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Hem1 promotes osteoclast fusion and bone resorption in mice

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Abstract (limit 150 words)

Hem1 (Hematopoietic protein 1), a hematopoietic cell-specific member of the Hem family of cytoplasmic adaptor proteins, is essential for lymphopoiesis and innate immunity and for the transition of hematopoiesis from the fetal liver to the bone marrow. However, the role of Hem1 in bone cell differentiation and bone remodeling is unknown. Here, we show that deletion of Hem1 resulted in a markedly increase in bone mass due to defective bone resorption in mice of both sexes. Hem1-deficient osteoclast progenitors were able to differentiate into osteoclasts, but the osteoclasts exhibited impaired osteoclast fusion and decreased bone-resorption activity, potentially due to cytoskeletal disorganization and decreased mitochondrial respiration. Transplantation of bone marrow hematopoietic stem and progenitor cells from wild-type into Hem1 KO mice increased bone resorption and normalized bone mass. These findings indicate that Hem1 plays a pivotal role in the maintenance of normal bone mass.

Introduction

Bone is a highly dynamic tissue that responds and adapts to changes in systemic signals and to mechanical forces. Bones regenerate periodically in discrete sites via a remodeling process through which old or damaged bone is resorbed by osteoclasts and is replaced with new bone by osteoblasts^{1,2}.

Osteoclasts differentiate from hematopoietic precursor cells of the monocyte/macrophage lineage in response to two critical osteoclastogenic cytokines—macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL)³. Osteoclasts develop in several steps, beginning with proliferation of hematopoietic progenitor cells that differentiate to mononuclear pre-osteoclasts that then fuse to become multi-nucleated mature osteoclasts³. These cells are uniquely capable of dissolving and digesting the bone matrix due to their ability to trigger actin polymerization in actin ring and to form a “podosome belt” that tightly adheres to the bone area that is targeted for removal, creating a sealed microenvironment into which the cells secrete protons and lysosomal enzymes^{3,4}. The high energy demands of these tasks are likely the reason for the abundance of mitochondria within osteoclasts, a distinct cellular feature of these cells^{5,6}. The balancing act of healthy bone remodeling is accomplished by osteoblasts refilling each resorption cavity with new bone. Osteoblasts derive from mesenchymal stem cells present in the bone marrow¹. Osteoblast differentiation occurs in response to osteoblast-formation signals, including matrix-derived factors released during bone resorption.

The contribution of osteoclast-derived signals or factors released during resorption to osteoblast generation is referred to as “coupling”^{7,8}.

Hematopoietic protein 1 (Hem1), also known as Nck-associated protein 1-like (NAP11 or Nckap11), is a member of the Hem family of cytoplasmic adaptor proteins. Orthologues of Hem1 in lower organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, are essential for cytoskeletal reorganization, embryonic cell migration, and morphogenesis⁹⁻¹¹. Studies in mice and in cell lines indicate that Hem1 is a component of the “WAVE (WASP [Wiskott-Aldrich syndrome protein]-family verprolin homologous protein)” complex, which signals downstream of activated Rac and upstream of the Arp2/3 complex to stimulate actin polymerization in response to immunoreceptor signaling^{9,12}. Recent work from our group has shown that mice with deletion of Hem1 exhibit growth retardation and premature death at about 6 weeks. These defects were associated with premature exhaustion of neonatal bone marrow hematopoietic stem cells, indicating that Hem1 is required for hematopoiesis to transition from the fetal liver to the bone marrow¹³. In contrast, Hem-1 is dispensable for the normal development and function of the other organ systems. Because osteoclasts differentiate from the hematopoietic lineage, we examined the roles played by Hem1 in the skeleton. Here, we show that deletion of Hem1 led to a defect in osteoclast maturation, a decrease in bone-resorption and a marked increase in trabecular bone volume. All these abnormalities could be corrected by transplantation of normal bone marrow hematopoietic stem and progenitor cells.

Results

Hem1 knockout mice have increased bone mass. To address the role of Hem1 in bone remodeling, we generated Hem1 knockout mice as described previously¹³. At 5.5 weeks of age, Hem1 knockout mice were smaller than their littermate wild-type controls, as evidenced by reduced body size, body weight, and femoral length (Fig. 1a and Supplementary Fig. 1), but the morphology of the femoral growth plate was not affected (Fig. 1b). Consistent with the reduced femoral length in Hem1 knockout mice, the femurs had lower cortical thickness, total area, and medullary area than wild-type littermates (Fig. 1c and d), as determined by micro-CT. In contrast, distal femurs of both male and female Hem1 knockout mice had elevated trabecular bone volume and bone mineral density (Fig. 1e, f and Supplementary Fig. 2) due to an increase in trabecular number and a decrease in trabecular spacing. No changes in trabecular thickness were detected in male (Fig. 1f) but slightly increased in female Hem1 knockout mice (Supplementary Fig. 2).

