RyRs) and the Shank family of scaffold proteins (Tu *et al.* 1998, 1999, Xiao *et al.* 1998, Feng *et al.* 2002, Shin *et al.* 2003, Yuan *et al.* 2003). Homer proteins were previously identified as synaptic proteins regulating synaptic activity in neurons. However, earlier studies have reported on the importance of the Homer family in other tissues (Brakeman *et al.* 1997, Tu *et al.* 1998, 1999, Xiao *et al.* 1998). In previous studies, it has been shown that Homer2 modulates the intensity of the GPCRs stimulus. Of note, this is done by regulating the GAP activity of RGS proteins and of PLC β in pancreas acinar cells (Shin *et al.* 2003). In the skeletal muscle, Homer2 enhances the NFATc1-dependent signaling pathway by increasing the release of RyR-dependent Ca^{2+} during myogenic differentiation (Stiber *et al.* 2005). It has been demonstrated that Homer3's deletion induces severe autoimmune phenotypes in the lung and liver. Additionally, Homer3's phosphorylation by the Ca^{2+} /calmodulin-dependent protein kinase (CaMK) II regulates Purkinje cells synaptic activity (Huang *et al.* 2008, Mizutani *et al.* 2008). Of note, Homer2 and Homer3 have been shown to regulate T cell activation through the interaction with NFATc2. Specifically, this is done by competing with calcineurin to bind with NFATc2 (Huang *et al.* 2008). Additionally, Homer2 identified a co-localization with NFATc1 at the neuromuscular junction. The latter is part of the calcineurin-NFATc1 signaling pathway (Salanova *et al.* 2011). However, to date, there is a poor understanding of the characteristics of the RANKL-induced NFATc1 signaling pathway during osteoclastogenesis and whether and how it is regulated by Homer proteins.

In the present study, we investigated the role of Homer proteins in the RANKL-induced NFATc1 signaling pathway, osteoclast differentiation and bone metabolism with the use of Homer2 and Homer3 (Homer2/3) double-knockout (DKO) mice.

Materials and methods

Cell culture and reagents

The Homer2/3 DKO mice were generously provided by Dr Paul F Worley (Johns Hopkins University School of Medicine, Baltimore, MD) (Huang *et al.* 2008) and all animal care and experimental procedures complied with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) in Yonsei University (IACUC approval no. 2014-0067). Primary cultured bone marrow-derived monocytes (BMMs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and α -minimum essential medium (α -MEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and incubated in 5% CO₂. To maintain BMMs, α -MEM was supplemented with 30 ng/mL M-CSF. Receptor activator of RANKL and M-CSF were purchased from KOMA Biotech (Seoul, Korea). Fura-2/AM was purchased from Teflabs (Austin, TX, USA). Pluronic F-127 was obtained from Molecular Probes. Anti-Homer2, anti-Homer3 and anti-NFATc1 antibodies were obtained from Santa Cruz Biotechnology. Anti-actin antibodies were purchased from Sigma-Aldrich.

Preparation of BMMs

The femur and tibia were isolated from 4–6-week-old mice as described previously (Kim *et al.* 2010). All cells derived from bone marrow of femur and tibia were collected and cultured in α -MEM medium containing 10% FBS and 30 ng/mL M-CSF. The following day, non-adherent cells in media were collected and seeded on adequate plates and treated with M-CSF (30 ng/mL). After 2 days non-adherent cells were washed out and adherent cells were used as BMMs.

Analysis of bone density and skeletal morphology

Tibias and femora were isolated from 6-week-old WT and Homer2/3 DKO mice. To exclude differences caused by threshold resolution set-up, samples from WT and Homer2/3 DKO were set in one soft X-ray apparatus. Bone density was measured with a 3D μ CT (SkyScan-1076 high resolution *in vivo* μ CT system; SkyScan, Aartselaar, Belgium) and analyzed with CTAn and Cone beam reconstruction software. Histological examination was performed as described elsewhere (Mayingi *et al.* 2007). Dynamic histomorphometric method was as described before (Kim *et al.* 2010). Briefly, 4-week-old

male mice were injected twice with calcein (15 mg/kg intraperitoneally) 2 days apart. The animals were killed on day 4, and undecalcified bones were embedded in methylnmethacrylate. Longitudinal sections (10 μ m thickness) of the tibias were prepared, and new bone formation was assessed by confocal microscopy (model LSM 510; Carl Zeiss) by capturing images of calcein green.

