Title

The tethering function of mitofusin2 controls osteoclast differentiation by modulating the Ca^{2+}-NFATC1 axis

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Abstract:
Dynamic regulation of the mitochondrial network by mitofusins (MFNs) modulates energy production, cell survival, and many intracellular signaling events including calcium handling. However, the relative importance of specific mitochondrial functions and their dependence on MFNs vary greatly among cell types. Osteoclasts have many mitochondria, and increased mitochondrial biogenesis and oxidative phosphorylation enhance bone resorption, but little is known about the mitochondrial network or MFNs in osteoclasts. Because expression of each MFN isoform increases with osteoclastogenesis, we conditionally deleted both MFN1 and -2 (dcKO) in murine osteoclast precursors, finding that this increased bone mass in young female mice, and abolished osteoclast precursor differentiation into mature osteoclasts, in vitro. Defective osteoclastogenesis was reversed by overexpression of MFN2 but not MFN1; therefore we generated mice lacking only MFN2 in osteoclasts. MFN2-deficient female mice had increased bone mass at 1 year and resistance to RANKL-induced osteolysis at 8 weeks. To explore whether MFN-mediated tethering or mitophagy is important for osteoclastogenesis, we overexpressed MFN2 variants defective in either function in MFN1/2 dcKO precursors and found that although mitophagy was dispensable for differentiation, tethering was required. Since the master osteoclastogenic transcriptional regulator nuclear factor of activated T cells 1 (NFATC1) is calcium-regulated, we assessed calcium release from the endoplasmic reticulum (ER) and store-operated calcium entry and found that the latter was blunted in dcKO cells. Restored osteoclast differentiation by expression of intact MFN2 or the mitophagy-defective variant was associated with normalization of store-operated calcium entry and Nfatc1 levels, indicating that MFN2 controls mitochondria-ER tethering in osteoclasts.

Introduction
Mitochondria exist within a highly regulated and dynamic network in most cell types. Termed mitochondrial dynamics, the linked processes of fission, fusion, and mitophagy play an important role in mitochondrial homeostasis. Changes in mitochondrial networks are frequent, and this architecture is varied depending on the developmental or metabolic needs of a cell (reviewed in (1, 2)). Mitofusin1 (MFN1) and Mitofusin2 (MFN2), transmembrane GTPases on the outer mitochondrial membrane, control mitochondrial architecture by tethering adjacent mitochondria and initiating fusion. MFN2 additionally mediates tethering of mitochondria to the ER, promotes mitophagy, and allows mitochondrial transport down axons. Both MFN1 and MFN2 are necessary for survival, as global deletion of either protein results in embryonic lethality prior to e12.5 in mice (3). Double deletion of MFN1 and MFN2 leads to even earlier mortality, and in mouse embryonic fibroblasts causes severe fragmentation of mitochondrial networks (3).

In humans, the nervous system is highly impacted by MFN2 defects, and MFN2 mutations...
are linked to neuropathies (reviewed in (4, 5)). The human syndrome most associated with mutations in MFN2 is Charcot-Marie-Tooth type 2A (CMT2A), a neurodegenerative disorder in which motor and sensory defects develop within the first two decades of life (6). Mice with homologous MFN2 mutations have sensory and/or motor defects and axonal degeneration accompanied by altered mitochondrial dynamics (7-9), and cultured human cells from CMT2A patients display decreased electrical properties and reduced mitochondrial membrane potential (10, 11). Models lacking mitofusins also yield conditions of myocardial hypertrophy, heart tube contractile dysfunction, vascular smooth muscle cell proliferation, and skeletal muscle atrophy, to name a few (12-15). To our knowledge, effects on the skeleton have not been reported in CMT2A mouse models or human patients. Additionally, no human syndromes with MFN1 mutations have been described.

Bone homeostasis relies on the coupled activity of osteoblast (OB) and osteoclast (OC) cells that build and resorb the bone matrix, respectively. OCs are rich in mitochondria and differentiation from bone marrow macrophage (BMM) precursors to OCs is associated with a significant increase in mitochondria DNA copy number, oxidative phosphorylation subunit protein levels, and oxygen consumption rates (16). Previous investigations of mitochondria in the OC lineage have largely focused on the role of mitochondrial biogenesis and oxidative phosphorylation in supporting OC function (17-19). We were prompted to explore the role of mitochondrial dynamics in the OC because this specialized cell type is mitochondria-rich and undergoes vast morphological changes to become a mature multinucleated cell. We hypothesized that mitochondrial fusion might accompany OC precursor fusion during osteoclastogenesis, and that tethering of mitochondria to each other or the ER is important for OC formation and/or function. To test this, we generated a mouse model in which MFN1 and MFN2 are conditionally deleted in the OC lineage with LysM-cre. We find that knockout of both homologs is associated with increased bone mass in female animals and blunted osteoclastogenesis in culture. Rescue experiments in vitro reveal that MFN2 more strongly induces osteoclastogenesis than MFN1, and in vivo, depletion of MFN2 is protective against age- and RANKL-induced bone loss. Lastly, we demonstrate mitochondrial tethering as the predominant function of MFN2 in OCs, associated with changes in Ca^{2+}-Nfatc1 signaling.

