Tmem64 Modulates Calcium Signaling during RANKL-Mediated Osteoclast Differentiation

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http://dx.doi.org/10.1016/j.cmet.2013.01.002

SUMMARY

Osteoclast maturation and function primarily depend on receptor activator of NF-κB ligand (RANKL)-mediated induction of nuclear factor of activated T cells c1 (NFATc1), which is further activated via increased intracellular calcium ([Ca2+]i) oscillation. However, the coordination mechanism that mediates Ca2+ oscillation during osteoclastogenesis remains ill defined. Here, we identified transmembrane protein 64 (Tmem64) as a regulator of Ca2+ oscillation during osteoclastogenesis. We found that Tmem64-deficient mice exhibit increased bone mass due in part to impaired osteoclast formation. Using in vitro osteoclast culture systems, we show here that Tmem64 interacts with sarcoplasmic endoplasmic reticulum Ca2+-ATPase 2 (SERCA2) and modulates its activity. Consequently, Tmem64 deficiency significantly diminishes RANKL-induced [Ca2+]i oscillation, which results in reduced Ca2+/calmodulin-dependent protein kinases (CaMk) IV and mitochondrial ROS, both of which contribute to achieving the CREB activity necessary for osteoclast formation. These data demonstrate that Tmem64 is a positive modulator of osteoclast differentiation via SERCA2-dependent Ca2+ signaling.

INTRODUCTION

Skeletal bone is maintained via continuous bone formation and destruction mediated by osteoblasts and osteoclasts (Zaidi, 2007; Zeilzer and Olsen, 2003). Imbalance of bone homeostasis causes various skeletal disorders (Teitelbaum and Ross, 2003). Congenital defects in the development and function of osteoclasts lead to osteopetrosis, which is characterized by high bone mineral density. In contrast, excessive osteoclast differentiation and activity causes osteoporosis, or low bone mineral density. Therefore, investigations of osteoclast differentiation and function aim to reveal the physiology and pathology of the skeletal system and to provide a molecular basis for designing therapeutic strategies for bone remodeling diseases (Karsenty and Wagner, 2002).

Since the discovery of RANKL as an essential osteoclast differentiation factor, many of the signaling pathways required for RANKL-induced osteoclast differentiation have been identified (Walsh et al., 2006). Binding of RANKL to RANK triggers TRAF6-dependent signaling, activating NF-κB, Akt, and MAP kinases (ERK, JNK, and p38). In other pathways, immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptors, Fc receptor common γ subunit (FcRγ) and DNAX-activating protein 12 (DAP12) deliver costimulatory signals through activation of PLCγ. Activated PLCγ leads to the generation of inositol-1,4,5-triphosphate (IP3), which mobilizes Ca2+ from the ER stores through inositol triphosphate receptors (IP3R) (Ferron et al., 2011; Kim et al., 2002; Koga et al., 2004; Kuroda et al., 2008), and subsequently generates Ca2+ oscillation, which is critical for the activation of CaMKIV and NFATc1. This RANKL-induced Ca2+ oscillation activates Ca2+/calmodulin-dependent protein kinases (CaMk) IV, followed by cAMP-responsive element binding protein (CREB) activation (Sato et al., 2006), which is also induced by mitochondrial reactive oxygen species (ROS) (Ishii et al., 2009). The CaMKIV/CREB/NFATc1 pathway is critical to osteoclast differentiation and function (Negishi-Koga and Takayanagi, 2009). Although Ca2+ oscillations are important triggers for efficient activation of NFATc1, the intra- and/or extracellular pathways that regulate Ca2+ oscillations during osteoclast differentiation are less well understood.

Sarco/endoplasmic reticulum Ca2+-ATPases (SERCAs) are a family of proteins reported to be involved in Ca2+ homeostasis in a broad range of cells (Feske, 2007; Fu et al., 2011). SERCA proteins are encoded by a multigenic family that includes SERCA1–3 (Atp2a1–3). Each gene encodes at least five splicing isoforms. SERCA1a and SERCA1b are mainly expressed in adult and neonatal fast-twitch skeletal muscles, whereas SERCA2a is
expressed in cardiac muscle. SERCA2b, which has a C-terminal extension, is ubiquitously expressed in smooth muscle tissues and nonmuscle tissues, including neurons. SERCA3 also has various 3’-end splice variants and limited expression in nonmuscle tissues (Daily et al., 2006). Recent studies have revealed that a number of proteins, such as ER protein 57 (also known as 1,25-MARRS, ERP57, ERP60, GRP58, or Pdia3) (Li and Camacho, 2004), calreticulin (John et al., 1998), sarcalumenin (Shimura et al., 2008), histidine-rich Ca2+-binding protein (HRC) (Arvanitis et al., 2007), and calumenin (Sahoo et al., 2009), associate with and regulate SERCA2B stability and activity, but the mechanisms are not completely understood. It has recently been reported that SERCA2 heterozygosity (SERCA2+/−) causes defects in osteoclast differentiation because of suppressed RANKL-induced [Ca2+]i oscillations, and is associated with bone abnormalities (Yang et al., 2009).

In this study, we report that Tmem64 is a regulator for RANKL-mediated Ca2+ signaling pathways via its direct association with SERCA2, which is critical for the RANKL-induced CREB activation and mitochondrial ROS generation necessary for proper osteoclast generation.

RESULTS

Tmem64 Deletion Increases Bone Mass in Mice

We have previously reported that ablation of the d2 isoform of vacuolar (H+) ATPase (v-ATPase) V0 domain (Atp6v0d2) in mice produces osteopetrotic bone morphogenesis due to impaired osteoclast fusion and enhanced bone formation (Lee et al., 2006). To understand the molecular bases of Atp6v0d2−/− osteoclast defects, we analyzed gene expression in wild-type (WT) and Atp6v0d2−/− osteoclast precursors using global mRNA profiles. Surprisingly, we found that only one gene, transmembrane protein 64 (Tmem64), exhibited markedly increased expression in RANKL-stimulated Atp6v0d2−deficient osteoclast precursors. Tmem64 mRNA is detected in various tissues (Figure S1A available online), its expression is upregulated during RANKL-induced osteoclastogenesis in vitro, and siRNA knockdown of Tmem64-inhibited osteoclast formation (Figure S1B). These findings suggest that increased Tmem64 expression in Atp6v0d2−/− BMMs is not likely responsible for defects in Atp6v0d2−/− osteoclasts and that Tmem64 expression may not be regulated directly by Atp6v0d2. Although this result disconfirmed further examination of an explicit relationship between Tmem64 and Atp6v0d2, we believed the unique expression profile and siRNA effects of Tmem64 merited additional examination with respect to potential function(s) in the context of osteoclast biology.