Hem1 knockout mice have more osteoclasts but less bone resorption than wild-type mice. To determine the cellular basis of the increase in trabecular bone mass, we conducted histological analyses to enumerate osteoblasts and osteoclasts in the distal femur. Consistent with the micro-CT results, trabecular bone volume and trabecular number were greater but trabecular spacing was smaller in Hem1 knockout mice than in wild-type controls (Fig. 2a). Surprisingly, the knockout mice had increased numbers of osteoclasts, as determined by tartrate-resistant acid phosphatase (TRAP) staining in

histological sections of the femur (Fig. 2b). These changes were associated with an increase in the number of myeloid cells in the bone marrow (Supplementary Fig. 3). However, bone resorption as determined by serum levels of CTX was severely reduced from that seen in wild-type controls (Fig. 2d).

Consistent with the observed reduction in bone resorption, osteoblast numbers were lower in Hem1 knockout mice (Fig. 2c). To establish whether the number of mesenchymal progenitors was affected by Hem1 deletion, we quantified the number of CFU-fibroblasts (CFU-F), CFU-osteoblasts (CFU-OB), and CFU-adipocytes (CFU-A) formed by mesenchymal progenitors from wild-type and Hem1 knockout mice in *ex vivo* bone marrow cultures. The numbers of CFU-F, CFU-OB, and CFU-A were greater in Hem1 knockout mice than in WT mice (Fig. 2e-g). These results indicate that the low osteoblast number in knockout mice are not due to reduced mesenchymal progenitors. Taken together, these findings suggest that the high bone mass of Hem1 knockout mice is due to a decrease in osteoclastic bone resorption.

Deletion of Hem1 decreases actin ring formation and osteoclast fusion in vitro. To investigate the role of Hem1 in osteoclastogenesis, bone marrow macrophages were cultured in the presence of M-CSF and RANKL. The Hem1 mRNA expression and protein levels was about 3-fold higher in mature osteoblast than macrophages or pre-osteoclasts from wild-type mice. (Fig. 3a-b). Macrophages from Hem1 knockout mice (Fig. 3c) failed to form multinucleated giant osteoclasts (with more than 10 nuclei) but formed a higher number of smaller osteoclasts (with 3–5 nuclei) than those from wild-

type mice (Fig. 3d). Levels of mRNA for markers of mature osteoclast differentiation (*TRAP*, *Cathepsin K*, *Calcitonin receptor*, *Oscar*) were significantly lower in cultures from Hem1-deficient mice than in those from wild-type littermates (Fig. 3e). In contrast, expression of NFATc1 and c-Fos, two essential transcription factors for early osteoclast differentiation, were not affected by Hem1 deletion (Fig. 3f), suggesting that Hem1 does not affect the early stage of osteoclast differentiation.

To determine whether osteoclasts from Hem1-deficient mice could resorb bone, we cultured osteoclasts from wild-type and Hem1 knockout mice on OsteoAssay plates and found that the area resorbed (Fig. 3g) and actin ring formation (Fig. 3h) by osteoclasts of Hem1-deficient mice were greatly diminished compared with the cells from wild-type mice. Consistent with this result, mRNA levels of *Dc-stamp* and *Atp6v0d2*, which are involved in osteoclast fusion and actin ring formation^{14,15}, were significantly decreased in pre-osteoclasts and osteoclasts from Hem1-deficient mice (Fig. 3i).

To confirm that the defects seen in bone resorption of Hem1 knockout mice were due to direct effects of Hem1 in macrophages, we expressed Hem1 in bone marrow macrophage cultures from Hem1 knockout mice (Fig. 4a). Expression of Hem1 increased osteoclast formation (Fig. 4b) and resorption capacity by 2 to 3-fold (Fig. 4c). The mRNA levels of osteoclast-related genes (*TRAP*, *Cathepsin K*, *Dc-stamp*, *Atp6v0d2*) also increased in response to Hem1 expression. (Fig. 4d).

Cells of the mesenchymal lineage including stromal cells are major producers of RANKL and other cytokines that support osteoclastogenesis. We also examined whether osteoclast differentiation in Hem1 knockout mice could be affected indirectly

by bone marrow–mesenchymal cells. To this end, we co-cultured bone marrow macrophages from wild-type mice with stromal cells from wild-type or Hem1 knockout mice. Stromal cells from either genotype equally supported osteoclast formation (Supplementary Fig. 4). These data further support to the idea that Hem1 contributes directly to the formation of multinucleated osteoclasts.

Deletion of Hem1 decreases mitochondrial respiration and c-Abl signaling in osteoclasts. WAVE activation of the Arp2/3 complex in yeast is indispensable for actin polymerization and cell migration and the accompanying enhanced mitochondrial function^{16,17}. However, little is known about the role of Hem1 in regulation of mitochondrial function in mammalian cells. To examine this potential role of Hem1 in osteoclasts, we performed extracellular flux analysis, comparing osteoclasts from Hem1 knockout mice with those from wild-type mice. In osteoclasts from Hem1 knockout mice, mitochondrial respiration was significantly diminished (Fig. 5a), and ATP-linked respiration, proton leakage, maximum respiration (the OCR when electron transport chain operates at maximum capacity), reserve respiratory capacity (a cell's flexibility to increase oxygen consumption during increased energy demands), and non-mitochondrial respiration were reduced (Fig. 5b–f). Consistent with these inhibitory effects of Hem1 deletion on respiration, osteoclasts from Hem1 knockout mice had decreased ATP production in response to RANKL stimulation (Fig. 5g).

