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The L-type amino acid transporter LAT1 inhibits osteoclastogenesis and maintains bone homeostasis through the mTORC1 pathway

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Abstract

L-type amino acid transporter 1 (LAT1), which is encoded by *solute carrier transporter 7a5* (*SLC7A5*), plays a crucial role in amino acid sensing and signaling in specific cell types, contributing to the pathogenesis of cancer and neurological disorders. Amino acid substrates of

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SUPPLEMENTARY MATERIALS

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LAT1 have a beneficial effect on bone health directly and indirectly, suggesting a potential role for LAT1 in bone homeostasis. Here, we identified LAT1 in osteoclasts as important for bone homeostasis. *Slc7a5* expression was substantially reduced in osteoclasts in a mouse model of ovariectomy-induced osteoporosis. The osteoclast-specific deletion of *Slc7a5* in mice led to osteoclast activation and bone loss in vivo, and *Slc7a5* deficiency increased osteoclastogenesis in vitro. Loss of *Slc7a5* impaired activation of the mechanistic target of rapamycin complex 1 (mTORC1) pathway in osteoclasts, whereas genetic activation of mTORC1 corrected the enhanced osteoclastogenesis and bone loss in *Slc7a5*-deficient mice. Last, *Slc7a5* deficiency increased the expression of nuclear factor of activated T cells, cytoplasmic 1 (*Nfatc1*) and the nuclear accumulation of NFATc1, a master regulator of osteoclast function, possibly through the canonical nuclear factor κ B pathway and the Akt–glycogen synthase kinase 3 β signaling axis, respectively. These findings suggest that the LAT1–mTORC1 axis plays a pivotal role in bone resorption and bone homeostasis by modulating NFATc1 in osteoclasts, thereby providing a molecular connection between amino acid intake and skeletal integrity.

INTRODUCTION

Amino acid transporters are transmembrane proteins that mediate the transfer of amino acids into and out of cells and are implicated in various cellular processes, including gene expression, protein synthesis, energy metabolism, and catabolic and anabolic reactions (1–3). Dysregulation or dysfunction of amino acid transporters contributes to various pathologies, such as cancer, neurodegenerative diseases, chronic kidney diseases, obesity, and diabetes, by inducing an imbalance in intracellular amino acid concentration (3–7). L-type amino acid transporter 1 (LAT1), encoded by *solute carrier transporter 7a5* (*Slc7a5*), is a Na⁺-independent amino acid transporter that mediates cellular uptake of large neutral amino acids, including the essential amino acids (EAAs) Leu, Ile, and Val (8, 9). LAT1 forms a heterodimer with the heavy chain of 4F2, a cell surface antigen encoded by *Slc3a2*, through a disulfide bond (8, 10). The intracellular concentration of Leu is an essential signal for promoting the activity of the mechanistic target of rapamycin complex 1 (mTORC1) in specific cell types, and its homeostasis is, at least in part, dependent on the uptake of Leu by LAT1 (11–14). LAT1 is reported to be present in a limited number of normal tissues, including the blood-brain barrier, placenta, and testes; however, genetic studies in mice have revealed functional LAT1 in T cells and skeletal muscle (15–18).

The development and integrity of the skeleton, as well as bone homeostasis, are coregulated by two cell types, bone-forming osteoblasts and bone-resorbing osteoclasts (19, 20). An imbalance in the sophisticated mutual regulation between osteoclasts and osteoblasts leads to pathogenesis and underlies the etiology of certain metabolic bone diseases including osteoporosis and osteopetrosis (21, 22). Adequate dietary protein and amino acid intake are widely acknowledged to be essential for proper skeletal development and bone homeostasis. In addition, amino acid supplementation can modify the progression of skeletal manifestations of certain genetic disorders of the bone such as Coffin-Lowry syndrome (23). Amino acid substrates of LAT1 are suggested to have a beneficial impact on bone health directly and indirectly. For example, EAA supplements that include Leu and Ile increase bone strength through modification of bone mineral density and bone microarchitecture in

ovariectomized osteoporotic rats fed a low-protein diet (24). Moreover, higher Leu intake is associated with greater bone mineral density in the spine and the forearm in a human study of female monozygotic twins discordant for amino acid intake (25).

Here, we found that the amino acid transport system involving LAT1 was active in both the osteoclasts and the osteoblasts of mice and that *Slc7a5* expression was reduced in osteoclasts in a mouse model of postmenopausal osteoporosis. Mouse genetic studies revealed that *Slc7a5* played a crucial role in bone resorption and bone homeostasis through its expression in osteoclasts. Subsequent analyses identified mTORC1 as a critical mediator of LAT1-dependent osteoclastogenesis, bone resorption, and bone homeostasis. Last, we found that the LAT1-mTORC1 axis controlled the nuclear accumulation of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), a master regulator of osteoclast differentiation, and *Nfatc1* expression, likely through the Akt–glycogen synthase kinase 3 β (GSK3 β) axis and the canonical nuclear factor κ B (NF- κ B) pathway, respectively. Our results demonstrate that the LAT1-mTORC1 axis may be a pivotal player in bone resorption and bone homeostasis by modulating NFATc1 in osteoclasts, thereby suggesting LAT1 as a novel potential target for metabolic bone diseases.