[Ca²⁺]_i measurement

The cells were seeded on cover glass in a 35 mm dishes (5 $\times$ 10⁴ cells/coverslip) and stimulated with RANKL (50 ng/mL) for the indicated times. The cells were loaded with 5 μ M Fura-2/AM and 0.05% Pluronic F-127 for 30 min in physiological salt solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, 10 mM glucose, 310 mosmol, pH 7.4). Fura-2 fluorescence intensity was measured using excitation wavelengths of 340 and 380 nm, and emitted fluorescence 510 nm (ratio = F₃₄₀/F₃₈₀) was collected and monitored at 2 s intervals using a CCD camera (Universal Imaging Co., Downingtown, PA, USA) as described previously (Yang *et al.* 2009). Images were digitized and analyzed by MetaFlour software (Universal Imaging).

Western blot

Whole-cell lysates were prepared using RIPA lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.01 mg/mL aprotinin, 5 μ g/mL leupeptin, 0.4 mM PMSF and 4 mM NaVO₄) and spun at 13,000 g for 10 min to remove insoluble material. Proteins (50–100 μ g/well) were subjected to 6–8% SDS-PAGE and were separated by size. Proteins were electro-transferred to a nitrocellulose membrane, blocked with 6% skimmed milk and probed with Abs against NFATc1 (1:1000) and actin (1:2000). Thereafter, blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h and finally detected by chemiluminescence (Amersham Pharmacia Biotech).

Immunoprecipitation

The immunoprecipitation was as before (Shin *et al.* 2003, Yang *et al.* 2014) with several modifications. In brief, microsomes were prepared from BMMs harvested from WT mice in a buffer containing 20 mM Mops (pH 6.7 with KOH), 250 mM sucrose, 1 mM EDTA, 1 mM MgCl₂, 10 mM benzamidine and 0.2 mM PMSF. The homogenates were centrifuged at 400 g for 10 min and the supernatants

were collected and re-centrifuged at 900g for 10 min at 4°C. Microsomes were lysed in a buffer containing 50 mM Tris (pH 6.8 with HCl), 150 mM NaCl, 3 mM EDTA, 2 mM EGTA and 0.5% Triton X-100 supplemented with protease inhibitors. The lysates were cleared by centrifugation at 14,000g for 15 min. About 150 µL of the extract (300 µg of protein/sample) was incubated for 10 days in a buffer containing 50 mM Tris (pH 6.8 with HCl), 150 mM NaCl, 3 mM EDTA, 2 mM EGTA. Protein A/G-agarose (Thermo Fisher Scientific) was added to each mixture, and rocking was continued overnight at 4°C. Protein A/G-agarose was pelleted at 1000g for 10 s and the beads were quickly washed with cold phosphate buffered saline (PBS). The immunoprecipitated proteins were separated by SDS-PAGE and probed with anti-Homer3 (1:1000) by overnight incubation at 4°C. To avoid protein degradation by digestive enzymes, immunoprecipitation was initiated immediately after completion of microsomal preparation.

Immunocytochemistry

Cells were seeded on coverslips (12 mm) and treated for 4 days with 50 ng/mL RANKL. After fixation in 4% paraformaldehyde (PFA) for 5 min, cells were sequentially incubated in blocking solution (0.1% gelatin, 1% BSA, 0.01% sodium azide, 5% goat serum) for 1 h, overnight in blocking solution containing Abs against NFATc1 (1:100), and finally were treated with Alexa 488-labeled anti-mouse IgG antibody (Molecular Probes) in blocking solution for 1 h. Nuclei were separately stained with TO-PRO3. F-actin was visualized with Alexa Fluor 488-Phalloidin.