Our recent work indicates that inactivation of c-Abl, a tyrosine kinase that regulates actin polymerization and cell migration¹⁸⁻²¹, contributes to the impairment of

hematopoietic stem cells in Hem1-deficient mice¹³. Here, we examined whether Hem1 activates osteoclast formation via c-Abl signaling. Compared to osteoclasts from wild-type mice, those from Hem1 knockout mice had less phosphorylation of the c-Abl downstream target Crkl but no changes in total protein levels of Crkl (Fig. 5h). Addition of imatinib, a synthetic small-molecule inhibitor of c-Abl, to cultures of osteoclast progenitors from wild-type C57BL/6 mice resulted in decreased formation of osteoclasts (Fig. 5i), and most of these osteoclasts were small (Fig. 5i), reminiscent of osteoclasts from Hem1 knockout mice.

Skeletal phenotype of Hem1 knockout mice is rescued by transplantation of bone marrow hematopoietic stem and progenitor cells from wild-type mice. Our previous studies showed that Hem1 knockout mice exhibited significant reductions in the numbers of total bone marrow nucleated cells, hematopoietic progenitor cells, hematopoietic stem cells, and cobblestone area forming cells, which could be corrected by transplantation of normal wild-type bone marrow hematopoietic stem and progenitor cells¹³. Therefore, we investigated whether transferring these cells could rescue the bone effects due to Hem1 deletion. We transplanted 1×10^6 Lin⁻CD45⁺ bone marrow cells from normal C57BL/6 congenic (CD45.1/GFP) mice into 3-week-old non-ablated CD45.2 wild-type and Hem1 knockout mice (Fig. 6a). Ten weeks after transplantation, most components of the hematopoietic system, including hematopoietic stem and progenitor cells, were comparable in wild-type and Hem1 knockout recipient mice (Supplementary Figure 5). In Hem1 knockout recipient mice, almost 90% of cells in

the peripheral blood and bone marrow were derived from CD45.1/GFP donor cells; however, in wild-type recipient mice, no donor cells were detected. These results indicate that the transplanted normal hematopoietic cells reconstituted the hematopoietic system in Hem1 knockout recipient mice. Transplantation with normal hematopoietic cells also restored body size (Fig. 6b). Although not fully rescued, femoral length (Fig. 6c), cortical thickness, total area, and medullary area were much improved (Fig. 6d and e) in transplanted Hem1 knockout mice than in non-transplanted Hem1 knockout mice (Fig. 1c and d). Remarkably, trabecular bone volume and microarchitecture in the distal femur (Fig. 6f and g) and serum levels of CTx in Hem1 knockout recipient mice were similar to wild-type (Fig. 6h). Consistently, in vitro bone-resorption ability was comparable for osteoclasts from both genotypes of recipient mice (Fig. 6i). In addition, osteoclast cultures from the bone marrow of transplanted Hem1 knockout mice had significantly increased osteoclast formation (Fig. 6j) and upregulated the expression of *TRAP*, *Cathepsin K*, *Calcitonin receptor*, and *Oscar* (Fig. 6k). Actin ring formation in osteoclasts from transplanted Hem1 knockout mice was comparable to that in wild-type mice, as was expression of *Dc-stamp*, *Oc-stamp*, *Atp6v0d2*, and *Nfatc1* (Fig. 6l and m).

Discussion

The pathways of osteoclastogenesis that regulate RANKL-induced osteoclast differentiation have been studied extensively, but less is known about osteoclast fusion, a unique feature of osteoclast maturation that is required for the proper bone resorption. Herein, we provide evidence that the hematopoietic-specific WAVE complex scaffold Hem1 is required for osteoclast fusion and bone-resorption activity and, thereby, contributes to the maintenance of bone mass in mice.

Deletion of Hem1 reduced osteoclast bone-resorption activity, in spite of increased numbers of osteoclasts on trabecular bone surfaces. A decrease in bone resorption has been also reported in mice with loss-of-function mutations in proteins important for lysosome biogenesis and functions in osteoclasts. For example, global or conditional deletion of ATP6i²², CLCN7²³, OSTM1²⁴, SNX10^{25,26}, or PLEKHM1^{27,28} leads to “osteoclast-rich” autosomal recessive osteopetrosis in which osteoclasts are abundant but have severely impaired bone-resorption activity.

A defect in hematopoiesis contributes to the osteopetrosis with abnormal bone remodeling^{29,30}. Transplantation of bone marrow hematopoietic stem and progenitor cells markedly improves severe osteopetrosis associated with the defect in bone marrow and offers the best chance of longer-term survival for individuals with this type of disease^{31,32}. Here, we showed that transplanting normal hematopoietic stem and progenitor cells into Hem1 knockout mice fully restored cancellous bone volume and microarchitecture in the distal femur, as well as osteoclast fusion and bone-resorption activity. More importantly, the transplantation also rescued growth retardation and

premature death of Hem1 knockout mice. Collectively, these findings suggest that Hem1 is essential during growth for bone remodeling and for hematopoietic stem cell maintenance.