RESULTS

LATs mediate amino acid uptake in bone cells

We first evaluated whether the functional amino acid transport system involving LATs was operational in osteoblasts and osteoclasts. To that end, we tested whether primary mouse osteoblasts and osteoclasts took up radiolabeled L-3-[¹²⁵I]iodo- α -methyltyrosine ([¹²⁵I]IMT), an artificial amino acid derived from the neutral amino acid Tyr (Fig. 1A), because its accumulation is mediated mainly by the LATs (26, 27). We synthesized [¹²⁵I]IMT at a radiochemical purity of 99% by high-performance liquid chromatography (HPLC). [¹²⁵I]IMT uptake was significantly higher at 37°C compared to 4°C in both primary osteoblasts and osteoclasts (Fig. 1B). Replacing the sodium chloride (NaCl) in the uptake buffer with choline chloride (ChoCl) reduced the uptake of [¹²⁵I]IMT at 37°C to about 70% in osteoblasts but not in osteoclasts (Fig. 1, C and D). Moreover, JPH203, a specific inhibitor of LAT1 (28), modestly but significantly reduced [¹²⁵I]IMT incorporation to about 60% in osteoblasts (Fig. 1E), whereas it reduced uptake to about 10% in osteoclasts (Fig. 1F). These results suggest that both osteoblasts and osteoclasts have a temperature-dependent, Na⁺-independent, and LAT1-dependent amino acid uptake system, whereas a Na⁺-dependent and JPH203-insensitive amino acid uptake system may be partially responsible for amino acid uptake in osteoblasts.

Slc7a5 expression in preosteoclasts is reduced in ovariectomized mice

To determine whether LATs are involved in the pathogenesis of metabolic bone disease, we examined the expression of LAT-encoding genes in bone cells in a mouse model of postmenopausal osteoporosis. C57BL6 mice underwent ovariectomy (OVX) or sham operation, and CD11b^{low/-}CX3CR1⁺Ly6C^{hi} cells (preosteoclasts), which comprise most of the osteoclast precursor activity in the bone marrow (29), and CD45⁺CD51⁺Sca1⁻ cells (osteoblasts), which represent a mixture of both immature and mature osteoblasts (30), were

subsequently isolated by fluorescence-activated cell sorting (FACS) for mRNA expression analysis (Fig. 2A). Expression of *Slc7a5*, *Slc7a8*, *Slc3a2*, and *Slc1a5* was higher in preosteoclasts than in osteoblasts relative to a reference RNA (*Actb*) (fig. S1). Among the transcripts tested, only *Slc7a5* mRNA was significantly reduced in the preosteoclasts of ovariectomized mice compared to sham-operated controls (Fig. 2B). In contrast, no significant alterations in amino acid transporter mRNA abundance were observed in the osteoblasts of ovariectomized mice (Fig. 2C).

Slc7a5 deficiency in osteoclasts causes bone loss and osteoclast activation

Our data revealed that LAT1 was produced and functional in osteoclasts and possibly associated with the pathogenesis of postmenopausal osteoporosis (Figs. 1 and 2); however, the physiological importance of LAT1 in the maintenance of bone homeostasis in vivo has not been demonstrated. Given that global *Slc7a5* knockout in mice is lethal between embryonic days 9.5 and 11.5 (E9.5 and E11.5) (16, 31), we generated osteoclast-specific *Slc7a5* knockout mice by crossing *Slc7a5*-floxed mice with *Tnfrsf11a-Cre* mice or *Lyz2-Cre* mice to determine whether LAT1 plays a role in bone homeostasis through *Slc7a5* expression in osteoclasts (Fig. 3A). *Slc7a5* mRNA abundance was decreased in osteoclasts cultured from mice lacking *Slc7a5* in *Tnfrsf11a*-expressing cells, hereafter referred to as *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice, compared with that in the control mice not expressing *Cre* (Fig. 3B and fig. S2A). The bone volume-to-tissue volume (BV/TV) ratio of *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice was significantly lower than that of the control mice in both the femurs (Fig. 3, C and D) and the vertebrae (Fig. 3, E and F). Bone histomorphometric analyses revealed that bone formation indices, such as number of osteoblasts/tissue area (N.Ob/T.Ar) and bone formation rate (BFR), were comparable between *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* and control mice (Fig. 3, G and H), whereas the osteoclast surface/bone surface (Oc.S/BS), an index of osteoclastic function, was significantly increased in *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice (Fig. 3I). Moreover, serum biochemical analyses revealed significantly higher circulating concentrations of the bone resorption marker CTx but a normal abundance of the bone formation marker P1NP in *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice (Fig. 3, J and K).

We used *Lyz2-Cre* transgenic mice to target *Slc7a5* deletion to osteoclasts. Similar to *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice, *Lyz2-Cre;Slc7a5^{fl/fl}* mice showed not only a significantly lower BV/TV ratio but also significantly higher osteoclast and bone resorption indices compared with those in control mice (Fig. 3, L to P). Collectively, these results suggest that LAT1 in osteoclasts might have an important role in bone resorption and bone homeostasis under physiological conditions in vivo.