To identify the in vivo function of Tmem64 in bone remodeling, we generated Tmem64 knockout mice using embryonic stem (ES) cells with a disruption of the Tmem64 gene (Figure 1A, left panel). Specific deletion of Tmem64 genomic DNA was confirmed by Southern blotting (Figure 1A, middle panel) and sequencing; mouse genotypes were verified by PCR (Figure 1A, right panel) and sequencing analysis (data not shown). Eight weeks after birth, Tmem64−/− mice showed normal appearance and growth, and most tissues exhibited no differences between groups. However, bone microstructure imaging by high-resolution microcomputed tomography (μCT) of Tmem64−/− mice revealed significantly increased bone mass, characterized by augmented bone indices including bone mineral density (BMD), bone mineral content (BMC), trabecular bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N), with concomitant decrease in trabecular spacing (Tb.Sp) (Figure 1B). Increased bone volume was observed starting at 6 weeks of age (Figure S1C). Bone sections with von Kossa staining also showed increased trabecular bone surface in Tmem64−/− versus WT mice (Figure 1C, left panel). Tartrate-resistant acidic phosphatase (TRAP)-stained bone sections exhibited a reduction in osteoclast numbers and osteoclast surface size in Tmem64−/− versus WT (Figure 1C, right panel). Serum TRACP-5b, an early marker of osteoclast formation, was consistently reduced in mutant mice (Figure 1D). Along with reduced in vitro osteoclast formation by Tmem64 knockdown (Figure S1B), these results suggested a potential link between Tmem64 ablation and an intrinsic defect in osteoclast differentiation. By contrast, Tmem64−/− mice showed marked increases in osteoblast surface area, bone formation rates (Figures 1C and 1E), and serum osteocalcin (Figure 1F). Tmem64 expression is downregulated during osteoblast differentiation (Figure S2A). In vitro osteogenic differentiation increased in Tmem64−/− calvaria-derived osteoblast precursors versus WT, as verified by increased activity of alkaline phosphatase (ALP) and visualized by alizarin red staining for mineralized nodule formation and calcium deposits (Figure S2B). This was also confirmed by upregulation of osteoblast marker genes, such as ALP, collagen type I (Col1a1), osteocalcin (OCN), Runx2, osterix (Ox), osteoprotegerin (Tnfsf11b), colony stimulating factor 1 (Csf1), and RANKL (Tnfsf11), and by enhanced activity of β-catenin in Tmem64−/− osteoblasts (as assessed by crossing to the BAT-GAL β-catenin reporter mouse) (Figures S2C and S2D). We have thus obtained strong genetic evidence, using Tmem64−/− mice, that Tmem64 is a regulator of bone metabolism; its absence results in reduced osteoclast numbers and increased bone formation in vivo. Although Tmem64 appears to affect both osteoclasts and osteoblasts, we have initially focused on dissecting the role of Tmem64 in osteoclast differentiation because we currently have a better understanding of the molecular pathways controlled by RANKL stimulation (and its attendant effects) when using a relatively homogenous population of bone marrow (BM)-derived osteoclast precursor cells.

Tmem64 Positively Regulates Osteoclast Differentiation

Our analysis of Tmem64−/− versus WT bones clearly showed reduced osteoclast numbers in vivo. Preliminary data for Tmem64 siRNA suggested that Tmem64 knockdown reduced RANKL-induced osteoclast generation. However, osteoclast defects in Tmem64−/− mice may have been due to crosstalk with abnormal Tmem64−/− osteoblasts. Therefore, it was necessary to determine (1) whether there were cell-intrinsic defects in the absence of Tmem64 during RANKL-induced osteoclast generation, and (2) at which stage(s) of osteoclast differentiation Tmem64−/− defects manifest. To determine the intrinsic cellular role of Tmem64 in osteoclast generation, we employed a standard in vitro osteoclast culture method using M-CSF and RANKL: bone marrow macrophages (BMMs) were generated by culturing BM cells with M-CSF only and Tmem64 knockout
reconfirmed by northern blot analysis (Figure 2A, left panel); then, BMMs were induced to differentiate into osteoclasts by RANKL. When purified BM cells were induced to become osteoclasts, Tmem64 mRNA levels increased (Figure 2A, right panel; real-time PCR and RT-PCR), and Tmem64/C0/C0 BMMs stimulated with RANKL exhibited significantly fewer TRAP+ multinucleated cells (MNCs), considered to be mature osteoclasts, in comparison to WT BMMs (Figure 2B). Moreover, Tmem64/C0/C0 BMMs showed impaired osteoclast generation whether they were cocultured with WT or Tmem64/C0/C0 osteoblasts (Figure S1D), and independent of RANKL expression levels (Figure S2E). These results strongly suggest that Tmem64 expression in osteoclast lineages is required for proper osteoclast differentiation. We also revealed that levels of Nfatc1, Oscar, ctsk, and pparaC1b, which are markers of differentiated osteoclasts, were significantly lower in Tmem64/C0/C0 cells (Figure 2C), whereas

Figure 1. Ablation of Tmem64 Enhances Bone Density in Mice
(A) Generation of Tmem64 knockout mice (left panel). E, EcoRI; TRAP cassette (Neo/i-galactosidase); SA, splice acceptor; pA, polyadenylation sequence. Homologous recombination was confirmed by Southern blot analysis of EcoRI-digested genomic DNA with a 3’ region probe (middle panel). PCR genotyping analysis of Tmem64+/+, Tmem64+/-, and Tmem64-/- mice (right panel).
(B) Microcomputed tomography (µCT) images of the proximal femur from WT and Tmem64-/- mice (top, axial view of the metaphyseal region; bottom, longitudinal view). BV/TV, trabecular bone volume per tissue volume; Tb.Th, trabecular thickness; BMD, bone mineral density; Tb.N, trabecular number; Tb.Sp, trabecular spacing; BMC, bone mineral content. Scale bar represents 1 mm.
(C) Histological analysis of tibias from 8-week-old WT and Tmem64-/- mice. Tibial sections were stained with Toluidine blue (left panels), von Kossa (middle panels), or for TRAP (right panels). N.Oc/BS, osteoclast number per bone surface; Oc.S/BS, osteoclast surface per bone surface; Ob.S/BS, osteoblast surface per bone surface; BV/TV, bone volume per tissue volume. Scale bar represents 50 µm.
(D) Serum TRAP-5b abundance in WT and Tmem64-/- mice.
(E) Dynamic histomorphometry of tibia from 8-week-old WT and Tmem64-/- mice. MS/BS, mineralizing surface per bone surface; BFR, bone formation (per trabecular surface); MAR, mineral apposition rate. Scale bar represents 10 µm.
(F) Serum osteocalcin abundance in WT and Tmem64-/- mice. *p < 0.01, **p < 0.05 between the indicated groups. NS, not significant. Data are represented as mean ± SD. See also Figures S1 and S2.
neither expression of c-fms nor RANK was altered in Tmem64<sup>−/−</sup> cells (Figure S3A). The proportion of osteoclast precursor cells (c-Fms<sup>+</sup>c-Kit<sup>+</sup>Mac-1<sup>low</sup>) to the total number of bone marrow cells was equivalent in WT and Tmem64<sup>−/−</sup> mice (Figure 2D). Proliferation and apoptosis of osteoclasts in vitro in WT and Tmem64<sup>−/−</sup> cells were also comparable (Figures 2E and 2F). In addition, when cultured on dentine slices, the pit area formed from Tmem64<sup>−/−</sup> osteoclasts was small, but no difference in the pit area per osteoclast size was observed (Figure 2G). These results suggest that Tmem64 is a crucial regulator of osteoclast differentiation; its absence reduced the number of mature osteoclasts, whereas their bone-resorption activity did not appear to be affected.