In line with our findings, studies of short interfering RNA oligonucleotides in an osteoclast cell line revealed that the Arp2/3 complex regulates actin polymerization and promotes bone resorption³³, and other studies showed that the Arp2/3 complex locates in the core of the podosomes that make up actin rings, where it co-localizes with cortactin, a known regulator of Arp2/3 complex activity^{34,35}. In yeast, the Arp2/3 complex promotes actin polymerization by regulating mitochondrial dynamics, but little is known about its role in mitochondria metabolism in mammalian cells^{16,17}. Here, we showed that in osteoclasts deletion of Hem1 decreased mitochondrial respiration. Overall, these findings support the notion that, Hem1, a component of the Rac-WAVE-Arp2/3 pathway, exerts effects in osteoclasts at least in part via an effect on mitochondrial function. Support for a link between Hem1, mitochondria, and bone resorption is provided by studies in which mitochondria activity has been attenuated in osteoclasts³⁶⁻³⁸. These studies indicate that normal mitochondria metabolism is critical for osteoclast fusion and bone resorption. Nonetheless, future work is required to identify the range of target proteins and mitochondrial pathways that are responsible for the effects of Hem1 on osteoclast development.

A decrease in bone resorption was, most likely, responsible for the increase in trabecular bone mass in Hem1 knockout mice. Nonetheless, changes in mesenchymal lineage cell number, specifically CFU-F, CFU-Ad, CFU-Ob, and osteoblast were also

noted. These changes are, most likely, indirect as the expression of Hem1 is very low in mesenchymal lineage cells (CD45⁻) from wild-type mice (Supplementary Fig. 6). Strikingly, Hem1 knockout mice exhibited low numbers of osteoblast on trabecular bone surfaces, which could not be explained by changes in progenitor cell numbers indicated by CFU assays. One possible explanation for the decrease in osteoblasts could be the decrease in growth factors released from the matrix during bone resorption^{7,8}. In view of the major decrease in osteoblast number in the Hem1 knockout mice, it is also possible that Hem1 plays a key role in supporting osteoblasts by stimulating osteoclasts to express coupling factors. Nonetheless, further biochemical and genetic studies are necessary to elucidate this possibility.

We previously reported that the defects in hematopoietic cells in Hem1 knockout mice are associated with decreased c-Abl signaling. Here, we found that c-Abl signaling is also decreased in osteoclast progenitors. Inhibition of c-Abl in wild type osteoclast progenitors caused defects in osteoclastogenesis similar to the ones caused by Hem1 deletion. These results suggest that c-Abl mediate the effects of Hem1 on osteoclast formation and function. Interestingly, c-Abl promotes mitochondrial dynamics in primary neurons via direct phosphorylation of target proteins such as Drp1³⁹. This, together with our present findings that Hem1 stimulates mitochondria function, supports the notion that c-Abl also mediates the effects of Hem1 on mitochondria of osteoclasts.

Based on the results of the present work, we propose that Hem1 directly regulates osteoclast fusion during bone development. Further studies with models of cell lineage-

specific deletion of Hem1 are needed to examine the role of Hem1 in adult bone both under physiological and conditions of increased bone remodeling such as estrogen deficiency.

Methods

Mice. Hem1 knockout mice were bred in our facility from pairs of mice heterozygous for the Hem1 mutant allele (C57BL/6 background), as described previously¹³. C57BL/6-Tg(CAG-EGFP) mice were purchased from Jackson Laboratories (Bar Harbor, MA, USA). All mice used in this study were housed under standard laboratory conditions with a 12-hour dark, 12-hour light cycle, a constant temperature of 23 °C, and humidity of 48%. A standard rodent diet (Envigo, Teklad 22/5) containing 22% protein, 1.13% calcium, and 0.94% phosphorus was provided to mice ad-libitum. All mice were maintained at the University of Arkansas for Medical Sciences (UAMS) animal facility, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Mice were randomly assigned to 4 or 5 mice per cage. All animal procedures were approved by the UAMS Institutional Animal Care and Use Committee.

Micro-CT analysis. Bone architecture was determined on dissected femurs that were cleaned of adherent soft tissues, fixed in Millonig's phosphate buffer (Leica Microsystems, Buffalo Grove, IL, USA), and gradually dehydrated in 100% ethanol. Bones were scanned with μ CT40 (Scanco Medical, Brüttiselen, Switzerland) at high resolution for obtaining images and at medium resolution for making quantitative determinations as described previously⁴⁰. Specifically, scans were performed at medium resolution (12 μ m isotropic voxel size) for quantitative determinations and integrated into 3-D voxel images (1024 x 1024 pixel matrices for each individual planar

stack). To reduce signal noise, a Gaussian filter ($\sigma = 0.8$, support = 1) was applied. Scano Eval Program v.6.0 was used to measure bone volume. Scan settings were applied to X-ray tube potential ($E=55$ kVp), X-ray intensity ($I=145$ μ A), and integration time (220 ms). Nomenclature conforms to recommendations of the American Society for Bone and Mineral Research⁴¹. Trabecular bone measurements of the distal femur were made on 151 transverse slices that were taken from the epicondyles, extending toward the proximal end of the femur; cortical bone and primary spongiosa were manually excluded from the analysis. All trabecular analyses were performed on contours of every 10 to 20 cross-sectional images and were measured at a threshold of 220 mg/cm³. Trabecular architecture was determined using sphere filling distance–transformation indices without assumptions about the bone shape as a rod or plate. Cortical dimensions were determined at the diaphysis (18 slices, midpoint of the bone length as determined in scout view) at a threshold of 200 mg/cm³.