Slc7a5 deficiency in osteoblasts does not affect the bone phenotype

We showed that the LAT1-dependent amino acid uptake system possibly could be functional in osteoblasts in addition to osteoclasts (Fig. 1). To determine the role of LAT1 in osteoblasts in bone homeostasis, we generated osteoblast-specific *Slc7a5* knockout mice by crossing *Slc7a5*-floxed mice with *Osterix (Ox)*-*Cre* mice, also known as *Ox-GFP::Cre*, which expresses the GFP::Cre fusion protein (Fig. 4A). *Slc7a5* expression was markedly decreased in the green fluorescent protein (GFP)-positive cells of osteoblast-specific *Slc7a5* knockout mice, termed *Ox-Cre;Slc7a5^{fl/fl}* mice (Fig. 4B and fig. S2B). The BV/TV ratio in

femurs of *Osx-Cre;Slc7a5^{fl/fl}* mice was indistinguishable from that of control mice (Fig. 4, C to F). Bone histomorphometric analyses revealed that the bone resorption index (Oc.S/BS) and bone formation indices (BFR and N.Ob/T.Ar) were comparable between *Osx-Cre;Slc7a5^{fl/fl}* and control mice (Fig. 4, G to K). These results indicate that LAT1 in osteoblasts is likely not required for bone formation and homeostasis under physiological conditions in vivo.

***Slc7a5* deficiency stimulates osteoclastogenesis in vitro**

We evaluated whether *Slc7a5* affected osteoclastogenesis in vitro. We infected bone marrow macrophages (BMMs) from *Slc7a5^{fl/fl}* mice with a retrovirus expressing Cre recombinase to induce *Slc7a5* knockout and differentiated the cells into osteoclasts by treating them with receptor activator of NF- κ B ligand (RANKL). The cells were then subjected to tartrate-resistant acid phosphatase (TRAP) staining and pit formation assays (Fig. 5A). The number of TRAP-positive multinucleated cells and the area of pit formation were both significantly increased in *Slc7a5*-deleted BMMs, indicating an enhancement of osteoclast differentiation and function (Fig. 5, B and C). Moreover, the expression of the osteoclast differentiation and fusion markers, *Nfatc1*, *transmembrane 7 superfamily member 4* (*Dcstamp*), *cathepsin K* (*Ctsk*), and *matrix metalloproteinase 9* (*Mmp9*), was markedly increased in *Slc7a5*-deleted osteoclasts compared to controls (Fig. 5D). We further determined the effect of *Slc7a5* deletion on proliferation and survival of osteoclasts. Flow cytometric measurements after BrdU incorporation revealed that the number of cells in S phase was comparable between control cells and *Slc7a5*-deficient BMMs, irrespective of RANKL treatment (Fig. 5E). Further-more, *Slc7a5* deficiency did not significantly affect the number of apoptotic cells in BMM cultures irrespective of RANKL treatment (Fig. 5F). These results show that LAT1 negatively regulates osteoclastogenesis in vitro without affecting cell proliferation or cell survival.

mTORC1 activation corrects bone loss and osteoclast activation in osteoclast-specific *Slc7a5* knockout mice

LAT1 mediates the transport of large neutral amino acids, including Leu, in many cell types, leading to activation of the mTORC1 pathway (11, 12), and mTORC1 in osteoclasts is essential for maintaining bone homeostasis and bone resorption (32, 33). Thus, we next examined whether the mTORC1 pathway might be implicated in LAT1-dependent negative regulation of osteoclastogenesis, bone homeostasis, and bone resorption.

Phosphorylation of p70S6 kinase (p70S6K) and 4E-BP1, both of which are downstream effectors of mTORC1, was significantly decreased in *Slc7a5*-deficient BMMs, irrespective of RANKL treatment, indicating reduced mTORC1 activity in osteoclasts (Fig. 6, A to C). Moreover, RANKL-dependent p70S6K phosphorylation tended to decrease in osteoclasts when the cells were deprived of Val, Ile, or Leu (fig. S3), whereas RANKL did not alter [¹²⁵I]IMT uptake in osteoclasts (fig. S4), indicating that Leu, Val, and Ile were all required for mTORC1 activation by RANKL. In contrast, phosphorylation of eukaryotic initiation factor-2 α (eIF2 α), which allows the preferential translation of the master regulator of amino acid metabolism activating transcription factor 4 (ATF4), was markedly increased in *Slc7a5*-

deficient cells (fig. S5), indicating the activation of the general amino acid control (GAAC) pathway in osteoclasts.