TRAP stain assay

BMMs were seeded in 96-well plates at a density of 3×10^5 cells per well and pretreated with the indicated compounds. Cells were then stimulated with 50 ng/mL RANKL. After 4 days, a TRAP stain assay was performed to evaluate the cell differentiation rate. TRAP staining was performed as described previously (Yang *et al.* 2009). Cells were fixed with 10% PFA and with ethanol/acetone (1:1) solution. 100 µL of TRAP staining solution were added to each well and stained for 5 min. TRAP-positive multinucleated cells (containing ≥ 3 nuclei) were then counted.

Measurement of bone resorption rate (pit assay)

Collected BMMs were seeded on Osteo assay plates (Corning) coated with a proprietary hydroxyapatite mineral surface. Following stimulation

(50 ng/mL M-CSF and sRANKL) of the BMMs for 6 days, the plates were washed with sodium hypochlorite solution for 1 h at room temperature. Pits formed on the surface of the hydroxyapatite mineral were imaged and calculated using MetaMorph software (Molecular Devices).

Alkaline phosphatase activity and Alizarin red S staining

After bone marrow stromal cells (BMSCs) were transduced, they were seeded into 48-well plates at a density of 5×10^5 cells per well. After osteogenic induction for indicated days, cells were rinsed two times with PBS. ALP and ARS (both from Sigma-Aldrich) were added into cells for the staining during 30 min. Staining agent was discarded and cells were rinsed 4–5 times with water and air-dried. Images of each sample were acquired using a CCD camera. To quantify matrix mineralization, cells were incubated in 100 mM cetylpyridinium chloride for ARS and alkaline phosphatase yellow for ALP. The concentration of ARS was measured using a microplate spectrophotometer at a 562 nm wavelength and ALP activity was measured at the wavelength of 405 nm.

Statistical analysis

All data were expressed as the mean $\pm$ S.E.M. Statistical significance was determined by using a paired Student's *t*-test. Statistical significance was set at $P < 0.05$ level.

Results

Decreased bone density in Homer2/3 DKO mice

Homer proteins have been shown to be regulators of Ca^{2+} signaling pathways and direct regulators of NFAT activation in T cells (Huang *et al.* 2008). In the present study, we examined whether the deletion of Homer proteins affects bone metabolism through bone remodeling. The tibias of WT and Homer2/3 DKO mice were dissected. Subsequently, we evaluated bone density by microradiographic and histological analyses. The tibias' bone density (bone volume/total volume, BV/TV) and the trabecular bone's thickness from Homer2/3 DKO mice were significantly reduced (~ 44.5 and $\sim 38.5\%$, respectively) vs WT (Fig. 1A). Histologically, Homer2/3 DKO mice exhibited an extensive resorption of trabecular bone below the growth plate and epiphysis of the tibia. Additionally, Homer2/3 DKO mice had a significantly

Figure 1

Decreased bone density in Homer2/3 DKO mice. (A) Microradiographic analyses of tibias in WT and Homer2/3 DKO mice. Note that in Homer2/3 DKO tibias, trabecular and cortical bone mass decreased (6 weeks). (B) Representative calcein-labeled sections of proximal tibias in WT and Homer2/3 DKO mice (scale bar, 20 μm). (C) Alkaline phosphatase (ALP) Alizarin red S (ARS) staining ($n = 4$). (D) μ CT data of proximal femur obtained from WT and Homer2/3 DKO mice. Each parameter, including BV/TV, Tb. Th, Tb. N and Tb. Sp were calculated and presented. (E) Histomorphometric parameters. The percentage of eroded surface (ES) on bone surface (BS) increased in Homer2/3 DKO mice (left, $n = 3$). (F) We observed no clear difference in mineral apposition rate (MAR) between Homer2/3 DKO and WT mice (middle, $n = 7$). (G) Following ALP activity and ARS staining, we found no difference in osteoblastogenesis between WT and DKO mice (right, $n = 4$). Data were normalized to bone volume in WT mice and expressed as means $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$ compared with WT.

increased erosion surface on bone by ~ 1.08 -fold (eroded surface/bone surface, ES/BS) (Fig. 1D).