**Results**

Bone mass is increased in female mice lacking MFN1 and MFN2 in the OC lineage

Since OCs undergo vast morphological changes to become mature multinucleated cells, we hypothesized that alterations in mitochondrial dynamics, and fusion in particular, accompany and modulate this event. To prevent mitochondrial fusion, we employed mice with floxed alleles for both Mfn1 and Mfn2, directing their excision to the OC lineage with LysM-cre. All of our mice were
mated onto a homozygous cre background \((LysMc/c)\) to increase recombination efficiency. We intercrossed double heterozygous \((LysMc/c; (Mfn1/2)^{+/+}=\text{ctrl})\) mice to generate double knockout \((LysMc/c; (Mfn1/2)^{fl/fl}=\text{dcKO})\) littermates, which were born at normal Mendelian ratios. Subsequently, these dcKO progeny were mated to the double heterozygous ctrl mice to produce our experimental groups. In parallel, double heterozygous ctrl mice were bred to cre-only \((LysMc/c; (Mfn1/2)^{+/+})\) mice to generate a cohort of ctrl and cre-only littermates. Importantly, there was no difference in bone mass parameters between ctrl and cre-only groups (Supp. Fig. 1 A-N), indicating that ctrl double heterozygous animals are an appropriate control group for dcKO mice.

dcKO mice showed no obvious gross phenotypes and had identical weights to ctrl littermates (Supp. Fig 2 A-B). Male and female animals were sacrificed at 2 months of age, a time of high OC activity, and basal bone morphology assessed by \(\mu\)CT of dissected femurs. We find a strong bone phenotype in female animals at this age, with all trabecular and cortical parameters consistent with a high bone mass (significantly increased bone volume per total volume, BV/TV, bone mineral density, BMD, and trabecular number, Tb. N.) Fig. 1 A-B)(Supp Fig 2 C-F). Interestingly, males show no significant differences between ctrl and dcKO groups in any of these parameters (Fig 1 C-D)(Supp Fig 2 G-J). Serum CTX-1 levels are decreased in dcKO females, indicating a decrease in bone catabolism (Fig 2 A). Histomorphometry shows that OC surface and number in dcKOs trend lower than in ctrls in female mice (Fig. 2 B-E). OB-OC coupling is not affected by loss of Mfn1 and Mfn2 in OCs as indices of bone formation such as serum P1NP, mineralizing surface, mineral apposition rate, and bone formation rate are unchanged between groups (Fig. 2 F-K).

Ostoclastogenesis is inhibited in MFN1/2 –deficient cultures

To more closely characterize the impact of mitofusin loss in the OC lineage, BMMs were expanded from the bone marrow of ctrl and dcKO mice. Compared to cre-only OCs with wild type mitofusin expression, double heterozygous ctrls produce less MFN1 and MFN2 protein that is further diminished in dcKO cells (Fig. 3A). dcKOs are unable to respond to RANKL and differentiate into OCs on plastic wells, while the differentiation of ctrls is intact (Fig 3B). When cultured on bone, resorption is also minimal (Fig 3C). Consistently, levels of OC markers \(Nfatc1\), \(Dc-stamp\), and \(Ctsk\) are reduced in dcKOs at the mRNA level (Fig 3 D-F). Western blots also demonstrate lower levels of CTSK and C-SRC proteins, which supporting a defect in OC differentiation (Fig 3G).

Levels of protein and mRNA for both MFN homologs increase throughout osteoclastogenesis in wild type cells (Fig 4 A-B). Therefore, we sought to determine whether MFN1 or MFN2 was dominant in contributing to the dcKO phenotype and attempted to rescue the defect in OC formation by retrovirally expressing either MFN1 or MFN2 in dcKO BMMs. We find that overexpression of MFN2 but not MFN1 restores full OC
differentiation, suggesting that MFN2 is the dominant mitofusin homolog that supports osteoclastogenesis (Fig 4 C-E).

MFN2 plays a role in OCs, in vivo

To assess whether loss of MFN2 alone in OC lineage cells would have an effect on bone mass in vivo, we generated littermate cohorts of mice with wild type Mfn2 (LysMc/c; Mfn2+/+ = cre-only), and conditionally targeted Mfn2 (LysMc/c; Mfn2fl/fl = Mfn2 cKO). We confirmed depletion of MFN2 by evaluating protein and mRNA expression (Fig. 5 A-B). While we observe upward trends in female Mfn2 cKOs at 2 and 4 months of age, the differences in BV/TV and BMD do not become statistically significant until 12 months of age; there is no difference in bone mass between male cKO and male cre-only mice at any age examined (Fig. 5 C-F, Table 1, Supp Table 1). The percent trabecular bone retained from 4 months to 12 months is 24% for ctrls vs. 46% for cKO females (64% vs. 63% for males). TRAP-stained 2 month old tibia sections show a trend toward lower Oc.N./BS and Oc.S/BS in female cKO mice (Supp Fig. 3). Thus, loss of MFN2 has limited effects on basal bone mass in young animals while it protects female mice from age-related bone loss.