Deletion of Tmem64 Exacerbates RANKL-Dependent Ca<sup>2+</sup> Oscillation and Mitochondrial ROS Generation

We next investigated the role of Tmem64 in the regulation of RANKL-dependent signaling pathways, including ERK, p38, JNK, NF-κB, and AKT, which are activated via the RANKL-TRAF6-dependent axis (Kim et al., 2009). As shown in Figure 3A, there was no significant difference in RANKL-dependent activation between WT and Tmem64<sup>−/−</sup> BMMs. Moreover, activation of the M-CSF-stimulated signaling pathways was also unchanged (Figure S3B). In contrast, expression levels of c-fos and NFATc1—key regulators of osteoclast differentiation—were decreased during osteoclast formation in Tmem64<sup>−/−</sup> BMMs (Figure 3B). CREB phosphorylation by RANKL was significantly suppressed in Tmem64<sup>−/−</sup> BMMs without a substantial change in protein expression (Figure 3B).

As previously reported (Sato et al., 2006), CREB is critical for RANKL-stimulated NFATc1 and c-fos induction in osteoclast precursors. To investigate whether enforced expression of CREB could rescue osteoclast formation in Tmem64<sup>−/−</sup> BMMs, we used retroviruses to introduce CREB or a dominant-negative inhibitor of CREB (A-CREB) into WT or Tmem64<sup>−/−</sup> BMMs. When CREB was introduced, the cells underwent normal osteoclast differentiation accompanied by significant induction of NFATc1 and c-fos (data not shown), whereas introduction of A-CREB led to strong suppression of osteoclast differentiation by RANKL (Figure 3C). These results indicate that Tmem64 is required for RANKL-mediated CREB activation during osteoclastogenesis.

RANKL-RANK signaling activates phospholipase C<sub>γ</sub>2 (PLC<sub>γ</sub>2) and leads to an increase in [Ca<sup>2+</sup>]<sub>i</sub>, via ITAM-harboring molecules DAP12 and FcγR, followed by activation of CaMKIV, which mainly contributes to activation of CREB (Sato et al., 2006; Takayanagi, 2007a; Wada et al., 2006). We examined whether Tmem64 affects PLC<sub>γ</sub>2 activation by RANKL stimulation (Figure 3D, upper panel) and found no differences between WT and Tmem64<sup>−/−</sup> activation of PLC<sub>γ</sub>2 in BMMs, whereas CaMKIV was barely activated in RANKL-stimulated Tmem64<sup>−/−</sup> BMMs.

Figure 2. Tmem64 Deficiency Inhibited Osteoclastogenesis and Bone Resorption

(A) Northern blot analysis of Tmem64 mRNA in BMMs from WT and Tmem64<sup>−/−</sup> mice (left panel), and real-time PCR (right upper) and conventional RT-PCR (right lower) of Tmem64 mRNA expression during osteoclast differentiation. (B) Osteoclast differentiation of the WT and Tmem64<sup>−/−</sup> cells. Right shows the number of TRAP<sup>+</sup> MNCs. Scale bar represents 100 μm. (C) Expression of osteoclast marker genes during osteoclastogenesis. Total RNA was collected on the indicated days and subjected to real-time PCR. (D) The percent frequency of a population of osteoclast precursor cells (c-Fms<sup>+</sup>c-Kit<sup>+</sup>Mac-1<sup>low</sup>) is shown as the mean ± SD. (E) Proliferation was assessed by the absorbance of incorporated BrdU in WT and Tmem64<sup>−/−</sup> BMMs. (F) Caspase-3 activity was measured in mature osteoclasts. (G) Bone resorption activity of WT and Tmem64<sup>−/−</sup> osteoclast. After differentiation of BMMs into osteoclasts on dentine slices, cells were stained with the TRAP Kit and pit area was analyzed. Scale bar represents 200 μm. *p < 0.01 between the indicated groups. NS, not significant. Data are represented as mean ± SD.
Based on these data indicating that Tmem64 regulates CaMKIV activation, we examined RANKL-induced Ca^{2+} oscillation, which is important for CaMKIV activation (Sato et al., 2006). As shown in Figure 3E, RANKL-induced Ca^{2+} oscillation was impaired in BMMs derived from Tmem64^{-/-} mice.

Because mitochondrial ROS produced downstream of RANK and ITAM during osteoclastogenesis activate CREB
(Ishii et al., 2009), we investigated whether ablation of Tmem64 affected the production of mitochondrial ROS by RANKL. Using a mitochondrial ROS-specific dye (MitoSOX), we showed that the production of mitochondrial ROS was substantially inhibited in the absence of Tmem64 after stimulation by RANKL for 6 hr (Figure 3F). Consistent with these data, PGC1α upregulation, which follows mitochondrial ROS production and CREB activation, was suppressed (Figure 3B) (Ishii et al., 2009). In order to verify the role of Tmem64, we infected Tmem64−/− BMMs with retroviral Tmem64 (MSCV-Tmem64). As expected, Ca²⁺ oscillation and mitochondrial ROS production were dramatically enhanced by RANKL stimulation (Figures 3G and 3H); transduced cells differentiated into mature osteoclasts (Figure S6B). Thus, Tmem64 is required for proper [Ca²⁺]i oscillation and optimal production of mitochondrial ROS induced by RANKL during osteoclast differentiation.

Tmem64 Associates with SERCA2

When transfected into HEK293 cells, Tmem64 was mostly associated with microsomal fractions containing endoplasmic reticulum (data not shown). Therefore, to further investigate the molecular mechanism of Tmem64, we immunoprecipitated the microsomal fraction of HEK293T cells transfected with Flag-tagged Tmem64 and identified the associated proteins by Coomassie blue staining followed by mass spectrometry (Figure S4; Table S1). Interestingly, Tmem64 associated with SERCA2, a protein essential for spiking Ca²⁺ oscillations (Müller et al., 2006). To specifically determine the SERCA subtype that mediated osteoclast differentiation, we performed real-time PCR. As shown in Figure 4A, Atp2a2 (encoding SERCA2) was strongly induced in comparison Atp2a3 (encoding SERCA3), whereas Atp2a1 (encoding SERCA1) was not detected and there was no difference in the expression of Atp2a2 and Atp2a3 between Tmem64−/− and WT cells. Immunoblotting of total proteins from RANKL+M-CSF-treated cells with an antibody specific for SERCA2 revealed that it was highly upregulated during osteoclast differentiation (Figure 4B). To confirm the association between Tmem64 and SERCA2, we cotransfected Myc-tagged SERCA2 and Flag-tagged Tmem64 into HEK293T cells. Coimmunoprecipitation (coIP) showed that Tmem64 and SERCA2 were strongly associated (Figure 4C). Then we performed communolabeling and confocal microscopy to clarify the subcellular localization of Tmem64 in HEK293T cells (Figure 4D). Confocal images indicated that Tmem64 colocalizes with SERCA2 in the ER; calreticulin was used as an ER marker. When retroviral Flag-tagged Tmem64 was reintroduced into Tmem64−/− BMMs, we found that endogenous SERCA2 coprecipitated with Tmem64-Flag from Tmem64-complemented BMMs (Figure 4E). SERCA2 is a critical regulator of RANKL-induced osteoclast formation by RANKL-mediated [Ca²⁺]i oscillations in the NFATc1 pathway (Yang et al., 2009), raising the possibility that suppression of SERCA2 activity may contribute to the phosphorylation of CREB by RANKL. Thus, we examined whether suppression of CREB phosphorylation impaired [Ca²⁺]i oscillations in SERCA2 heterozygotes (SERCA2+/−). As shown in Figure 4F, CREB activation was severely inhibited in SERCA2+/−; we also observed that osteoclast differentiation was completely rescued by retroviral expression of CREB (Figure 4G). Consistent with these data, the production of mitochondrial ROS 6 hr after RANKL stimulation was suppressed in SERCA2−/− cells (Figure 4H, left panel). We also confirmed that Tmem64 siRNA repressed the generation of mitochondrial ROS in SERCA2 WT cells (Figure 4H, right panel) and, conversely, the retroviral expression of Tmem64 rescued osteoclast differentiation in SERCA2−/− cells (Figure 4I). These data indicate that Tmem64 interaction with SERCA2 is critical in the Ca²⁺ signaling cascade for RANKL-induced CREB activation and mitochondrial ROS generation.