Bone Histology. The terminology used in this report is that which is recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research⁴². The femoral bones were fixed for 24 h in Millonig's phosphate buffer, transferred to 100% ethanol, and embedded undecalcified in methyl methacrylate. For histomorphometric measurements, 5- μ m-thick longitudinal sections were cut in the medial-lateral plane. To visualize osteoclasts, sections were stained with naphthol AS-MX and Fast Red TR salt (Sigma-Aldrich, St. Louis, MO, USA) to detect TRAP activity. Sections also were stained with 0.3% toluidine blue in phosphate

buffered citrate, pH 3.7, to visualize osteoblasts, osteoid, and cement lines. Standard histomorphometric parameters including the number of TRAP-positive cells and osteoblasts on trabecular bone surface of longitudinal section were measured using an Olympus BX53 microscope and Olympus DP73 camera (Olympus America, Inc., Center Valley, PA, USA) interfaced with a digitizer tablet with OsteomeasureTM software version 4.1.0.2 (OsteoMetrics Inc., Decatur, GA, USA). One section per sample was analyzed by a histopathologist blinded to the study groups.

CTx ELISA. Circulating CTx in serum was measured using a mouse RatLaps (CTx-I) EIA kit (Immunodiagnostic Systems, Boldon, United Kingdom) according to the manufacturer's directions. Blood was collected into 2.0 mL microcentrifuge tubes by retro-orbital bleeding. Blood was then kept on ice for 1 hour and centrifuged at 10,000 x rpm for 10 min to separate serum from cells.

Osteoclast differentiation. Bone marrow macrophages were obtained as described previously⁴³. Briefly, bones (i.e., femurs and tibiae) were isolated and cleared of soft tissues. After red blood cells were removed using ACK buffer (0.01 mM EDTA, 0.011 M KHCO₃, and 0.155 M NH₄Cl, pH 7.3), the remaining cells were cultured with 10% fetal bovine serum, 100 units ml⁻¹ of penicillin, and 100 µg ml⁻¹ of streptomycin in the presence of 10 ng ml⁻¹ M-CSF (R&D Systems, Minneapolis, MN, USA.) for 24 h. Non-adherent bone marrow cells were collected and cultured in Petri dishes with 30 ng ml⁻¹ M-CSF for 3 days to generate bone marrow macrophages; adherent bone marrow

macrophages were harvested as osteoclast progenitors.

To generate pre-osteoclasts and osteoclasts, bone marrow macrophages were cultured in 6-well (for RT-PCR and Western blot analysis), 24-well (for resorption assays), or 48-well (for TRAP staining) plates in α -MEM complete medium with 30 ng ml⁻¹ M-CSF and 30 ng ml⁻¹ RANKL (R&D Systems) for 2 and 4 days, respectively. Imatinib (Selleckchem, Houston, TX, USA, #S2475) was used in some of experiments at the indicated concentrations. For co-culture systems, bone marrow-derived stromal cells and bone marrow macrophages were seeded in 48-well plates with 1 α ,25(OH)₂D₃ (10⁻⁸ M; Sigma) and prostaglandin E₂ (10⁻⁸ M; Sigma) for 5–6 days in α -MEM complete medium.

To enumerate osteoclasts, cells were fixed with 10% neutral-buffered formalin for 10 min, permeabilized with 0.1% Triton-X 100 in PBS for 5 min, and stained for TRAP with the Leukocyte Acid Phosphatase Assay Kit (Sigma-Aldrich), following the manufacturer's instructions. Pre-osteoclasts were identified as round mononuclear TRAP-positive cells; osteoclasts were identified as multinucleated (>3 nuclei) TRAP-positive cells.

Retroviral gene transduction. To prepare retroviral particles, Plat-E retroviral packaging cells were plated on a 10-mm culture dish and transfected with MSCV vectors encoding Hem1 using Lipofectamine™ 2000 (Invitrogen). After 3 days, the medium containing retroviruses was harvested and passed through a syringe filter (0.2 μ m pore diameter). Bone marrow macrophages were infected with retroviruses for 8

hours with $6\text{ }\mu\text{g ml}^{-1}$ polybrene (Sigma-Aldrich) in the presence of 30 ng ml^{-1} M-CSF. After washing with fresh medium, the cells were cultured for 2 days in the presence of $2\text{ }\mu\text{g ml}^{-1}$ puromycin (Sigma-Aldrich) with 30 ng ml^{-1} M-CSF. Puromycin-resistant Bone marrow macrophages were studied.

Colony-forming unit (CFU) assay. CFU-F (fibroblast), CFU-AD (adipocyte), and CFU-OB (osteoblast) number was determined as previously described⁴⁴, 15% FBS, and 1 mM ascorbate-2-phosphate. Half of the medium was replaced every 5 days. CFU-Fs were enumerated at 10 days of culture after staining for AP, and CFU-OBs were enumerated at 25 days of culture after von Kossa staining. CFU-AD was enumerated at 6 days of culture in presence of 1 μM rosiglitazone after Oil-Red O staining.