Our group and others have previously observed that challenging animals with a stimulus for pathological bone loss can unmask a robust OC phenotype even when basal changes are not prominent (25, 31-33). We hypothesized this may be the case for Mfn2 cKO animals, and therefore induced acute osteolysis in 2-month-old animals. Male and female cre-only and cKO mice were challenged with intraperitoneal RANKL injection, which stimulates OC activity and induces significant loss of trabecular bone in 2 days. While cre-only males, male cKOs, and cre-only females lost 30-45% of trabecular bone fraction following RANKL injection, female cKOs were protected from induced osteolysis, showing no significant change (Fig. 5 G-I, Supp Fig. 4). Therefore, even with a strong OC stimulus, male mice are not affected by loss of MFN2, while female mice require MFN2 in the OC to respond.

Mitochondrial tethering is the dominant MFN2 mechanism contributing to osteoclastogenesis, in vitro

MFN2 has two primary molecular functions – tethering/fusion and mitophagy – that can be selectively disabled via mutation of threonine and serine residues to constitutively activate or ablate the signal for mitophagy (27). MFN2 T111E-S442E (MFN2-EE) mimics phosphorylation by Pink1 kinase, driving spontaneous mitophagy and preventing fusion, leading to a highly fragmented mitochondrial network. In contrast, MFN2 T111A-S442A (MFN2-AA) has the opposite effect; the non-phosphorylatable MFN2 residues eliminate mitophagy but allow normal tethering and fusion (Fig. 6A) (27, 34). To examine the ability of either MFN2 function to impact OCs, we retrovirally overexpressed each mutant, along with empty vector (V) and wild type MFN2 (MFN2-WT) controls, in female dcKO BMMs that are defective in producing OCs (Fig 6B). To verify mutants were
functioning properly we stained transduced BMMs with Mitotracker Green and Lysotracker Red prior to live cell confocal microscopy (Fig 6C). Mitochondrial colocalization with the lysosomes, an indication of mitophagy, was minimal under all conditions. Similar to previous studies with these constructs, MFN2-WT and MFN2-AA increase tethering and fusion as manifested in increased mitochondrial aspect ratios (mito length/mito width) compared to less fused V and MFN2-EE samples (Fig 6D). Following 6 days of RANKL exposure, cultures were fixed and stained for TRAP (Fig 6E-F). We find that transduction of dcKO BMMs with MFN2-AA restores osteoclastogenesis to a comparable level as MFN2-WT, indicating that the mitophagy function of MFN2 is dispensable for osteoclastogenesis. In contrast, transduction with MFN2-EE has only a minimal effect on osteoclastogenesis, suggesting that tethering/fusion by MFN2 is necessary for osteoclastogenesis to occur efficiently (Fig. 6 C-F). Because MNF1 was absent from these cells, this reveals mitochondrial tethering by MFN2 contributes to OC formation in a way that MFN1 cannot.

**Ca\(^{2+}\)** signaling is perturbed in pre-OCs with impaired MFN2-mediated tethering

To further explore the mechanism through which MFN2 controls osteoclastogenesis, we assayed cytoplasmic Ca\(^{2+}\) levels with Fura-2 basally, following ATP stimulation (to release ER calcium stores) and after addition of extracellular calcium (2 mM) to activate store operated calcium entry (SOCE). In pre-OCs generated with 48 hrs of RANKL exposure, dcKOs have higher basal Ca\(^{2+}\) levels and a similar ATP-mediated spike from baseline, but a muted intake of Ca\(^{2+}\) following SOCE activation, compared to ctrls, as assessed by area under curve (AUC) quantification (Fig 7A-C). We used the same assay in dcKO BMMs overexpressing MFN2-WT, EE, and AA. Compared to the vector control, MFN2-WT and MFN2-AA increase responsiveness to extracellular Ca\(^{2+}\), while MFN2-EE does not, suggesting that the tethering function of MFN2 regulates SOCE (Fig 7 D-F). Congruent with the restoration of osteoclastogenesis, the cells with improved SOCE (MFN2-WT and -AA) have increased NFATC1 levels compared to dcKOs with V or MFN2-EE transduction, linking the MFN2 tethering function to the Ca\(^{2+}\)-NFATC1 axis during OC differentiation (Fig 7G).

**Discussion**

The abundance of mitochondria has been deemed important for function of the OC, and increases in mitochondrial proteins are associated with differentiation from myeloid precursors (16, 35, 36). Mitochondrial biogenesis, mediated by PGC1\(\alpha\) and PGC1\(\beta\), plays an important role as global deletion of either of these factors leads to increased trabecular bone mass (17, 37). Further, conditional PGC1\(\beta\) deletion in the OC results in impaired cytoskeletal organization and bone resorption (19). OCs have been reported to utilize both oxidative phosphorylation and aerobic glycolysis, and it has been suggested that the
primary function of mitochondria may not be for ATP generation at the ruffled border (38).