Tuberous sclerosis complex 1 (Tsc1), also known as hamartin, is a critical negative regulator of mTORC1 because it acts as a guano-sine triphosphatase-activating protein for the small G protein Ras homolog enriched in the brain (Rheb) (34, 35). Introduction of a constitutively active form of Rheb, the upstream activator of mTORC1, significantly inhibited the enhanced osteoclastogenesis observed in *Slc7a5*-deficient BMMs (Fig. 6, D and E). To confirm this finding in vivo, we generated *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice lacking one copy of the floxed *Tsc1* allele, hereafter referred to as *Tnfrsf11a-Cre;Slc7a5^{fl/fl}; Tsc1^{fl/+}* mice. The low bone mass phenotype of *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice was rescued in *Tnfrsf11a-Cre;Slc7a5^{fl/fl}; Tsc1^{fl/+}* mice as a result of the normalization of osteoclast activation (Fig. 6, F and G). In line with our in vitro findings, these results suggest that LAT1, at least in part, negatively regulates bone resorption and bone homeostasis by modulating mTORC1 signaling in osteoclasts in vivo.

Slc7a5 deficiency increases nuclear accumulation of NFATc1 in osteoclasts

To elucidate the potential mechanisms underlying osteoclastogenesis inhibition by the LAT1-mTORC1 axis, we first examined the protein abundance of NFATc1, a master transcription factor required for osteoclastogenesis, in *Slc7a5*-deficient cells. The whole-cell abundance of NFATc1 was significantly increased in *Slc7a5*-deficient cells in the presence of RANKL (Fig. 7, A and B), in accordance with the observed increase in *Nfatc1* mRNA abundance in *Slc7a5*-deficient cells (Fig. 5D). Moreover, the nuclear abundance of NFATc1 was also significantly increased in *Slc7a5*-deficient cells in the presence of RANKL (Fig. 7, A and C), suggesting that *Slc7a5* deficiency leads to an increase in the nuclear accumulation of NFATc1, in addition to its transcriptional enhancement, in osteoclasts.

Slc7a5 deficiency induces nuclear NFATc1 accumulation through the Akt-GSK3 β axis and Nfatc1 expression through the canonical NF- κ B pathway in osteoclasts

To determine which signaling pathways might be involved in the LAT1-dependent regulation of *Nfatc1* expression and nuclear NFATc1 accumulation, we analyzed various signaling pathways known to regulate osteoclastogenesis and NFATc1 abundance. RANKL-induced phosphorylation of mitogen-activated protein kinase (MAPK) family members Erk1 and Erk2 (Erk1/2), Jun N-terminal kinases 1 and 2 (JNK1/2), and p38, all of which are immediately downstream of RANKL signaling in osteoclasts, was comparable between the control and *Slc7a5*-deficient cells (Fig. 7, D to I).

In contrast to the MAPK family members, Akt phosphorylation was markedly increased in *Slc7a5*-deficient cells irrespective of RANKL stimulation (Fig. 8, A and B). Akt phosphorylates and inhibits GSK3 β , thus preventing GSK3 β from promoting NFATc1 translocation from the nucleus into the cytoplasm, leading to enhanced osteoclastogenesis by nuclear NFATc1 (36). In accordance with an increase in nuclear NFATc1 accumulation (Fig. 7, A and C), GSK3 β phosphorylation was also significantly increased in RANKL-stimulated *Slc7a5*-deficient cells compared to RANKL-treated control cells (Fig. 8, A and C),

indicating an increase in nuclear NFATc1 accumulation by *Slc7a5* deficiency, at least in part, through the Akt-GSK3 β axis.

RANKL stimulation causes the activation of inhibitor of κ B (I κ B) kinase (IKK), which induces phosphorylation and proteasomal degradation of I κ B. This results in the nuclear translocation of NF- κ B, composed of p65 and p50 subunits, and the transcription of target genes, including *Nfatc1* (37–39). Nuclear accumulation of p65, as well as the phosphorylation of both IKK α / β and I κ B α , was significantly increased in *Slc7a5*-deficient cells in the presence of RANKL (Fig. 8, D to H), indicating the activation of the canonical NF- κ B pathway by *Slc7a5* deficiency.

The enhanced osteoclastogenesis phenotype of *Slc7a5*-deficient cells was significantly inhibited by treatment with either the Akt inhibitor X or the IKK inhibitor BMS-345541, suggesting the functional importance of the Akt and NF- κ B pathways in the LAT1-dependent inhibition of osteoclastogenesis (Fig. 8, I and J, and fig. S6, A and B). Collectively, these results suggest that the LAT1-mTORC1 axis negatively regulates not only the nuclear accumulation of NFATc1 through the Akt-GSK3 β axis but also *Nfatc1* expression through the canonical NF- κ B pathway, thereby inhibiting osteoclastogenesis (Fig. 8K).