Tmem64 Modulates SERCA2 Activity by Association with Its Regulatory C-Terminal Region during Osteoclastogenesis

To gain insight into the SERCA activity critical for Ca²⁺ signaling modulation by Tmem64, we measured intrinsic Ca²⁺-ATPase activity, which represents SERCA activity (Randriamboavonjy et al., 2008), in BMMs from WT and Tmem64−/− mice using an enzyme-coupled assay. As shown in Figure 5A, Ca²⁺-ATPase activity was reduced by ~60% in Tmem64−/− BMMs versus WT BMMs. Ionomycin, an intracellular calcium-elevating compound, stimulates calcineurin/NFATc1 signal transduction in BMMs, which leads to enhanced osteoclast differentiation. These data indicate that both ER Ca²⁺ release and reuptake by ER-process proteins, such as IP₃Rs and SERCA, are critically important to the Ca²⁺ signaling cascade. Consequently, decreased Ca²⁺ concentrations in the ER trigger activation of the store-operated Ca²⁺ entry (SOCE) pathway, thus activating SERCA in the ER (Takayanagi, 2007b), but it was unclear whether this occurred through increased activation or conductance of Ca²⁺ release channels, or from enhanced store filling. To clarify the mechanism of activation, we examined whether functional coordination of Ca²⁺ release and sequestration is required for RANKL-dependent osteoclastogenesis. We used a loss-of-function approach for SERCA2, employing thapsigargin, a specific inhibitor of SERCA. As shown in Figure 5B, ionomycin-induced intracellular Ca²⁺ influx synergistically enhanced TRAP⁺ MNC formation induced by RANKL; and this effect was completely abolished by thapsigargin in WT cells without cytotoxicity (Figure S5A). In addition, ionomycin did not fully restore the osteoclast differentiation defects in the absence of Tmem64. Thus, modulation of SERCA2 activity by Tmem64 (and subsequent calcium uptake) is necessary for the RANKL-induced osteoclast differentiation.

One remaining question was how Tmem64 regulates SERCA2 activity. One possible mechanism is through a direct interaction with the regulatory region of SERCA2. The Camacho group demonstrated that the intraluminal loop (L7–8) domain of SERCA2 plays a critical role in the regulation of ER Ca²⁺ homeostasis in Xenopus oocytes (Li and Camacho, 2004). Overexpression of ERP57 specifically inhibits SERCA2 activity through association with the L7–8 domain of SERCA2. Based on this observation, we hypothesized that Tmem64 binds and/or modulates the L7–8 domain of SERCA2, which switches SERCA2 to its active form. To investigate this hypothesis, we generated deletion mutants of SERCA2 (Figure 5C) and looked for binding to Tmem64. As shown in Figure 5D, in vitro deletion mapping indicated that Tmem64 binds directly to the 257 amino acid C-terminal segment of SERCA2, which contains the L7–8 domain (SERCA2F2). Immunoprecipitation experiments...
confirmed that SERCA2F1 (1–787), which lacks the C-terminal segment, did not bind to Tmem64-Flag. Further evaluation of the specificity of the Tmem64-SERCA2 association was addressed in competition assays using ERp57, which is moderately expressed during osteoclast differentiation without substantial changes in Tmem64−/− versus WT cells (Figure S5B). We confirmed the association between the SERCA2 C terminus region and ERp57 (Figure S5C). As shown in Figure 5E, reciprocal coimmunoprecipitation performed using Tmem64-Flag and SERCA2-Myc, in addition to increasing the amount of...
ERp57-HA, demonstrated association between Tmem64 and SERCA2 is reduced by ERp57 in a dose-dependent manner, and that Tmem64 association with the L7–8 domain in the C terminus region of SERCA2 was required for SERCA2 Ca²⁺ pump activation. Consistently, we also showed that ERp57 inhibited CREB phosphorylation and suppressed osteoclast differentiation (Figures S5D and S5E). Finally, to explore the relevance of our results to the modulation of SERCA2 activity, we examined whether Tmem64 is required for SERCA2 activation. As shown in Figure 5F, the activity of Ca²⁺-ATPase was fully recovered in MSCV-Tmem64-introduced Tmem64+/− BMMs. Taken together, the results demonstrate

Figure 5. Tmem64 Modulates RANKL-Stimulated Ca²⁺ Signal via Interaction with SERCA2 C Terminus Region
(A) Comparison of Ca²⁺-ATPase activity from WT and Tmem64−/− BMMs. Details are described in Experimental Procedures.
(B) Induction of Ca²⁺ by ionomycin only partially rescued osteoclast differentiation of Tmem64−/− BMMs. BMMs were cultured with thapsigargin (5nM) and ionomycin (0.5 μM) for 3 days with medium containing M-CSF and RANKL.
(C) Schematic representation of SERCA2 and the series of SERCA2 deletion constructs.
(D) Tmem64 interacts with the SERCA2 C terminus. Flag-tagged Tmem64 coimmunoprecipitates with the Myc-tagged SERCA2 deletion series. HEK293T cells were transfected as indicated with Tmem64-Flag, SERCA2-Myc, SERCA2F1-Myc, and SERCA2F2-Myc. Cells were lysed and Tmem64 was immunoprecipitated with anti-Flag antibody. Coimmunoprecipitated SERCA2 was detected by anti-Myc antibody; *nonspecific band.
(E) Competitive interaction between Tmem64 and ERp57. HEK293T cells were transfected with a constant amount (1 μg) of pcDNA3.1-Tmem64-Flag and pcDNA3.1-SERCA2-Myc and increasing amounts (0.1, 0.2, 0.5, and 1 μg) of pcDNA3.1-ERp57-HA; empty vector was added to ensure a similar amount of DNA was transfected in each sample. After 24 hr, cell lysates were incubated with Flag and HA-affinity agarose gel beads and western blotted with the indicated antibodies.
(F) Retroviral Tmem64 introduction rescued Ca²⁺-ATPase activity in Tmem64−/− BMMs. *p < 0.01 between the indicated groups. Data are represented as mean ± SD. See also Figure S5.
that Tmem64 is required as a modulator of SERCA2 activity and mediates RANKL-dependent Ca\textsuperscript{2+} signaling during osteoclast differentiation.