To better understand the role of mitochondria and put into context the elongated organelles observed in human OCs (36), we aimed to perturb mitochondrial network organization. We find that dual depletion of key mitochondrial dynamics proteins MFN1 and MFN2 in the OC lineage leads to increased bone mass in female mice in vivo, which is accompanied by a decrease in serum CTX-1 levels. This corresponds to an inability of OCs derived from these dcKO animals to form in culture. Previous work in the field has focused primarily on how changes in mitochondrial content and oxidative phosphorylation impact OC resorption (16, 19, 39, 40). Our model expands the relationship between mitochondria and OCs by linking disruption of mitochondrial dynamics to inhibition of OC formation.

To determine whether MFN1 and MFN2 contribute differentially to OC formation, we overexpressed each homolog in dcKO BMMs in culture, finding that osteoclastogenesis was much more efficient with MFN2 than MFN1 addition. Further, loss of MFN2 alone leads to increased bone mass in vivo, albeit delayed compared to the doubly deficient cohort. Similarly, work in the heart and skeletal muscle have revealed that loss of both MFN1 and MFN2 produces more severe phenotypes than single knockout of either mitofusin (14, 41). It is likely that the mitochondrial fusion function of MFN2 plays some role in the OC, which allows compensation by MFN1 to reduce the severity of bone accrual in MFN2 singly deficient mice, though at least one MFN2-unique role is also important.

MFN2, but not MFN1, functions in mitophagy, mitochondrial transport in axons, and ER-mitochondrial interactions. Phenotypic effects of specific mutations reveal significant cell type specificity in these various roles. For example, fibroblasts derived from CMT2A patients show differences in ER-mitochondria spacing that correlates with disease severity, suggesting that alteration of ER-mitochondria tethering contributes to pathogenicity in humans (42). In contrast, mitophagy is a requisite for metabolic reprogramming of mammalian hearts during perinatal development, but tethering by MFN2 is dispensable for this process (27). We utilized point mutants separating tethering and mitophagy functions to probe which MFN2 function is important in the OC lineage, and find that the tethering function of MFN2 is necessary for osteoclastogenesis.

The master OC transcription factor NFATc1 is dependent on cytoplasmic Ca\textsuperscript{2+} fluxes and requires dephosphorylation by calcineurin for translocation to the nucleus (43), leading to amplification due to binding at its own promoter. We find that Nfatc1 mRNA levels are diminished in our dcKO cells, suggesting alterations in Ca\textsuperscript{2+} handling. The ER-mitochondria juxtaposition, regulated by the tethering function of MFN2 but not MFN1, is known to impact Ca\textsuperscript{2+} signaling (9, 42, 44). We therefore examined cytoplasmic Ca\textsuperscript{2+} levels, finding that dcKOs had a significantly reduced magnitude of SOCE. A similar blunting of
SOCE was found in C2C12 myoblasts with MFN2 knockdown (45). Interestingly, our dcKOs showed higher basal cytoplasmic Ca$^{2+}$, while MFN2-depleted C2C12 cells and BMMs have lower basal cytoplasmic Ca$^{2+}$ than controls (45, 46). It is possible that the presence of MFN1 in the experiments with MFN2 knockdown plays a role in the basal levels, while MFN2 is more important for SOCE regulation. On our MFN1/MFN2-deficient background, we further found that the tethering competent/mitophagy deficient MFN2-AA, but not the tethering deficient/mitophagy activated MFN2-EE, restored SOCE along with NFATc1 induction and osteoclastogenesis. Previously we found that cells lacking TMEM178, which show increased NFATc1 and osteoclastogenesis, have elevations in both basal cytoplasmic Ca$^{2+}$ and SOCE (47, 48). Thus, it is likely that the magnitude of Ca$^{2+}$ excursions, rather than the basal level, is more important for osteoclastogenesis. Mechanistically, we propose that MFN2-mediated restoration of ER-mitochondrial contacts, which regulate mitochondrial Ca$^{2+}$ uptake, are responsible for the ability of cells to generate the large swings in cytoplasmic Ca$^{2+}$ (i.e. high amplitude oscillations) that drive NFATc1 activation.