DISCUSSION

Appropriate dietary protein and amino acid intake is widely accepted as essential for adequate accumulation of bone tissue during growth and for maintenance of skeletal structural integrity throughout life, although a high-protein diet has long been considered to have a negative impact on bone health through an increase in bone breakdown, leading to an increase in urinary calcium (40, 41). Although amino acid substrates for LAT1 are suggested to have a beneficial impact on bone health directly and indirectly, a potential direct role of LAT1 in bone cells in the maintenance of bone homeostasis had not been previously described. The main relevance of the present findings is that the cell surface amino acid transporter LAT1 is present in both osteoblasts and osteoclasts and is functionally required in osteoclasts for proper amounts of bone resorption to maintain bone homeostasis. The fact that *Slc7a5* transcripts decreased in osteoclasts of ovariectomized mice (Fig. 2) suggests a possible inverse association between osteoclast *Slc7a5* expression and the pathogenesis of postmenopausal osteoporosis, although the mechanisms governing *Slc7a5* expression in osteoclasts currently remain unclear. Given that *Slc7a5* global knockout in mice causes early embryonic lethality at E9.5 to E11.5 (16, 31), the in vivo role of LAT1 (*Slc7a5*) on bone development is currently unknown. *Slc7a5* deficiency in osteoblasts did not alter bone volume or bone formation parameters in vivo (Fig. 4), irrespective of the JPH203-sensitive LAT1-dependent amino acid uptake in osteoblasts (Fig. 1), suggesting that LAT1 might contribute to intracellular amino acid homeostasis and various cellular processes in osteoblasts to a lesser extent compared with other members of the LAT family. Alternatively, compensatory activation of other members of the LAT family might be occurring in *Slc7a5*-deficient osteoblasts, similar to that reported in muscle-specific *Slc7a5* knockout mice (16). Although further examination is necessary to completely exclude the possible involvement of LAT1 in bone formation and bone remodeling through its activity in osteoblasts, LAT1 is

likely the primary amino acid sensor involved in the regulation of bone resorption and bone remodeling in the osteoclast lineage.

There are two major intracellular amino acid sensing mechanisms: the GAAC pathway, which is mediated by general control nonderepressible 2 and the transcription factor ATF4, and the mTORC1 pathway (42–44). Various independent lines of investigation using pharmacological and genetic strategies demonstrate that mTORC1 signaling is important for osteoclast differentiation and function in vitro and in vivo; however, the results of these studies have been inconsistent (32, 33, 45, 46). For example, bone mass was increased or decreased by osteoclast-specific *Raptor* deletion depending on the driver that was used to generate the deficiency (*Ctsk-Cre* or *Lyz2-Cre*, respectively) (32, 33, 45). This discrepancy might be caused by *Raptor* deletion in different cell types—mature osteoclasts in the case of *Ctsk-Cre* and preosteoclasts with *Lyz2-Cre*. Given that genetic activation of mTORC1 almost completely corrects both the lower bone mass and the higher bone resorption phenotypes of *Slc7a5*-deficient mice (Fig. 6), our findings suggest that mTORC1 might be a pivotal downstream effector of LAT1 in osteoclasts. In contrast, ATF4, the major transcription factor in the GAAC pathway, activates osteoclastogenesis through the induction of *Nfatc1* (47). We, therefore, cannot exclude the possibility that the GAAC pathway, which is a well-known alternative major amino acid sensing pathway, might also contribute to LAT1-dependent regulation of bone homeostasis. The increase in eIF2 α phosphorylation that we observed upon *Slc7a5* deficiency is consistent with the involvement of the GAAC pathway (fig. S5).

Our findings suggest that the LAT1-mTORC1 axis inhibits osteoclastogenesis through the negative control of the NF- κ B-NFATc1 pathway and the Akt-GSK3 β axis (Fig. 8); the latter enhances osteoclastogenesis by dephosphorylation and nuclear accumulation of NFATc1 (36). Given that mTORC1 directly phosphorylates NFATc1, leading to a decrease in its nuclear accumulation and therefore inhibiting osteoclastogenesis (33), the possibility that the LAT1-mTORC1 axis inhibits osteoclastogenesis through negative regulation of the NFATc1 function by promoting direct mTORC1-mediated phosphorylation of NFATc1 should not be ruled out.

In conclusion, the current findings indicate that the amino acid transporter LAT1 functions in osteoclasts to repress osteoclastogenesis during bone resorption and bone homeostasis, thereby providing a molecular connection between a nutritional signal and skeletal integrity. LAT1 has been proposed as a promising target for both diagnostic cancer imaging (for positron emission tomography or single-photon emission computed tomography) and anticancer therapeutics (48–51). Our findings suggest that LAT1 is also a potential target for the discovery and development of drugs for diagnostic and therapeutic approaches in various metabolic bone diseases relevant to abnormal osteoclastogenesis, such as osteoporosis, rheumatoid arthritis, and metastatic bone cancer in addition to congenital skeletal dysplasias such as Coffin-Lowry syndrome.

MATERIALS AND METHODS

Mice

The protocol used here meets the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. *Tsc1^{fl/fl}* mice were obtained from the Jackson laboratory. *Slc7a5^{fl/fl}* and *Tsc1^{fl/fl}* mice were crossed with *Osx-Cre*, *Tnfrsf11a-Cre*, or *Lyz2-Cre* mice (52–55). These mutant mice were backcrossed more than five generations with C57BL/6J. Mice were bred under standard animal housing conditions at 23° ± 1°C with humidity of 55% and a 12-hour light/12-hour dark cycle, with free access to food and water. *Osx-Cre* mice were maintained in the absence of doxycycline. Genotyping was performed by polymerase chain reaction (PCR) using tail genomic DNA. The numbers of animals used per experiment are stated in the figure legends. For OVX, 8-week-old mice were anesthetized by an intraperitoneal injection of pentobarbital and subjected to OVX or sham operation under aseptic environments as described previously (56). Mice were killed by decapitation 28 days after operation.