Bone resorption assay. Bone marrow macrophages were isolated as described above and were stimulated with RANKL to form osteoclasts on 24-well Osteo Assay Surface plates (Corning Life Sciences, Corning, NY, USA), which are coated with an inorganic bone biomaterial surface. Cells were removed with a 2% hypochlorite solution for 5 min, washed with distilled water, and dried at room temperature. For Von Kossa staining, wells were incubated for 20 min in darkness with 5% (w/v) aqueous silver nitrate solution (150 $\mu\text{l/well}$). Plates then were washed for 5 min with distilled water and incubated in darkness with 5% (w/v) sodium carbonate (150 $\mu\text{l/well}$) in 10% formalin solution. Wells were washed twice with PBS, rinsed with distilled water, and dried for 30 min at 50 °C. Each well was imaged with a microscope; the resorbed areas

are white, and the unresorbed mineralized surface are black. Three wells were assessed for each group.

Cellular bioenergetics. Application of extracellular flux analysis was performed as we previously described^{45,46}. Bone marrow macrophages were plated and treated with 30 ng ml⁻¹ M-CSF and 30 ng ml⁻¹ RANKL for 3 days. The media in the wells was replaced with XF assay media and the plate was kept in a non-CO₂ incubator for 20 minutes at 37 °C. After recording three total cellular respiration measurements with the XF96 analyzer, 10 µg ml⁻¹ oligomycin was added to inhibit mitochondrial ATP synthase and measure the decrease in the oxygen-consumption rate that is linked to ATP turnover. To determine the maximal respiration potential of the cells, 10 µM FCCP (an uncoupler of oxidative phosphorylation) was used. The amount of non-mitochondrial oxygen consumption was determined by inhibiting the respiratory chain activity with an antimycin A and 10 µM rotenone cocktail. These data were used to calculate the mitochondrial basal respiration, ATP-linked respiration, reserve respiratory capacity, and proton leak.

Hematopoietic cell transplantation. Bone marrow mononuclear cells were isolated from 8-week-old GFP mice and resuspended at the concentration of 10⁷ cells ml⁻¹. Cells were incubated with purified lineage antibodies (CD11b, Gr1, CD3e, B220, Ter119, BD) for 30 min at 4 °C, and then washed twice with 2% FBS/HBSS. The labelled mature cells were depleted by incubation with goat anti-rat IgG magnetic beads (Thermo Fisher

Scientific, Waltham, MA, USA) in a magnetic field. The isolated Lin⁻ cells were washed and incubated with APC-Cy7-conjugated anti-mouse CD45 antibody. Lin⁻ CD45⁺ cells were sorted with an Aria II cell sorter (BD Biosciences, San Jose, CA, USA). 1×10^6 GFP⁺Lin⁻CD45⁺ cells were retro-orbitally transplanted into a 3-week-old Hem1 knockout mouse or wild-type littermate control. Body weights were measured every week after transplantation. Mice were analyzed at 10 weeks after transplantation. Femurs and tibias were isolated, and bone marrow macrophages and bone marrow mononuclear cells were collected for subsequent analysis.

RNA isolation and quantitative RT-PCR analysis. Total RNA was purified from cultured BMMs or bone marrow stromal cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's directions. From 2 µg of total RNA extract, cDNA was obtained with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. TaqMan quantitative real-time PCR was performed with the following primers from Applied Biosystems: TRAP (Mn00475698_m1), Ctsk (Mm00484039_m1), Calcr (Mm00432271_m1), Oscar (Mm00558665_m1), DC-Stamp (Mm04209236_m1), Atp6v0d2 (Mm01222963_m1), Oc-Stamp (Mm00512445_m1), and NFATc1 (Mm00479445_m1). To measure Hem1 expression, the SYBR assay kit was used (Applied Biosystems). Briefly, 1 µl cDNA was mixed with 7.5 µl SYBR Green PCR Master Mix and 0.2 µl of primers. Samples were then added into 6.30 µl of water (for a total volume of 15 µl). qPCR conditions were as

follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60°C for 1 min, 95 °C for 15 min, 60 °C for 60 min, and 95 °C for 15 min. All reactions were run in triplicate, and target gene expression was calculated by normalizing to the housekeeping gene ribosomal protein S2 (Mm00475528_m1) or HPRT (Forward; 5'-AGCAGTACAGCCCCAAAATGGTTA-3' and Reverse; 5'-TCAAGGGCATATCCAACAACAAAC-3) with the Δ Ct method⁴⁷.

Western blot analysis. Cells were lysed with a buffer containing 20 mM Tris-HCL, 150 mM NaCl, 1% Triton X-100, protease inhibitor mixture, and phosphatase inhibitor cocktail (Sigma-Aldrich). After incubation on ice for 30 min, the cell lysates were centrifuged at 13,200 rpm for 15 min at 4 °C. Protein concentrations of cell lysate samples were determined with the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). The extracted proteins (40 µg per sample) were separated electrophoretically on 8–10% SDS-PAGE gels and then transferred onto PVDF membranes. The membranes were blocked in 5% fat-free milk/Tris-buffered saline for 90 min and incubated with each primary antibody, followed by secondary antibodies conjugated to horseradish peroxidase. Monoclonal antibodies against NFATc1 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-7294, 1/500 dilution), CrkL (Cell Signaling Technology, Danvers, MA, USA, #3182, 1/1000 dilution), and β -actin (Santa Cruz Biotechnology, sc-81178, 1/2000 dilution) were used to detect their corresponding protein levels. Also, we used rabbit polyclonal antibodies for Hem1 (Novus Biologicals, Littleton, CO, USA, NBP2-13643, 1/1000 dilution), c-Fos (Santa Cruz Biotechnology; sc-7202, 1/500 dilution),

and p-CrkL (Cell Signaling Technology, Danvers, MA, USA, #3181, 1/1000 dilution). to analyze their protein levels. Band intensities in the autoradiograms were quantified with a VersaDoc imaging system (Bio-Rad).