We find striking sexual differences in our bone phenotypes, and to our knowledge this is the second report of differential male vs. female responses to mitofusin loss. Conditional ablation of MFN2 in brown adipose tissue leads to a sex-specific remodeling of mitochondrial function; females displayed increased ATP-synthesizing fatty acid oxidation while males experienced increased glycolytic capacity (49, 50). Although loss of MFN2 in brown adipose tissue caused distinct abnormalities in males and females, we only see changes in females with loss of mitofusins in the OC lineage. We have previously found higher OC numbers in the trabecular compartment of female mice, possibly explaining the greater impact on bone mass with more abundant OCs in this sex (51). One explanation could be that there is a threshold effect for mitofusins on OC formation, and females are more sensitive to mitofusin depletion than males. Further, the high bone mass phenotype may be more severe in female dcKOs compared to single Mfn2 cKOs because residual MFN1 expression partially compensates for loss of MFN2. Consistent with this threshold hypothesis, we see no differences in bone mass between cre-only and double heterozygous ctrl animals, despite protein levels differing by about half. Since residual mitofusin proteins were detected in our lysM-cre models, greater effects, including increased bone mass in male mice, might be achieved with more complete recombination in the OC lineage. Future investigations can utilize RANK-cre to target a similar early developmental stage or Cathepsin K-cre to deplete mitofusins in mature OCs.

In sum, our studies highlight the importance of mitofusin-mediated organelle tethering in OC lineage cells and suggest that mitochondrial dynamics are important in the control of bone resorption, at least in females. Preclinical experiments have suggested that downregulation of MFN2 may have beneficial effects in cardiac ischemia (52, 53), and our work opens the
possibility that it may also preserve bone mass in women.

**Experimental Procedures**

**Mice**

Double floxed Mfn1<sup>fl/fl</sup>; Mfn2<sup>fl/fl</sup> animals (20), which delete exon 4 of MFN1 and exon 6 of MFN2 upon cre-recombination, were mated to C57Bl/6 mice harboring the LysozymeM-cre recombinase (LysM-cre) allele (21) to place all animals on a homozygous LysM-cre background. To generate dcKOs, double heterozygous (Mfn1<sup>fl/+</sup>; Mfn2<sup>fl/+</sup>; LysM<sup>c/c</sup>) mice were first bred together and dcKO (Mfn1<sup>fl/fl</sup>; Mfn2<sup>fl/fl</sup>; LysM<sup>c/c</sup>) female progeny mated to double heterozygous males to generate the cohort of double heterozygous controls and dcKOs. In parallel, cre-only (Mfn1<sup>fl/+</sup>; Mfn2<sup>fl/+</sup>; LysM<sup>c/c</sup>) females from the original double heterozygous matings were bred to double heterozygous males to generate the cohort of cre-only and control animals. The single Mfn2 cohort was generated using heterozygous breeding pairs so that progeny genotypes included cre-only (Mfn2<sup>fl/+</sup>; LysM<sup>c/c</sup>) and cKOs (Mfn2<sup>fl/fl</sup>; LysM<sup>c/c</sup>).

Mice were housed communally with ad libitum access to fresh chow and water in a pathogen-free temperature controlled barrier facility. Daily observation and weekly cage change was provided by staff of the Division of Comparative Medicine. Lab members coordinated animal husbandry and breeding, and the DCM veterinarian assessed all health concerns. All protocols were approved by Washington University School of Medicine’s Animal Studies Committee (protocol #20170025).

**Micro-computed tomography**

Following dissection of right femurs from 2, 4, and 12-month old animals, bones were fixed in 10% neutral buffered formalin (Di Ruscio & Associates, Inc., Fenton, MO) for 24 hours, and then stored in 70% ethanol. Trabecular and cortical scans were acquired proximal to the distal growth plate and at the mid-shaft of femurs, respectively (µCT40, Scanco, Brüttisellen, Switzerland) (10 µm resolution, 55kVp, 145 µA, 300 seconds integration time). Thresholds were determined in a blinded manner and analyzed with reference to accepted guidelines (22, 23).

**CTX1 and P1NP**

Serum was collected from animals fasted overnight via mandibular bleed (BD Microtainer), and stored at -80°C. RatLaps CTX-1 and P1NP EIA assays were conducted according to manufacturer’s instructions (Immunodiagnostic Systems, Gaithersburg, MD, USA; AC-06F1 and AC-33F1, respectively).

**Histomorphometry**

2 month-old mice were injected intraperitoneally (IP) with 10mg/kg calcein (C0875; Sigma, USA) and 30 mg/kg alizarin red (A3882; Sigma, USA) seven and two days prior to sacrifice, respectively. Intact femur/tibias were fixed in 10% neutral buffered formalin for 24 hrs prior to storage in 70% ethanol. Tissues were embedded in
methylmethacrylate and sectioned sagittally by the Washington University Musculoskeletal Histology and Morphometry Core. Unstained and TRAP-stained (Sigma, St. Louis, MO, USA) slides were imaged at 20X high-resolution using a NanoZoomer 2.0 with brightfield and FITC/TRITC (Hamamatsu Photonics, Japan). Images were then analyzed via Bioquant Osteo software according to manufacturer’s instructions and published standards (v18.2.6; Bioquant Image Analysis Corp., Nashville, TN, USA)(24).