**DISCUSSION**

Osteoclasts are highly specialized, multinucleated cells that tightly regulate skeletal homeostasis. They are differentiated from hematopoietic stem cells by macrophage colony-stimulating factor (M-CSF, also known as Csft), which stimulates the proliferation and survival of osteoclast precursor cells and RANKL (also known as TNFSF11), a key cytokine for osteoclastogenesis and a member of the tumor necrosis factor (TNF) family. Recent studies have determined the function of NFATc1, which is induced by RANKL and plays a crucial role in differentiation, fusion, maturation, activation, and survival of osteoclasts (Ferron et al., 2011; Kim et al., 2008; Takayanagi, 2007b; Takayanagi et al., 2002). Transcription of NFATc1 is mainly regulated by Ca\textsuperscript{2+} signals, which are activated by costimulatory signaling via FcγRI and DAP12. Although the mechanism has not been clearly elucidated in osteoclasts, Ca\textsuperscript{2+} oscillation induced by RANKL is thought to be important for efficient activation of NFATc1 via the Ca\textsuperscript{2+}-dependent phosphatase calcineurin, which lies downstream of FcγRI and DAP12. After Ca\textsuperscript{2+} stimulation, activated NFATc1 has a short half-life. Thus, a \textit{“cycling hit”} such as continuous Ca\textsuperscript{2+} spiking is required for long-lasting transcriptional activation of NFATc1 during osteoclastogenesis (Negishi-Koga and Takayanagi, 2009; Yang and Li, 2007). A Ca\textsuperscript{2+} cycling hit requires tight regulation of the reduction and refilling of ER Ca\textsuperscript{2+} stores. IP\textsubscript{3}R-mediated signaling has an IP\textsubscript{3}R2- and IP\textsubscript{3}R3-dependent physiological role in ER Ca\textsuperscript{2+} release during osteoclast differentiation (Kuroda et al., 2008). Recently, it has been reported to regulate Ca\textsuperscript{2+} entry processes during osteoclastogenesis by plasma membrane-localized Ca\textsuperscript{2+}-permeable channels, such as Orai1 and TRPV4 (Masuyama et al., 2008; Robinson et al., 2012). However, the mechanisms or molecules involved in Ca\textsuperscript{2+} reuptake into ER stores by RANKL-dependent Ca\textsuperscript{2+} signaling are largely unknown. Here, we determined that ablation of Tmem64 impaired osteoclast differentiation, causing strong suppression of RANKL-triggered CREB activation and c-fos and NFATc1 induction. Further, Tmem64\textsuperscript{-/-} BMMs exhibited attenuated RANKL-induced Ca\textsuperscript{2+} oscillations. Consistent with these data, introduction of CREB, c-fos, or Tmem64 into Tmem64\textsuperscript{-/-} BMMs were sufficient to rescue osteoclastogenesis (Figures 3C and S6).

Tmem64 has seven predicted transmembrane domains and a conserved SNARE domain by TMPREED program analysis, but its function has been completely unknown. In this study, we showed that Tmem64 is associated mainly with ER. Intriguingly, in vitro pull-down assays and coimmunoprecipitation analyses revealed that Tmem64 associates with SERCA2, which impairs Ca\textsuperscript{2+} oscillations and leads to defective osteoclast differentiation in heterozygotes. Furthermore, we provide mechanistic clarification of both the suppression of CREB phosphorylation and the downregulation of NFATc1 during osteoclastogenesis (Figure 4F). Indeed, we observed decreased SERCA2 activity (by ~60%) in Tmem64\textsuperscript{-/-} BMMs compared with WT cells. However, it is not known how SERCA2 regulates intracellular Ca\textsuperscript{2+} homeostasis for Ca\textsuperscript{2+} oscillations during osteoclastogenesis. We found that Tmem64 specifically associated with the L7–8 domain of SERCA2 (SERCA2F2) by competing with Erap7, which is necessary for Ca\textsuperscript{2+} oscillations, whereas Tmem64 is required for their activation. Posttranslational modifications, such as SUMOylation, are critical regulators of protein function and play an important role in various cellular processes (Geiss-Friedlander and Melchior, 2007). Kho et al. (2011) determined that SERCA2a function is regulated by SUMO1-dependent modulation in heart failure. SUMOylation of SERCA2a elevated its stability and activity, thereby improving heart function. We also identified E3 SUMO-protein ligase RanBP2 as a Tmem64-associated factor via coimmunoprecipitation (Table S1). RanBP2 binds Ubc9 and SUMO1 and enhances SUMOylation of HDAC4, Sp100, and PML (Kirsh et al., 2002; Wilkinson and Henley, 2010); however, the relationship between Tmem64, SERCA2, and RanBP2 requires further investigation.

One of the most noteworthy findings of this study is that RANKL-induced Ca\textsuperscript{2+} signaling is dependent on SERCA2 activity, which is critically mediated by Tmem64. Our study provides important insight into the coordinated mechanism of SOCE and Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels in osteoclasts. Furthermore, significant suppression of SERCA2 activity by RANKL correlated with the generation of mitochondrial ROS. Involvement of mitochondrial ROS in NFATc1 induction has been clearly demonstrated (Ishii et al., 2009; Ke et al., 2006). However, these data are subject to different interpretations with respect to the cause-effect relationship between Ca\textsuperscript{2+} signaling and mitochondrial ROS generation by RANKL. Our study shows that Ca\textsuperscript{2+} oscillations lead to the production of mitochondrial ROS, which is reversed by full-length Tmem64 (Figures 3G and 3H).

Interestingly we also showed that cyclosporin A treatment significantly inhibited RANKL-induced Tmem64 mRNA expression (Figure S3C), suggesting that NFATc1 is mediating the upregulation of Tmem64 mRNA expression during osteoclast differentiation. Taken together with its importance to Ca\textsuperscript{2+} oscillation/NFATc1 activation, Tmem64 is likely to control the positive feedback controlled regulation of NFATc1-mediated osteoclastogenesis.

Although not studied in detail here, the effect of Tmem64 on osteoblasts appears to be cell-intrinsic, as shown in calvarial or bone marrow stromal cells cultured in osteogenic media. However, the detailed mechanism of how Tmem64 regulates osteoblast differentiation needs future study. Moreover, although we clearly showed that there was decreased osteoclast activity and increased osteoblast activity in Tmem64\textsuperscript{-/-} mice, and Tmem64 has a cell-intrinsic role in both cell types, it is not clear in which cell type Tmem64 is more important to the Tmem64\textsuperscript{-/-} bone volume phenotype. This will require future studies involving cell-type-specific deletion of Tmem64.