Actin ring staining. Bone marrow macrophages were cultured in 6-well plates with 30 ng ml⁻¹ M-CSF and 30 ng ml⁻¹ RANKL to form osteoclasts. The cells were fixed with 4% paraformaldehyde in PBS for 15 min and blocked in 1% BSA in PBS for 1 h. Actin fibers and nuclei were stained with phalloidin-rhodamine conjugate (Invitrogen) and DAPI (Vector Laboratories Burlingame, CA, USA), respectively. The stained cells were viewed and photographed with an Axioplan research microscope (Carl Zeiss Inc., Jena, Germany) equipped with a 100-W mercury light source. Images were captured with a Dage CCD100 integrating camera (Dage-MTI, Michigan, USA) and a Flashpoint 128 capture board (Integral Technologies, Indianapolis, Indiana, USA). The captured images were processed with Image Pro Plus software (Media Cybernetics, Rockville, Maryland, USA).

Statistical Analyses. The data were analyzed by analysis of variance (ANOVA) or Student's *t*-test (independent samples, two-sided) with GraphPad Prism 9 from GraphPad Software, after determining that the data were normally distributed and exhibited equivalent variances. In the event that ANOVA justified post hoc comparisons between group means, these were conducted with Tukey's multiple-comparisons test.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

M.A., D.Z., L.S., and H.N.K. conceived and designed the experiments; L.S., X.W., and A.W. generated mice and performed animal experiments; H.N.K., L.S., X.W., K.K., and N.A.B. generated and analyzed the data; M.A., L.S., D.Z., and H.N.K. prepared the manuscript. R.H. provided the Hem1 knockout mice and advised on some of the experimental designs. All authors reviewed and revised the manuscript.

Competing Interests

All the authors have declared that no conflict of interest exists.

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Fig. 1: Deletion of Hem1 increases trabecular bone mass in mice. Micro-CT imaging and quantification of femoral bones from male Hem1 knockout mice and wild-type littermates at 5.5 weeks of age. **(a)** Representative images of whole femur (left) and length measurement (right). **(b)** Histological sections of distal femurs stained with toluidine blue (scale bar: 100 μ m). **(c)** Representative images of cortical bone at midshaft (scale bar: 100 μ m). **(d)** Cortical thickness and cortical perimeter at midshaft. **(e)** Representative images of trabecular bone (scale bar: 1 mm) and **(f)** bone volume per tissue volume (BV/TV), bone mineral density (BMD), and microarchitecture of trabecular bone. Lines and error bars represent mean $\pm$ SD; $n = 4\text{--}5$ animals/group. P values determined with Student's t -test.

Fig. 2: Deletion of Hem1 in mice decreases bone resorption. **(a)** Standard histomorphometric parameters quantifying trabecular architecture (BV/TV, trabecular number, thickness, and spacing) in histologic sections of 5.5-week-old male mice. **(b)** Representative photomicrographs non-decalcified femur sections stained for TRAPase activity and number of osteoclasts (N.Oc/B.Pm), and **(c)** number of osteoblasts (N.Ob/B.Pm) per endocortical bone surface of non-decalcified femur sections stained for TRAPase activity (red arrows) from 5.5-week-old male Hem1 knockout mice and wild-type littermates ($n = 4\text{--}5$ animals/group) (scale bar: 100 μ m). **(d)** ELISA analysis of serum concentration of a collagen degradation product (CTX) in 5.5-week-old male Hem1 knockout mice and wild-type littermates ($n = 5\text{--}6$ animals/group). **(e–g)** Photomicrographs show representative colonies (top). CFU-F stained for alkaline

phosphatase after 10 days, CFU-Ad stained with Oil Red O after 7 days, and CFU-Ob stained with von Kossa after 25 days to detect mineral (bottom) (triplicate cultures) (scale bar: 1 cm). Lines and error bars represent mean $\pm$ SD. *P* values determined with Student's *t*-test.