Next, we aimed at analyzing the impact of Homer2/3 deletion on bone formation rates. To this end, we performed dynamic histomorphometric measurements on 4-week-old mice double-labeled with sequential doses of calcein. We did not observe a clear difference in bone-formation rate and mineral apposition rate (MAR) between Homer2/3 DKO and WT mice (Fig. 1B and E). Of note, the results for osteoblastogenesis by alkaline phosphatase (ALP) activity and alizarin red S (ARS) staining showed no difference between DKO and WT mice (Fig. 1C and F).

Homer2/3 deletion does not affect RANKL-induced $[\text{Ca}^{2+}]_i$ oscillation

It has been previously shown that RANKL-induced $[\text{Ca}^{2+}]_i$ oscillations are essential for osteoclast differentiation (Takayanagi *et al.* 2002). As a consequence, we analyzed Ca^{2+} signaling in RANKL-treated BMMs obtained from WT and Homer2/3 DKO mice. We initiated $[\text{Ca}^{2+}]_i$ oscillations after 24 h of RANKL treatment in both WT and Homer2/3 DKO BMMs (Fig. 2A and B).

We observed that the number of responding cells and the frequency of $[\text{Ca}^{2+}]_i$ oscillations were not altered by the deletion of Homer2/3 (Fig. 2B and C).

Key role of Homer2/3 proteins in RANKL-induced NFATc1 expression and osteoclastogenesis

It has been previously shown that Homer2 and Homer3 interfere with the interaction of NFAT with calcineurin (Huang *et al.* 2008, Salanova *et al.* 2011). Therefore, we examined the effects of Homer2/3 proteins on NFATc1 expression and translocation into the nucleus in BMMs after RANKL treatment. We observed that the expression of Homer proteins was not affected by RANKL's treatment of WT BMMs (Fig. 3A). RANKL induced a transient and moderate increase in NFATc1 protein levels after 12 h of treatment. Subsequently, it caused a sustained upregulation, which persisted for several days in WT and Homer2/3 DKO BMMs (Fig. 3B). The RANKL-induced upregulation of NFATc1 in Homer2/3 DKO cells was significantly higher after 48 and 72 h vs WT BMMs (Fig. 3C). Of note, we found that NFATc1 translocation into the nucleus was markedly increased in Homer2/3 DKO BMMs (Fig. 3D and E).

Figure 2

RANKL-induced $[Ca^{2+}]_i$ oscillations in WT and Homer2/3 DKO BMMs. (A) Oscillatory change in $[Ca^{2+}]_i$ in WT and Homer2/3 DKO BMMs before and after RANKL treatment. (B) Quantitative analysis of the number of cells with $[Ca^{2+}]_i$ oscillation induced by RANKL in WT and Homer2/3 DKO BMMs ($n = 3$). (C) Oscillation frequency at 24, 48 and 72 h. Data are expressed as mean $\pm$ s.e.m.

Earlier studies have reported that T cells and skeletal muscle Homer proteins interact with and sequester NFAT in the cytosol (Huang *et al.* 2008, Salanova *et al.* 2011). We aimed at examining the interaction of Homer proteins with NFATc1 in BMMs and determining if it is affected by the induction of osteoclastogenesis. As a consequence, we examined both whether Homer proteins selectively bind NFATc1 and RANKL stimulation affects the interaction in WT and Homer2/3 DKO BMMs. Figure 3F shows the

co-immunoprecipitation with NFATc1 of Homer2 and Homer3 prior to RANKL stimulation. Importantly, RANKL stimulation significantly reduced the interaction aimed at dissociating the NFATc1/Homers complexes. Of note, following treatment with the specific calcineurin inhibitor cyclosporine A, Homer proteins and NFATc1's interaction were restored and complex formation was enhanced.

Next, we aimed at confirming the role of Homer proteins in RANKL-mediated osteoclasts differentiation.