Cell sorting

Femur and tibia were crushed and digested with 0.2% collagenase in phosphate-buffered saline (PBS), followed by depletion of red blood cells by soaking in 0.15 M NH₄Cl for 5 min. Cells were then resuspended in PBS containing 2% fetal bovine serum and incubated for 30 min at 4°C with a cocktail of antibodies for preosteoclast-like cell markers [allophycocyanin-conjugated anti-CD11b antibody (M1/70) (BioLegend), fluorescein isothiocyanate (FITC)-conjugated anti-CX3CR1 antibody (SA011F11) (BioLegend), and phycoerythrin (PE)-Cy7-conjugated anti-Ly6C (AL-21) (BD Pharmingen)] or a cocktail of antibodies for osteoblast-like cell markers [FITC-conjugated anti-CD45 antibody (30-F11) (BD Pharmingen), PE-conjugated anti-CD51 antibody (RMV-7) (BioLegend), and PE-Cy7-conjugated anti-Sca1 antibody (D7) (BD Pharmingen)] and 7-Aminoactinomycin D for dead cell exclusion. Immunostained cells were washed and analyzed on a FACS Aria II cell sorter (BD Biosciences) and then sorted for CD11b^{low/-}/CX3CR1⁺/Ly6C^{hi} cells (preosteoclasts) and CD45⁻/CD51⁺/Sca-1⁻ cells (osteoblasts) (29, 57, 58).

Preparation of [¹²⁵I]IMT

[¹²⁵I]NaI (629 GBq/mg as iodine) was obtained from PerkinElmer. No-carrier-added [¹²⁵I]IMT was prepared using the conventional chloramine-T method from L- α -methyltyrosine as a precursor. Briefly, chloramine-T (Nacalai Tesque) aqueous solution (2 mg/ml) was added to a mixture of L- α -methyltyrosine (1.7 mg/ml) dissolved in 0.1 M phosphate buffer (pH 7.4) and carrier-free [¹²⁵I]NaI (925 to 1100 kBq). After incubation for 10 min, the reaction was quenched by adding sodium bisulfite aqueous solution (2 mg/ml). [¹²⁵I]IMT was identified by HPLC (Shimadzu), and IMT, which was prepared using nonradioactive iodine, was identified by mass spectrometry (JMS-T100TD, JEOL). HPLC was performed with a COSMOSIL 5C₁₈-AR-II column (4.5 mm by 150 mm; Nacalai Tesque) at a flow rate of 1 ml/min with a gradient mobile phase of 20% methanol in water with 0.1% trifluoroacetic acid (TFA) to 40% methanol in water with 0.1% TFA for 20 min. The column temperature was maintained at 40°C.

[¹²⁵I]IMT uptake assay

Cultured cells were washed twice with Hanks' balanced salt solution (HBSS) [125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM Hepes, and 5.6 mM D-glucose (pH 7.4)] and subsequent incubation in HBSS at 37°C for 10 min in a 5% CO₂ incubator. Cells were then incubated with [¹²⁵I]IMT (176.5 pM) at 4° or 37°C for 30 min. Cells were treated with JPH203 at 30 μM. The reaction was terminated by the aspiration of buffer, followed by superficial rinsing with ice-cold HBSS containing 1 mM unlabeled L-α-methyltyrosine at 4°C three times to remove extracellular [¹²⁵I]IMT. The cells were lysed using 0.1 M NaOH. The radioactivity was measured by a γ-counter (ARC-7010, Hitachi Ltd.). Protein concentration was determined with a Protein Assay Bicinchoninate Kit (Nacalai Tesque).

Bone histomorphometric analysis, x-ray micro-computed tomography (μCT) analysis, and serum biochemical analysis

Bone histomorphometric analyses were performed on uncalcified long bones and vertebrae in accordance with previously described methods (59). Briefly, the bone was fixed with 10% formalin, followed by dehydration in an ethanol series and subsequent embedding in methyl methacrylate resin, according to standard protocols. The BV/TV ratio was measured by von Kossa staining. Osteoblast and osteoclast parameters were analyzed by staining with toluidine blue and TRAP, respectively. Analyses were performed using the OsteoMeasure analysis system (OsteoMetrics), according to standard protocols (60). Trabecular architecture was assessed in long bones using a μCT system (Comscan), and the BV/TV ratio was measured using TRI/3D-BON software (RATOC) (61, 62). Calcein, which has a high calcium affinity, was injected twice into mice with an interval of 3 days, and then, mice were killed 2 days after the last injection. The new-forming bone area was marked with the calcein-calcium complex. Concentrations of CTx and PINP in serum were determined by enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Immunodiagnostic Systems).