Fig. 3: Deletion of Hem1 decreases osteoclast fusion. (a and b) Bone marrow macrophages were isolated from 6-month-old C57BL/6 wild-type mice and were cultured with M-CSF (30 ng mL⁻¹, bone marrow macrophages) or with M-CSF and RANKL (30 ng mL⁻¹) for 2 days (pOC) or 5 days (mOC). Levels of Hem1 (a) mRNA (qPCR assay) and (b) protein (Western blot) during osteoclastogenesis. (c–i) Bone marrow macrophages were isolated from 5.5-week-old male Hem1 knockout mice and wild-type littermates and were cultured with M-CSF (30 ng mL⁻¹) and RANKL (30 ng mL⁻¹) for (c) 24 h, (d, g, and h) 5 days, or (e, f, and i) indicated time periods. (c) Hem1 mRNA levels (qPCR assay) during osteoclastogenesis. (d) Representative pictures (left) and number (right) of TRAP-positive multinucleated osteoclasts generated from bone marrow macrophages (scale bar: 500 μ m). (e) mRNA levels (qPCR assay) of osteoclast markers during osteoclastogenesis. (f) Protein levels (Western blot) in bone marrow macrophage cultures. (g) Representative pictures of Von Kossa-stained bone biomaterial surface (scale bar: 500 μ m) (*n* = 4/group). (h) Representative pictures of DAPI (blue; nuclei) and phalloidin (red; actin rings) staining of osteoclast cultures. (scale bar: 500 μ m). (i) mRNA levels (qPCR assay) of osteoclast fusion markers during osteoclastogenesis. All cultures were completed in triplicate. Lines and error bars

represent mean $\pm$ SD. *P* values were determined with (a) 1-way ANOVA or (c–e, i) Student's *t*-test.

Fig. 4: Rescue of cell fusion in Hem1-deficient osteoclasts by retroviral transduction of Hem1. Bone marrow macrophages lacking Hem1 were transduced with retroviral vectors expressing either empty vector (MSCV) or Hem1 (MSCV-Hem1) and were cultured with M-CSF and RANKL for (a) 24 h or (b–d) 5 days. (a) Protein levels (Western blot) of Hem1 in bone marrow macrophage cell cultures. (b) Representative pictures (left) and number (right) of TRAP-positive multinucleated osteoclasts with more than 10 nuclei, generated from bone marrow macrophages (scale bar: 500 μ m). (c) Representative pictures (left) and resorbed areas (right) of Von Kossa-stained bone biomaterial surface (scale bar: 500 μ m) (*n* = 4/group). (d) mRNA levels (qPCR assay) of osteoclast markers in mOCs. All cultures were completed in triplicate. Lines and error bars represent mean $\pm$ SD. *P* values determined using Student's *t*-test.

Fig. 5: Deletion of Hem1 attenuates respiration by suppressing c-Abl signaling. (a–f) Bone marrow macrophages from indicated genotypes were cultured with M-CSF and RANKL for 3 days. Different fractions of mitochondrial and non-mitochondrial respiration per 10000 cells, in osteoclasts, was measured by Seahorse XF96 (*n* = 11–12 wells/group). (g–h) Bone marrow macrophages lacking Hem1 were cultured with M-CSF and RANKL for (g) 3 days or (h) indicated time points. (g) ATP levels in osteoclasts (RLU, relative luminescence units; *n* = 4/group). (h) Protein levels (Western

blot) in bone marrow macrophage cultures. (i) Osteoclasts developed in cultures of bone marrow macrophages from 6-month-old male C57BL/6 mice in the presence or absence of imatinib (5 μ M). Representative pictures (left) and number (right) of TRAP-positive multinucleated osteoclasts with more than 10 nuclei (scale bar: 500 μ m) (triplicate cultures). Lines and error bars represent mean $\pm$ SD. *P* values were determined with (a–g) Student's *t*-test or (i) 1-way ANOVA.

Fig. 6: Rescue of bone phenotype in Hem1-null mice by transplantation with wild-type hematopoietic cells. Hematopoietic stem cells from GFP⁺Lin⁻CD45⁺ wild-type mice efficiently repopulated the hematopoietic system of non-ablated Hem1-deficient mice after bone marrow cell transplantation. Femurs and bone marrow were analyzed 10 weeks after transplantation (*n*=5). (a) Schematic model of bone marrow transplantation. (b) Representative images of whole body (left) and longitudinal weight measurement (right). (c) Representative images of whole femur (left) and length measurement in 13-week-old male recipient Hem1 knockout mice and wild-type littermates (right). (d) Representative images of cortical bone at midshaft (scale bar: 100 μ m). (e) Cortical thickness and cortical perimeters at the midshaft. (f) Representative images of trabecular bone (scale bar: 1 mm) and (g) bone volume per tissue volume (BV/TV), bone mineral density (BMD), and microarchitecture of trabecular bone. (h) Serum concentration of a collagen degradation product (CTX) by ELISA. (i) Representative pictures (left) and resorbed areas (right) of Von Kossa-stained bone biomaterial surface (scale bar: 500 μ m) (triplicate cultures). (j–m) Bone

marrow macrophages were isolated from 13-week-old recipient Hem1 knockout mice and wild-type littermates and cultured with M-CSF and RANKL for (**g**, **i**, and **j**) 5 days or (**k** and **m**) 3 days. (**j**) Representative pictures (left) and number (right) of TRAP-positive multinucleated osteoclasts with more than 10 nuclei (scale bar: 500 μ m) ($n = 6$). (**k**) mRNA levels (qPCR assay) of osteoclast markers during osteoclastogenesis (triplicate cultures). (**l**) Representative pictures of DAPI (blue, nuclei) and phalloidin (red, actin rings) staining in osteoclast cultures (scale bar: 500 μ m). (**m**) mRNA levels (qPCR assay) of fusion markers during osteoclastogenesis (triplicate cultures). Lines and error bars represent mean $\pm$ SD. P values determined with Student's t -test.

● WT ● Hem1 KO

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

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