In summary, our study suggests that Tmem64 in the ER of BMMs contributes to the efficient activation of SERCA2 (Figure 6). Impairment of Ca\textsuperscript{2+} signaling pathways in the absence of Tmem64 reduces SERCA2 activity and mitochondrial ROS production, resulting in inefficient osteoclast differentiation and function. Identification and elucidation of key mediators of Ca\textsuperscript{2+} oscillation by RANKL should aid in the development of therapeutic strategies for the treatment of skeletal diseases.
mineral deposition rate were performed as previously described. For details, Measurement of Mineral Deposition Rate Microcomputed Tomography, Histological Analysis, accredited facility using protocols approved by the Animal Care and Use mice, and all animal work was performed with veterinary supervision in an reported by our group (Prasad et al., 2005). We used 6- to 8-week-old male described (Song et al., 2012). Cells were cultured for 3 days in Bone marrow-derived macrophages (BMMs) were obtained from cultures of were fixed with 3.7% formaldehyde in PBS for 10 min and stained for TRAP M-CSF (60 ng/ml) and RANKL (150 ng/ml). After culture for 3 days, the cells were counted. To analyze bone pit formation, BMMs (1 × 10⁴ cells) containing three or more nuclei and an actin ring were were multiplied by an in-frame fusion between the 5' exons of the trapped gene and a reporter, β-geo (a fusion of β-galactosidase and neomycin phosphotransferase I). To determine the location of the genomic insertion site in the SYA242 stem cell line, genomic DNA was extracted from the embryonic stem cells by using the DNeasy blood and tissue kit (QIAGEN). PCR was then performed using primers P1 (within intron 2-3 of Tmem64, 5'-AGTCCATCGGCCTCAAGTGG-3') and P3 (within the β-geo gene of the gene-trapping vector, 5'-AGATATCGGCCTCAGGAAGATCG-3'), and the PCR product was sequenced to verify the insertion site. The embryonic stem cells were injected into C57BL/6 blastocysts to create chimeric mice, PCR was then performed using primers P1 (within intron 2–3 of Tmem64, 5'-GCATGCACTGTAGAC-3'), and P3 were used for multiplex genotyping thereof all three the gene-trapping vector, 5'-AGTCCATCGGCCTCAAGTGG-3') and P3 (within the insertion site in the SYA242 stem cell line, genomic DNA was extracted from the embryonic stem cells by using the DNeasy blood and tissue kit (QIAGEN). PCR was then performed using primers P1 (within intron 2–3 of Tmem64, 5'-AGTCCATCGGCCTCAAGTGG-3') and P3 (within the β-geo gene of the gene-trapping vector, 5'-AGATATCGGCCTCAGGAAGATCG-3'), and the PCR product was sequenced to verify the insertion site. The embryonic stem cells were injected into C57BL/6 blastocysts to create chimeric mice, which were bred with C57BL/6 mice to generate heterozygous Tmem64-deficient mice. The heterozygous mice were interbred to generate all Tmem64-deficient genotypes. Primers P1, P2 (5'-GCATGCACTGTAGAC CAGGTGC-3') and P3 were used for multiplex genotyping thereof all three genotypes, and an animal was born previously reported by our group (Prasad et al., 2005). We used 6- to 8-week-old male mice, and all animal work was performed with veterinary supervision in an accredited facility using protocols approved by the Animal Care and Use Committee of the University of Pennsylvania. All other reagents were acquired from Sigma.

Microcomputed Tomography, Histological Analysis, and Measurement of Mineral Deposition Rate Microcomputed tomography, histological analysis, and measurement of mineral deposition rate were performed as previously described. For details, see the Supplemental Experimental Procedures (Lee et al., 2006).

Osteoclast Differentiation Bone marrow-derived macrophages (BMMs) were obtained from cultures of bone marrow collected from 6- to 8-week-old male C57BL/6 mice and from femurs as described (Song et al., 2012). Cells were cultured for 3 days in α-MEM containing M-CSF (60 ng/ml) and RANKL (150 ng/ml). After culture for 3 days, the cells were fixed with 3.7% formaldehyde in PBS for 10 min and stained for TRAP using the acid phosphatase, leukocyte (TRAP) kit (Sigma). TRAP-positive multinucleated cells containing three or more nuclei and an actin ring were counted. To analyze bone pit formation, BMMs (1 × 10⁴ cells/well in 96-well plates) were seeded onto dentine slices and allowed to differentiate into osteoclasts in the presence of M-CSF and RANKL for 5 days, with a change of medium after 2 days. Cells on the dentine slice were removed by washing with PBS, and stained with 1% toluidine blue (Sigma-Aldrich); pit formation was analyzed using the VIA-160 video image-maker measurement system (Boeckeler Instruments).

Osteoclast Proliferation and Apoptosis Assay Proliferation was measured by absorbance at 450 nm by using the BrdU Cell Proliferation Assay Kit (Cell Signaling) according to the manufacturer's protocol. Osteoclast apoptosis was assayed as previously described (Kim et al., 2009). Briefly, purified osteoclasts were cultured without RANKL for 9 hr and then measured with a caspase-3 colorimetric assay kit (R&D Systems).

Mitochondrial ROS Measurement ROS were measured by flow cytometry using the MitoSOX Red mitochondrial superoxide indicator. Cells were treated as indicated, stained with 5 μM MitoSOX Red for 10 min, and washed with media three times for 2 min per wash. Cells were detached with enzyme-free cell dissociation solution (Millipore, Bedford, MA). Detached cells were washed once with PBS and resuspended in PBS containing 2% FBS and 0.7 mM EDTA (disodium salt). Fluorescence was recorded on the PE channel (excitation 510 nm, emission 580 nm) of a FACSCalibur (BD Biosciences, Heidelberg, Germany). For each analysis, 1 × 10⁴ cells were counted. Mean values of the log fluorescence in individual samples were recorded and normalized to control cells.

SERCA2 Activity SERCA2 activity was measured with an enzyme-coupled assay according to previously described methods (Randriamboavonjy et al., 2008).

[Ca²⁺]i Oscillation A total of 5 × 10⁴ BMM cells were seeded on the coverslips in the bottom of a 24-well plate and cultured with RANKL (150 ng/ml) in the presence of M-CSF (60 ng/ml) for 48 hr. Cells were then incubated in the presence of 5 μM fluo-4 AM, 10 μM Fura Red AM, and 0.05% Pluronic F-127 (Invitrogen) for 30 min in serum and phenol red-free α-MEM (Invitrogen). Cells were washed twice with α-MEM and postincubated in α-MEM with 10% FBS, and 10 ng/ml RANKL for 20 min. The dye-loaded cells were washed twice with α-MEM and Hank’s buffered salt solution. Cells were viewed on the inverted stage of a confocal microscope (Leica). The loading medium consisted of 115 mM NaCl, 20 mM HEPES-NaOH, 5.4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, and 10 mM glucose (pH 7.4). At an excitation wavelength of 488 nm, emission at 493–539 nm for fluo-4 and 653–742 nm for Fura Red was analyzed simultaneously at 5 s intervals. The ratio of fluo-4 to Fura Red was calculated to estimate intracellular Ca²⁺ influx concentrations in single cells (Sato et al., 2006).

Statistical Analysis Data were analyzed by using Student’s two-tailed t test and are presented as mean ± SEM or ± SD, as indicated. Means were checked for statistical differences using the Student’s t test with error probabilities of *p < 0.01 and **p < 0.05.

SUPPLEMENTAL INFORMATION Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.01.002.