Bone marrow macrophage (BMM) isolation and osteoclast differentiation

BMMs were harvested by collecting bone marrow from dissected long bones of 2 month old female animals via centrifugation at 10,000 rpm, passed through a 40µm filter and cultured with α-MEM (Sigma M0894, St. Louis, MO, USA) containing 10% FBS (Gibco, Grand Island, NY, USA), 100 IU/ml penicillin/streptomycin, and 1:10 dilution of CMG 14-12 cell supernatant (containing equivalent of 100ng/ml M-CSF) (25, 26).

Expanded BMMs were plated at a density of 9,000 and 300,000 cells per well for 96 and 6 well plates, respectively. Purified GST-RANKL at 30ng/ml was added to α-MEM containing 10% FBS, 100 IU/ml penicillin/streptomycin, and 1:50 dilution of CMG 14-12 cell supernatant. Media was changed every alternate day until control wells were filled with multinucleated OCs. TRAP stains were employed following 10 minute fixation with 4% paraformaldehyde (Polysciences, Warrington, PA, USA) and 0.1% Triton X-100 in PBS according to manufacturer’s instructions (Sigma 387A, St. Louis, MO, USA).

Resorption Assay

Expanded BMMs were seeded on bovine bone slices and differentiated to OCs with purified GST-RANKL as above. At maturity, bone slices were fixed for 10 min with 4% paraformaldehyde and 0.1% Triton X-100 in PBS. Bone slices were incubated in 0.5 NaOH for 30 seconds and kept hydrated in PBS. Cells were scraped off bone slices with a cotton swab, and bone slices were incubated with 20µg/ml peroxidase-conjugated wheat germ agglutinin in PBS (Sigma 61767, St. Louis, MO, USA) for 30 min. Subsequently, slices were washed 3x in PBS and incubated with a DAB chromogen kit (Biocare Medical BDB2004H) at 37˚C for 30 min. Slices were left to air dry and imaged under brightfield at 20X.

Quantitative real-time PCR

Following collection of cells with TRIzol (Life Technologies, Carlsbad, CA, USA), solutions were centrifuged at 12,000xg and aqueous layer extracted with phenol:chloroform. An equal volume of 70% ethanol was added, and remaining RNA isolation done via NucleoSpin RNA II kit (Clontech Laboratories, Palo Alto, CA, USA, 740955.50). 1µg of RNA was input into cDNA Ecodry premix kit prior to qPCR program run on ABI QuantStudio 3 with iTaq Universal SYBR Green Supermix (BioRad Laboratories 1725121, Hercules, CA, USA). Each reaction proceeded at 50˚ for 2 min, 95˚ for 10 min, then 40 cycles of 95˚
for 15 sec and 60° for 1 min. Relative expression was calculated as \(100 \times 2^{-(\text{target CT-B2M CT})}\). Primer sequences -

**Mfn1**: F – TTGGCAGGACAAGTAGTGGC, R – AGCAGTTGGTTGTGTAACCA

**Mfn2**: F – AAGCACTTTGTCACTGCAAG, R – TTGTCCCAGAGCATGAGCATTG

**Nfatc1**: F – GGTAACTCTGTCTTTCTAACCTTAAGCTC, R – GTGATGACCCCAGCATGCACCAGTCACA

**DC-STAMP**: F – ACAAAACAGTCCAAAGCTTGC, R – TCCTGGGTCTTCCAGCTTC

**Ctsk**: F – AGGCAGCTAAATGCAGAGGGTACA, R – AGCTTGCATCGATGGACACAGAGA

**B2m**: F – CTGCTACGTAACACAGTTCCACCC, R – CATGATGCTTGATCACATGTCTCG

**Western Blot**

RIPA buffer (20mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na3VO4, 1mM NaF) with 1:50 HALT protease and phosphatase inhibitor (Thermo Fisher Scientific 1861280, Waltham, MA, USA) was used to lyse BMM and OC cells following 2x washes in PBS. Lysates were vortexed, centrifuged at 700xg and supernatant quantified with BCA assay (Bio-Rad Laboratories, Hercules, CA, USA), and 20 µg loaded to 10% SDS PAGE gel. Gels were run at 80 V for 2 hrs, and transferred to nitrocellulose by wet transfer at 100V for 90 minutes. Blocking was done in TBS+0.1% Tween 20 with 5% milk. Membranes were probed with 1° antibodies overnight at 4°C (1:500 Mfn1 - Santa Cruz Biotechnology sc-50330, Dallas, TX, USA; 1:1,000 Mfn2 - Abcam ab56889, Cambridge, UK, 1:1,000 CTSK - EMD Millipore MAB3324, Burlington, MA, 1:1,000 c-Src – gift monoclonal antibody developed by S. Teitelbaum, St. Louis, MO, USA). Actin was used as a loading control at 1:10,000 (Sigma-Aldrich A228, St. Louis, MO, USA). Imaging was done using SuperSignal West Femto substrate (Thermo Fisher Scientific 34095, Waltham, MA, USA) and a chemiluminescence imager (Syngene, Cambridge, UK).