Figure 3

RANKL-induced NFATc1 expression and translocation depend on the interaction of Homer proteins with NFATc1. (A) Expression levels of Homer proteins (45 kDa) and β -actin (42 kDa) in WT BMMs after RANKL stimulation. (B) Expression level of NFATc1 (approximately 90–110 kDa) and β -actin in WT and Homer2/3 DKO BMMs following RANKL stimulation. (C) Mean NFATc1 expression levels in WT and Homer2/3 DKO BMMs. We analyzed by densitometry the expression levels in experiments similar to those in B. Specifically, we observed that NFATc1's expression levels between 48 and 72 h were significantly increased in Homer2/3 DKO cells ($n = 6$). Data were normalized to expression level in cells from WT mice and expressed as the mean $\pm$ s.e.m. (D) NFATc1 protein's immunostaining in WT and Homer2/3 DKO BMMs post 72 h RANKL stimulation. Of note, NFATc1's nuclear translocation was significantly higher in Homer2/3 DKO vs WT BMMs. Arrows indicate nuclear localized NFATc1. TO-PRO3 counterstaining was performed on the nuclei (scale bar, 20 μ m). (E) Quantification of cell number with nuclear NFATc1. Homer2/3 DKO cells had a significant increase in NFATc1 translocation ($n = 7$). (F) Homer proteins and NFATc1 co-immunoprecipitation in cell lysates prepared from WT BMMs before and after 24-h RANKL stimulation. A reduced interaction of NFATc1 with Homer3 and Homer2 was found following RANKL stimulation. As a control, inhibition of calcineurin with cyclosporine A (CsA, 0.01 μ g/mL) to inhibit NFATc1 dephosphorylation increased NFATc1's interaction with Homer3 and Homer2 and reversed RANKL stimulation's effect. * $P < 0.05$ and ** $P < 0.01$ compared with WT.

To this end, we used TRAP staining with the goal of determining the difference between the morphology of differentiated Homer2/3 DKO osteoclasts and that of WT cells. We found that a 4-day RANKL stimulation increased the formation of TRAP⁺ MNCs such that the increase was higher in cells from Homer2/3 DKO. Additionally, the morphological features characteristic of osteoclast differentiation (i.e., the number of nuclei and area of MNCs) were enhanced in the RANKL-induced Homer2/3 DKO BMMs vs WT BMMs (Fig. 4A). We then examined Homer2/3's effect on osteoclast function by performing pit formation and actin ring formation assays. Osteoclasts were generated on a bone resorption assay plate. We observed that the percentage of bone resorption area increased in the RANKL-induced Homer2/3 DKO cells. Additionally, the actin staining results demonstrated that area of MNCs is higher in Homer2/3 DKO cells (Fig. 4B and C).

Discussion

In the present study, we report a novel interaction between Homer2/3 and NFATc1 for the regulation of osteoclastogenesis and bone metabolism. Our study demonstrates that inhibition of Homer2/3 expression strongly facilitates osteoclastogenesis. Homer2/3 DKO mice had an osteoporotic phenotype. The latter was caused by increased osteoclast differentiation with a wider bone erosion surface, independent of the rate of osteoblastic bone formation. We traced the aberrant bone metabolism to reduced Homer2/3 expression responsible for inducing NFATc1 with a sustained increase in its levels several days from RANKL treatment and a marked increase in its nuclear localization. These findings suggest that Homer2 and Homer3 are key regulators of RANKL-mediated osteoclastogenesis by linking RANKL-induced NFATc1 activation to the last stages of osteoclast differentiation.

Figure 4

RANKL-induced osteoclastogenesis is enhanced in Homer2/3 DKO BMMs. (A) RANKL-induced osteoclast differentiation *in vitro* in WT and Homer2/3 DKO BMMs (TRAP staining; scale bar, 50 μ m). Columns show quantitative analysis of the number (middle) and the area (right) of TRAP-positive (TRAP⁺) multinucleated cells (MNCs; ≥ 3 nuclei) in WT and Homer2/3 DKO mice. The number and area of TRAP⁺ MNCs are significantly higher in Homer2/3 DKO mice ($n = 4$). (B) Pits area during osteoclastogenesis is higher in Homer2/3 DKO cells ($n = 5$). (C) Actin staining of TRAP⁺ MNCs in WT and Homer2/3 DKO cells (scale bar, 20 μ m). The area of actin ring formation in TRAP⁺ MNCs during osteoclastogenesis is significantly higher in Homer2/3 DKO cells ($n = 6$). We normalized the data to the number and area of MNCs in RANKL-treated BMMs of WT mice. Results were expressed as the mean $\pm$ s.e.m. * $P < 0.05$ and *** $P < 0.001$ compared with RANKL-treated WT.