Culture of osteoclasts, TRAP staining, pit formation assay, cell cycle assay, and cell death assay

BMMs were cultured in the presence of macrophage colony-stimulating factor (M-CSF) (20 ng/ml) and RANKL (20 ng/ml) for 5 days, and TRAP staining and the pit formation assay were performed as previously described (63, 64). Cells were treated with BMS-345541 (Abcam) at 0.16 μM or Akt inhibitor X (Cayman) at 0.2 μM followed by TRAP staining. Cells were cultured with 10 μM BrdU for 45 min and were prepared for analysis of BrdU incorporation using the FITC BrdU Flow Kit (BD Biosciences) by flow cytometric analysis using the FACSVerse (BD Biosciences). Cell death assay was performed with FITC–Annexin V (BD Biosciences) and propidium iodide by FACSVerse. Data were analyzed by FACSuite software (BD Biosciences).

Generation of retroviral vectors and infection

pMX-CA-Rheb was generated by subcloning into the pMX vector from the pcDNA3-FLAG-Rheb-N153 T vector (Addgene plasmid no. 19997). Retroviral vectors were transfected into

Plat-E cells using the calcium carbonate method. Virus supernatants were collected 48 hours after transfection, and then, cells were infected with virus supernatants for 72 hours in the presence of polybrene (4 µg/ml). Cells were then subjected to selection by culture with puromycin (1 µg/ml) for 3 days before use in experiments.

Real-time qPCR

Total RNA was extracted from cells, followed by synthesis of complementary DNA (cDNA) with reverse transcriptase and oligo-dT primer. The cDNA samples were then used as templates for real-time PCR analysis, which was performed on an Mx3005P instrument (Agilent Technologies), by using specific primers for each gene (table S1). The levels of the genes examined were normalized using *Actb* as an internal control for each sample.

Immunoblotting analysis

Cultured cells were solubilized in lysis buffer containing 1% NP-40. Samples were then subjected to SDS–polyacrylamide gel electro-phoresis, followed by transfer to polyvinylidene difluoride membranes and subsequent immunoblotting assay. Quantification was performed by densitometry using ImageJ. The primary antibodies used were anti-phospho-p70S6k1 (no. 9205), anti-p70S6k1 (no. 2708), anti-phospho-4E-BP1 (no. 2855), anti-4E-BP1 (no. 9644), anti-eIF2α (no. 5324), anti-phospho-eIF2α (no. 9721), anti-NFATc1 (no. 8032), anti-phospho-Erk1/2 (no. 4370), anti-Erk1/2 (no. 4695), anti-phospho-JNK (no. 4668), anti-JNK (no. 9252), anti-phospho-p38 (no. 4511), anti-p38 (no. 8690), anti-phospho-Akt (no. 4060), anti-Akt (no. 4685), anti-phospho-GSK3β (no. 9323), anti-GSK3β (no. 9315), anti-phospho-IKKα/β (no. 2697), anti-IKKα (no. 2689), anti-phospho-IκBα (no. 2859), anti-IκBα (no. 4812), and anti-p65 (no. 8242) (Cell Signaling Technologies); anti-β-actin (no. sc-47778) (Santa Cruz Biotechnology); and anti-LaminB1 (MABS492) (EMD Millipore).

Data analysis

Results are all expressed as the means ± SE, and statistical significance was determined by the two-tailed Student's *t* test or the one-way or two-way analysis of variance (ANOVA) with Bonferroni and/or Dunnett post hoc test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. A LAT-dependent amino acid uptake system operates in both osteoclasts and osteoblasts. (A) Schematic diagram of the procedure for generating $[^{125}\text{I}]\text{IMT}$. (B) Primary osteoblasts and osteoclasts cultured from wild-type (WT) mice were incubated with $[^{125}\text{I}]\text{IMT}$ at 4° or 37°C for 30 min in HBSS buffer. $n = 4$ cell cultures from different mice. (C to F) Primary cells were incubated with $[^{125}\text{I}]\text{IMT}$ at 37°C for 30 min in HBSS buffer in which NaCl was replaced with equimolar ChoCl (C and D) or in HBSS buffer containing JPH203 (E and F). $n = 4$ cell cultures from different mice. Data were analyzed by the two-tailed Student's t test. * $P < 0.05$ and ** $P < 0.01$, significantly different from the value obtained in cells incubated at 4°C (B), cells incubated in HBSS buffer with NaCl (C and D), or cells incubated in HBSS buffer with dimethyl sulfoxide (DMSO) (E and F).

Fig. 2. *Slc7a5* expression in preosteoclasts is reduced in ovariectomized mice.

(A) C57BL/6 mice were subjected to ovariectomy (OVX) or sham operation and subsequent isolation of CD11b^{low/-}CX3CR1⁺Ly6C^{hi} cells (preosteoclasts) and CD45⁻CD51⁺Sca1⁻ cells (osteoblasts) by flow cytometry. (B and C) Quantification of mRNAs encoding amino acid transporters in preosteoclasts (B) and osteoblasts (C) by qPCR. $n = 5$ mice. All data were analyzed by the two-tailed Student's t test. $**P < 0.01$, significantly different from the value obtained for sham-operated mice. U.D., under detectable values.