ACKNOWLEDGMENTS We are thankful to Dr. Daewon Jeong (Youngnam University, Korea) and Dr. Matthew C. Walsh (University of Pennsylvania) for helpful discussions and critical reading of the manuscript. This work was in part supported by the Korea Institute of Oriental Medicine (KIOM) from Ministry of Education, Science and Technology (MEST) (K12050 to T.K., Y.C.), an AHA Beginning Grant-in-Aid award (11BGIA772005 to V.P.), the National Research Foundation of Korea (NRF) Grant funded by the Korea government (MEST) (2011-0030719 to S.H.L.) and by NIH (to Y.C.).


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Supplemental Information

Tmem64 Modulates Calcium Signaling during RANKL-Mediated Osteoclast Differentiation
Hyunsoo Kim, Taesoo Kim, Byung-Chul Jeong, Il-Taeg Cho, Daehee Han, Noriko Takegahara, Takako Negishi-Koga, Hiroshi Takayanagi, Jae Hee Lee, Jai-Yoon Sul, Vikram Prasad, Seoung-Hoon Lee, and Yongwon Choi
Figure S1 (Related to Figure 1). Defective Osteoclast Differentiation in the Absence of Tmem64

(A) Northern blot analysis of tissue distribution of Tmem64 mRNA. (B) Tmem64 positively regulates RANKL-induced osteoclast differentiation. BMMs were infected with retroviruses encoding Tmem64 and control siRNA. Infected BMMs were cultured for 3 days in the presence of RANKL (150 ng/ml) and M-CSF (60 ng/ml). Cells were stained with a TRAP-staining kit and positively stained MNCs were counted. (C) Change in bone volume in WT and Tmem64−/− mice at different ages. (D) Osteoclast differentiation from WT or Tmem64−/− BMMs induced by co-cultures with WT or Tmem64−/− osteoblasts (Ob). TRAP+ osteoclasts after co-culture with osteoblasts for 6 days are shown and counted. Scale bar, 100 µm. *P < 0.01 between the indicated groups. Data are presented as means ± SD.
Figure S2 (Related to Figure 1). Ablation of Tmem64 Enhances Osteoblast Differentiation

(A) Expression of Tmem64 during osteoblast differentiation. (B) WT and Tmem64<sup>−/−</sup> BMSCs were cultured with osteogenic medium, and then stained with alkaline phosphatase (ALP-S) or alizarin red (AR-S). ALP activity was quantified at 7 days. (C) Expressions of positive marker genes in osteoblast differentiation from WT and Tmem64<sup>−/−</sup> BMSCs. *Alp*, alkaline phosphatase; *Col1a*, collagen type I; *Ocn*, osteocalcin; *Runx2*, Runt-related transcription factor 2; *Osx*, osterix; *Tnfrsf11b*, OPG; *Csf1*, colony-stimulating factor 1 and *Tnfsf11*, RANKL. Osteoblast marker expression at 7 day was analyzed by real-time PCR. (D) Calvarial cells from *Tmem64<sup>+/+</sup>::BAT-GAL and Tmem64<sup>−/−</sup>::BAT-GAL mice were lysed and assayed for β-gal activity using chromogenic substrate, which was normalized to protein concentration. X-gal
staining of WT and Tmem64<sup>−/−</sup> carrying the BAT-Gal reporter reflecting β-catenin transcriptional activity. (E) Serum concentration of RANKL was measured by ELISA kit (R&D systems). *P < 0.01 between the indicated groups. Data are presented as means ± SD.
Figure S3 (Related to Figure 3). Tmem64 mRNA Expression during Osteoclast Differentiation

(A) Expression of RANK and c-fms during osteoclast differentiation from WT and Tmem64−/− BMMs. (B) M-CSF-induced ERK, p38, JNK and AKT phosphorylation in BMMs derived from WT and Tmem64−/− mice. BMMs were stimulated with M-CSF (30 ng/ml) prior to western blotting. (C) Tmem64 mRNA expression is regulated by NFATc1 during osteoclast differentiation. BMMs were differentiated to osteoclasts by M-CSF and RANKL in the presence of various inhibitors for ERK (U0126), p38
(SB203580), JNK (SP600125), NF-κB (SN50) and NFATc1 (CsA) for 3 days. Total RNA was isolated and subjected to real-time PCR analysis. Data are presented as means ± SD.
Figure S4 (Related to Figure 4). Isolation of Tmem64-Associated Proteins

HEK293T cells transiently expressing Flag-Tmem64 were lysed and subjected to immunoprecipitation using anti-Flag antibody. Proteins were separated by SDS-PAGE, visualized with Comassie Blue R-250, and subjected to MS/MS mass spectrometry.
Figure S5 (Related to Figure 5). ERp57 Inhibits Osteoclast Differentiation via its Interaction with SERCA2 C Terminus Region
(A) Cytotoxicity of thapsigargin during osteoclast differentiation. BMMs were induced to differentiate with thapsigargin (0–5 nM) for 3 days in the presence of RANKL (150 ng/ml) and M-CSF (30 ng/ml). Cytotoxicity was determined using MTT. Data are presented as means ± SD. (B) Expression of ERp57 during osteoclast differentiation. Cells were cultured with RANKL and M-CSF; total RNA was isolated and subjected to reverse-transcription real-time PCR. Data are presented as means ± SD. (C) ERp57 interacts with the SERCA2 C-terminus region. HA-tagged ERp57 coimmunoprecipitates with Myc-tagged SERCA2 deletion mutants. HEK293T cells were transfected with ERp57-HA, SERCA2-Myc, SERCA2F1-Myc and SERCA2F2-Myc. Cells were lysed and ERp57 was immunoprecipitated with anti-HA antibody. Coimmunoprecipitated SERCA2 was detected by anti-Myc antibody. (D) ERp57 inhibits osteoclast differentiation. Data are presented as means ± SD. (E) Suppression of CREB phosphorylation by ERp57. BMMs were infected with a retroviral vector encoding HA-tagged ERp57 or with the empty vector and were differentiated with RANKL. Cell lysates were subjected to western blot analysis with the indicated antibodies.
Figure S6. Restoration of Osteoclast Differentiation with Tmem64 and c-Fos in Tmem64<sup>−/−</sup> BMMs

(A and D) BMMs from WT and Tmem64<sup>−/−</sup> mice were infected with retroviral Tmem64-Flag, c-fos-Flag or empty vectors (EV). Cell lysates were immunoblotted with anti-Flag antibody. (B and E) Osteoclast differentiation rescue by retroviral expression of Tmem64-Flag, c-fos-Flag, or empty vector in WT and Tmem64<sup>−/−</sup> BMMs. TRAP<sup>+</sup> MNCs were counted. (C and F). After introduction of Tmem64-Flag or c-fos-Flag into Tmem64<sup>−/−</sup> BMMs, NFATc1 expression was analyzed by real-time PCR. Scale bar, 200 µm. *P < 0.01 between the indicated groups. Data are presented as means ± SD.
Table S1. Identification of TMEM64 associated proteins by ms/ms spectrometry.

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Supplemental Experimental Procedures

RNA interference
RNA interference oligonucleotides (TMEM64, 5′-GTGGAATTGAATGCAGCTA-3′) were synthesized by Integrated DNA Technologies and cloned into the retroviral small interference RNA (siRNA) vector pSuper retro Puro (OligoEngine). BMMs were infected with retroviruses and cultured with M-CSF (60 ng/ml) and sRANKL (150 ng/ml) for 3–5 days to generate osteoclasts. For osteoclast analyses, osteoclasts were fixed with 10% formalin and stained for TRAP activity, whereas bone slices were stained with 0.5% toluidine blue as described previously (Lee et al., 2006).