It has been shown that Ca^{2+} oscillations have a prominent role in osteoclastogenesis (Takayanagi *et al.* 2002, Yang *et al.* 2009, Kim *et al.* 2010). Furthermore, Homer proteins were reported to interact with several Ca^{2+} signaling proteins (Shin *et al.* 2003, Worley *et al.* 2007, Yang *et al.* 2014). However, we found that the increase in long-lasting $[\text{Ca}^{2+}]_i$ oscillations occurring between 24 and 72 h from RANKL stimulation (Fig. 2) occurred independently in Homer2/3 expressions. Therefore, it appears that Homer2 and Homer3 have another role in BMMs. Homer1 is the major regulator of Ca^{2+} signaling (Yuan *et al.* 2003), while Homer2 modulates only GPCR by affecting Gq-related signaling through RGS2 (Shin *et al.* 2003) and Homer3 has no known role in Ca^{2+} signaling. However, Homer2/3 competes with calcineurin for interaction with NFAT and may, therefore, regulate NFAT-dependent transcription (Huang *et al.* 2008). This accounts for the lack of effect of the DKO on RANKL-mediated Ca^{2+} oscillations. Specifically, we believe that they regulate the NFATc1 system's function. As a consequence, the expression and translocation of NFATc1 appeared 12 h post RANKL treatment. Of note, these effects were enhanced in the RANKL-induced Homer2/3 DKO BMMs.

Previous reports showed that NFAT activation driving osteoclast differentiation requires NFAT expression's induction via NF- κ B and AP-1 and posttranslational activation induced by ITAM-associated immunoreceptors (Koga *et al.* 2004, Mocsai *et al.* 2004, Kuroda *et al.* 2008). Several studies suggest that NFAT binds Homer proteins participating in skeletal muscle differentiation and T cell activation (Stiber *et al.* 2005, Huang *et al.* 2008, Salanova *et al.* 2011). Huang and colleagues also reported that Homer2 and Homer3 play the action as a selective inhibitor to calcineurin-NFAT activation which the action was not related with AP1 and NF- κ B pathway (Huang *et al.* 2008). Such mode of NFATc1 regulation appears to operate in both BMMs and osteoclasts. This is based on Homer proteins and NFATc1's interaction, which is dependent on calcineurin and markedly inhibited by RANKL stimulation during osteoclastogenesis (Fig. 3F). Therefore, our study shows that Homer proteins are necessary for the control of RANKL-mediated NFATc1 induction and translocation. Additionally, they represent key molecules of the NFATc1 signaling pathway and auto-amplification during osteoclastogenesis. Accordingly, the deletion of Homer2/3 facilitated the formation of MNCs and bone resorption in late-stage osteoclastogenesis to reduced bone density.

In summary, these results provide strong evidence for the regulation of NFATc1 availability for transcriptional

regulation by the Homers in osteoclasts and that the Homers are major regulators of osteoclasts differentiation and bone metabolism. Specifically, our findings indicate that Homer proteins constitute a node at which NFATc1 activity, formation of MNCs, and physiological activity of osteoclasts are sequentially regulated. We believe that our findings may have a clinical relevance in pathological conditions of the skeletal system. We demonstrated essential roles of Homer proteins in the RANKL-induced NFATc1 signaling pathway during osteoclasts differentiation. Such observations suggest a specific and potent therapeutic target for various bone diseases caused by the abnormal formation of osteoclasts.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A S, Y M Y and D M S designed the study. A S, Y M Y, S Y O and N K performed experiments. A S and Y M Y contributed to data acquisition and interpretation. K W K, Y M Y and D M S drafted and revised the manuscript. All authors approved the final version of the manuscript.

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