**Retroviral overexpression**

MFN1-Myc and MFN2-Myc were obtained from Addgene (plasmids #23212 and #23213, respectively). MFN2-WT, MFN2-EE, and MFN2-AA were generated by Gerald Dorn (27). Each gene was cloned into the pMX retroviral vector and transfected into Platinum-E (platE) cells by calcium phosphate precipitation (28, 29). Each insert was also sequenced to verify the presence or absence of the desired point mutations. PlatE supernatant was harvested 48 and 72 hrs post-transfection, filtered through 0.45µm and added to BMMs with α-MEM, 10% FBS, and 100 IU/ml Penicillin/Streptomycin, 1:10 CMG, and 8µg/ml polybrene (Sigma H9268, St. Louis, MO, USA) on 2 consecutive days. 24 hrs following the final viral addition, 1µg/ml blasticidin (Sigma 203350, St. Louis, MO, USA) was added to select for infected BMMs.

**Induced Osteolysis**
2 month old ctrl and Mfn2 cKO animals were injected intraparitoneally (IP) with 1mg/kg body weight GST-RANKL twice, 24 hrs apart, as previously published (30). 50 hrs after the first injection, animals were sacrificed, and femoral trabecular bone was evaluated by µCT.

Confocal imaging

Cultured BMMs transduced with Vector, MFN2-WT, MFN2-EE, and MFN2-AA retroviruses were plated on 25mm glass round coverslips at 500,000 cells/well, washed with phenol red-free DMEM (Thermo Fisher, Waltham, MA, USA) and stained with 200nM Mitotracker Green FM and 200nM Lysotracker Red (M7514 and L7528, Molecular Probes, Eugene, OR, USA) for 30 min at 37˚C. Following incubation, cells were washed again and kept in DMEM with 10% FBS, 10% CMG, and 10µg/ml Hoechst (Thermo Fisher, Waltham, MA, USA). Confocal imaging was obtained on a Nikon Ti confocal microscope using a 60X 1.3 NA oil immersion objective with the coverglass loaded onto a chamber (Warner instrument RC-40LP, Hamden, CT, USA) in modified Krebs-Henseleit buffer (138 mM NaCl, 3.7 mM KCl, 1.2 mM KH₂PO₄, 15 mM glucose, 20 mM HEPES, 1 mM CaCl₂).

Cytoplasmic Ca²⁺ measurement

BMMs were plated in 29 mm glass bottom plates at 300,000 cells/plate (Cellvis D29-14-1.5-N, Mountain view, CA, USA), and exposed to 30 ng/ml RANKL for 48 hrs. Cells were washed and incubated at 37°C for 30 min in Hanks’ Balanced Salt Solution (HBSS) containing 2 mM CaCl₂, 1 mM MgCl₂, 10 ng/ml hM-CSF (Biolegend, CA, USA), 30 ng/ml RANKL, and 2 µM Fura-2. Cells were washed and kept in HBSS with 1 mM MgCl₂ without Ca²⁺, 10 ng/ml hM-CSF, 30 ng/ml RANKL for imaging. 35-85 cells/plate were measured at wavelengths of 340 and 380 every 2 seconds on an Olympus IX-71 inverted microscope with Lamda-LS illuminator, at the Washington University Hope Center and the Center for Investigation of Membrane Excitability Diseases Live Cell Imaging Facility. 100 µM ATP and 2 mM CaCl₂ were used as stimuli, as indicated.

Statistics

Two-way ANOVA with Tukey’s multiple comparisons and one- or two-tailed unpaired t-tests with Welch’s correction were performed with GraphPad Prism built-in statistical analysis (GraphPad Software, Inc., La Jolla, CA, USA). All data are represented as mean ± standard deviation with 3+ biological replicates. P values are designated as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Data Availability

All data are contained within the manuscript.

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Fig 1. Trabecular and cortical bone mass is increased in 2 month old female mice lacking MFN1 and MFN2 in the OC lineage.
BV/TV (A) and Cort. Th (B) are significantly elevated in female dcKOs compared to ctrls in the distal femur at 2 months of age, by µCT. Representative reconstructions of analyzed regions above the growth plate and at mid-shaft are shown at right, scale=200µm. Two month old males were evaluated for BV/TV (C) and Cort.Th (D). **p<0.01, unpaired t-test with Welch’s correction.
**Fig 2: Female Mfn1/2 dcKOs have decreased OC activity, but no change in bone formation.**

(A) Serum CTX-1 assay shows decreased bone resorption in 2 month old dcKO females. Histomorphometry of TRAP stained tibias reveals modest decreases in dcKO Oc.N/BS (B) and Oc.S/BS (C). Representative TRAP stained ctrl and dcKO femurs are shown in (D) and (E), respectively. No differences in bone accrual between groups are observed by serum P1NP (F) or dynamic histomorphometric analysis of trabecular bone for MS/BS (G), MAR (H), and BFR/BS (I) in tibiae. Representative fluorescence images of ctrl and dcKO femurs are shown in (J) and (K), respectively. Scale = 400µm (low power) and 40 µm (insets), *p<0.05, one-tailed unpaired t-test with Welch’s correction.
Fig 3. Osteoclastogenesis is defective in BMMs derived from dcKO bone marrow.