Real-time PCR and protein analyses
Total RNA was isolated from cells using TRIzol Reagent (Invitrogen); cDNA was synthesized by reverse transcriptase (Invitrogen). To quantify gene expression, real-time PCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems) using TaqMan™ probes. For protein analysis, cells were lysed in RIPA buffer containing 25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor and phosphatase inhibitor cocktail (Roche). Protein samples (20-40 µg) were subjected to SDS-PAGE on a 10% NuPAGE gel (Invitrogen) and visualized by peroxidase solution and Luminol Enhancer solution (Thermo Scientific). The following primary antibodies were used: Actin (A2066; Sigma); NFATc1 (7294; Santa Cruz); CaMKIV (610276; BD Bioscience); PGC1β (18-003-44008; GenWay); SERCA2 (a gift from Dr. Jonathan Lytton; University of Calgary); phospho-CaMKIV Thr196 (28442; Santa Cruz); phospho-JNKThr183/Tyr185 (612541; BD Bioscience); phospho-ERK Thr202/Tyr204 (9101), phospho-p38 Thr180/Tyr182 (9211), phospho-IκBα (2859), phospho-AKT (9271), phospho-PLCγ2 (3874), ERK (9102), p38 (9212), JNK (9252), IκBα (9242), AKT (9272), CREB (4820) and PLCγ2 (3872), and c-Fos (2250) from Cell Signaling Technology.

Retroviral gene transduction
To prepare retroviral particles, PLAT-E packaging cells (3 × 10^6) or GP2-293 were plated on a 10-mm culture dish and transfected with pMX or MSCV vectors encoding
Flag-tagged Tmem64, c-Fos, PGC1β, CREB, and a dominant-negative inhibitor of CREB, A-CREB, using Lipofectamine™ 2000 (Invitrogen). After 3 days, the medium containing retroviruses was harvested and passed through a syringe filter (0.2 μm pore diameter). BMMs or primary osteoblasts were infected with retroviruses for 8 hours with hexadimethrine bromide (8 μg/ml) in the presence of M-CSF (120 ng/ml). After washing with fresh medium, the cells were cultured for 2 days in the presence of puromycin (2 μg/ml) with M-CSF (120 ng/ml). Puromycin-resistant BMMs or osteoblasts cells were studied.

**Osteoblast differentiation and osteoclast/osteoblast co-culture**

Primary osteoblasts were prepared from the bone marrow stromal cells (BMSCs), which are also known as mesenchymal stem cells (MSC), of 6–8 week-old male mice. Osteoblasts (1 × 10^5 cells/well in 48-well plates) were cultured in osteogenic medium [α-MEM supplemented with 10% FBS, 10 mM β-glycerophosphate, and ascorbic acid (50 μg/ml)] for 15 days. Mineralized nodule formation was assessed on days 6, 9 and 15 by staining with alizarin red S solution (2%, pH 4.2). To quantify calcium deposition, cells were washed with distilled water, the dye was eluted with 10% cetylpyridinium chloride, and absorbance was measured with a microplate reader (Vmax, Molecular Devices) at 570 nm. In the co-culture experiment, BMMs (1 × 10^5 cells/well in 96-well plates) were cultured with calvarial osteoblast cells (5 × 10^3 cells/well) in the presence of 10 nM 1α,25-dihydroxy vitamin D₃ and 1 μM prostaglandin E₂ for 6 days.

**Tmem64-associated protein identification using mass spectrometry**

HEK293T cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, CA), with 10% fetal bovine serum (HyClone, Logan, UT) and antibiotics at 37°C with 5% CO₂. For transfection, 1.5 × 10⁷ cells were plated in 150-mm plates (30–50% confluence) on the previous day pcDNA3.1 (+)-flag and pcDNA3.1 (+)-TMEM64-flag (20 μg) were transfected into cells by polyethylenimine (PEI, Polysciences Inc. Warrington, PA). Cells were harvested after 48 hours, resuspended in buffer A (20 mM HEPES-NaOH, pH 7.4, 0.25 mM Sucrose, 1 mM EDTA, and protease inhibitors), and kept on ice for 10 min.
The swollen cells were disrupted by a dounce homogenizer (tight pestle) with 30 strokes and spun down at 1,000 × g for 10 min. The precipitate was suspended in buffer A and dounce-homogenized with 30 strokes and ultracentrifuged at 100,000 × g for 1h at 4 °C. The precipitates (insoluble fraction) was resuspended in buffer B (50 mM Tris-Cl, pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 1 mM EDTA, and protease inhibitors) and sonicated (Sonic Dismembrator, Fisher Scientific). The lysate was cleared by centrifugation at 13,000 × g for 15 min at 4 °C and the supernatant was incubated with ANTI-FLAG M2 Affinity Gel (Sigma) for 3 h at 4 °C. The beads were washed in buffer B and TMEM64-flag-associated proteins were eluted in buffer B containing 3x FLAG peptide (100 µg/ml). Eluted proteins were separated in 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250. MS/MS spectrometry analyses of excised bands was performed at the Proteomics Core Facility, University of Pennsylvania Perelman School of Medicine. Scaffold (version Scaffold 3.2.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications.

Cytotoxicity assay
Cytotoxicity was assessed as previously described using the MTT (Sigma) assays (Kim et al., 2009). The BMMs (5 × 10⁴ cells/well, 24-well plate) were cultured in the presence of M-CSF (60 ng/ml) for 3 days. MTT was added (500 µg/ml) and incubated for 30 min; cells were washed with PBS and formazan was solubilized with DMSO. Absorbance was measured at 570 nm using a microplate reader. All experiments were repeated for 3 times.

BAT-GAL mice
BAT-Gal (Maretto et al., 2003) mice were purchased from The Jackson Laboratory. The transgenic line BAT-GAL was bred with the TMEM64⁻/⁻ line to generate TMEM64⁻/⁻::BAT-GAL animals. Mice were bred on a C57BL/6J background.

ALP activity
Bone marrow stromal cells were cultured with osteogenic medium containing AA (50 µg/ml) and β-GP (5 mM). At the designated time point, the cell homogenates were reacted with an ALP assay mixture containing 0.1 M 2-amino-2-methyl-1-propanol
(Sigma), 1 mM MgCl₂, and 8 mM p-nitrophenyl phosphate. After incubation at 37°C, the reaction was quenched by adding 0.1 N NaOH, and the absorbance was measured at 405 nm.

X-Gal staining
X-gal staining was performed with the β-galactosidase staining kit (Mirus). Briefly, calvarial cells were fixed, washed and incubated with β-galactosidase substrate in the dark at 37°C. Four hours later, the substrate was removed, and cells were washed and photographed.

β-Gal activity assay
β-Galactosidase activity (β-gal) was assayed by using the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG). Isolated primary calvarial cells were lysed in lysis buffer (Cell Signaling) and then incubated with ONPG buffer at 37°C for 12 hours. Optical density was measured at 420 nm.

Supplemental References