(A) MFN1 and MFN2 protein levels are progressively decreased in RANKL-treated ctrls and dcKOs compared to RANKL-treated cre-only BMMs. (B) BMMs cultured with RANKL for 5 days form OCs in ctrls but not in dcKOs. Scale = 400µm. (C) Bone resorption is congruently inhibited with resorption pits outlined in dotted white. Scale = 200µm. mRNA levels of OC markers NFATc1 (D), DC-Stamp (E), and Ctsk (F) are diminished in dcKOs after 3 and 5 days of differentiation in RANKL, correlating with C-SRC and CTSK protein levels during osteoclastogenesis (G) *p<0.05, ***p<0.001, ****p<0.0001, unpaired t-test with Welch’s correction. N=3 biological replicates.
Fig 4. Though both homologs increase during OC formation, addition of MFN2 more efficiently drives osteoclastogenesis in vitro.
Protein (A) and mRNA (B) expression through 6 days of osteoclastogenesis in wild type BMMs shows parallel increases of MFN1 and MFN2 expression. (C) dcKO BMMs were transduced with retroviral pMX-Vector, V, pMX-MFN1, MFN1, or pMX-MFN2, and mitofusin proteins were detected via western blot. (D-E) Following RANKL treatment for 6 days, cultures were stained with TRAP and OCs enumerated, revealing robust osteoclastogenesis in MFN2-treated but not MFN1 treated cells. Graph represents 3 biological replicates. Scale=400µm, *p<0.05, **p<0.01, ****p<0.0001, by one-way ANOVA.
Fig 5. Female mice lacking MFN2 alone in the OC lineage are protected from bone loss with age and RANKL-induced osteolysis.

MFN2 expression is decreased in Mfn2 cKO OCs, in vitro, compared to cre-only at the protein (A) and RNA (B) levels. Femurs from cre-only and Mfn2 cKO mice at 12 months of age were analyzed by mCT for (C) BV/TV in females, (D) BV/TV in males, (E) BMD in females, and (F) BMD in males. Trabecular bone mass is increased in female cKOs but not males. (G-I) Acute bone loss was induced by intraperitoneal RANKL injection at 2 months of age in cre-only and Mfn2 cKO mice, and assessed by mCT at the distal femur. BV/TV is not decreased with RANKL treatment in female Mfn2 cKO mice (G). BV/TV decreases to a similar degree in male Mfn2 cKO mice compared to cre-only controls (H). (I) Representative post-RANKL μCT reconstructions from G-H, scale=200μm. The untreated cohorts are the same as those listed at 2 months of age in Tables 1 and S1. *p<0.05, **p<0.01, unpaired t-test with Welch’s correction in B-F, ordinary two-way ANOVA with multiple comparisons in G-H.
**Fig 6. Restoration of MFN2 tethering function rescues osteoclastogenesis in dcKO BMMs.**

(A) MFN2 functions in each rescue condition. (B) dcKO BMMs were transduced with retroviral pMX-Vector (V), pMX-MFN2-WT (WT), pMX-MFN2-EE (EE), and pMX-MFN2-AA (AA), and MFN2 protein is detected via western blot in BMMs. (C) Cultures of transduced BMMs were stained with MitoTracker Green and LysoTracker Red, scale = 10 µm (whole cells) and 2.5 µm (insets). Mitochondrial aspect ratio (mito length/width) is quantified in (D). (E) BMMs were TRAP-stained following 6 days of RANKL exposure, revealing robust osteoclastogenesis with Mfn2-AA, but not Mfn2-EE. Scale = 400 µm. (F) Quantification of OC numbers in 3 biological replicates. ****p<0.0001, ordinary one-way ANOVA.
Figure 7

Fig 7. Blunted Ca$^{2+}$ entry in dcKO preOCs is restored with MFN2-AA overexpression.

(A-C) Fura-2 staining shows elevated basal intracellular Ca$^{2+}$ but blunted store operated calcium entry after addition of extracellular Ca$^{2+}$ in dcKO pre-OCs. Area under the curve quantification following Ca$^{2+}$ (B) and ATP (C). (D-E). Overexpression of MFN2-WT or MFN2-AA restores Ca$^{2+}$ entry in dcKO pre-OCs, while MFN2-EE does not. Area under the curve is quantified in (F). (G) Corresponding Nfatc1 levels evaluated by q-rt-PCR in mutant OCs correlate with SOCE responsiveness. *p<0.05, ***p<0.001, ****p<0.0001, by unpaired t-test (B,C) or ordinary one-way ANOVA (F, G).
The tethering function of mitofusin2 controls osteoclast differentiation by modulating the Ca2+-NFATC1 axis
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