Fig. 3. *Slc7a5* in osteoclasts is essential for bone resorption and bone homeostasis in vivo. (A) Schematic diagram of the generation of osteoclast-specific *Slc7a5* knockout mice using the osteoclast-specific Cre drivers *Tnfrsf11a* and *Lyz2*. (B) *Slc7a5* expression in cultured osteoclasts from *Slc7a5^{fl/fl}* (control) and *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice as determined by PCR. *Actb* is a control. *n* = 3 mice per genotype. (C) μ CT analysis and (D) bone volume-to-tissue volume (BV/TV) ratio measurements of femurs from *Slc7a5^{fl/fl}* and *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice. (E) von Kossa staining, (F) BV/TV, (G) number of osteoblasts/tissue area (N.Ob/T.Ar), (H) bone formation rate (BFR), and (I) osteoclast surface/bone surface (Oc.S/BS) measurements from vertebrae and (J) P1NP and (K) CTx concentrations in serum of *Slc7a5^{fl/fl}* and *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice at 12 weeks of age. *n* = 5 to 7 *Slc7a5^{fl/fl}* mice; *n* = 6 to 9 *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice. (L) von Kossa staining, (M) BV/TV, and (N)

Oc.S/BS of vertebrae and (O) P1NP and (P) CTx concentrations in serum from *Slc7a5^{fl/fl}* and *Lyz2-Cre;Slc7a5^{fl/fl}* mice at 12 weeks of age. $n = 7$ *Slc7a5^{fl/fl}* mice; $n = 8$ *Lyz2-Cre;Slc7a5^{fl/fl}* mice. All data were analyzed by the two-tailed Student's t test. * $P < 0.05$ and ** $P < 0.01$, significantly different from the value obtained for control mice. Scale bars, 1 mm (C) and 200 μ m (E and L).

Fig. 4. *Slc7a5* in osteoblasts is dispensable for bone formation and bone homeostasis in vivo. (A) Schematic diagram of the generation of osteoblast-specific *Slc7a5* knockout mice. (B) *Slc7a5* expression in the GFP-positive cells of control and *Osx-Cre;Slc7a5^{fl/fl}* mice as determined by PCR. *n* = 3 mice per genotype. (C) μ CT analysis, (D) BV/TV ratio as determined by μ CT, (E) von Kossa staining, (F) BV/TV as determined by von Kossa staining, (G) TRAP staining, (H) Oc.S/BS ratio, (I) calcein labeling, (J) BFR, and (K) N.Ob/T.Ar of femurs from control and *Osx-Cre;Slc7a5^{fl/fl}* mice at 12 weeks of age. *n* = 6 *Osx-Cre* mice; *n* = 8 *Osx-Cre;Slc7a5^{fl/fl}* mice. All data were analyzed by the two-tailed Student's *t* test. The double-headed white arrow in (I) indicates the distance between calcein double labeling. Scale bars, 1 mm (C and E), 100 μm (G), and 10 μm (I).

Fig. 5. *Slc7a5* deficiency stimulates osteoclastogenesis in vitro.

BMMs from *Slc7a5^{fl/fl}* mice were retrovirally infected with Cre recombinase and subsequently stimulated with RANKL to induce osteoclast differentiation. Cells were then analyzed for (A to C) the number of TRAP-positive multinucleated cells MNCs and pit formation area, (D) expression of osteoclast marker genes, (E) cell proliferation ratio, and (F) cell survival. $n = 4$ to 6 mice per genotype. Data were analyzed by the two-tailed Student's t test (B to D) or the two-way ANOVA with Bonferroni and/or Dunnett post hoc test (E and F). * $P < 0.05$ and ** $P < 0.01$, significantly different from the value obtained in control cells. Arrows in (A) indicate representative resorption pits. Scale bar, 50 μm .

Fig. 6. mTORC1 activation rescues *Slc7a5* deficiency-induced osteoclast activation in vitro and in vivo.

(A to C) BMMs from *Slc7a5*^{fl/fl} mice were retrovirally infected with Cre recombinase and subsequently stimulated with RANKL, followed by immunoblotting and densitometric quantification of phosphorylation of the indicated downstream effectors of mTORC1 signaling. β-Actin is a loading control. *n* = 4 mice per genotype. (D and E) BMMs from *Slc7a5*^{fl/fl} mice were retrovirally infected with Cre recombinase and constitutively active (CA) Rheb, an upstream activator of mTORC1, and subsequently treated with RANKL, followed by determination of the number of TRAP-positive multinucleated cells. *n* = 5 mice per genotype. E.V., empty vector “pMX-Control”. (F) μCT analysis and (G) BV/TV ratio and Oc.S/BS ratio of femurs and P1NP and CTx concentrations in serum of *Tnfrsf11a*-

Cre;Slc7a5^{fl/fl};Tsc1^{fl/+} mice at 12 weeks of age. $n = 6$ control mice (*Slc7a5^{fl/fl};Tsc1^{fl/+}* mice or *Slc7a5^{fl/+};Tsc1^{fl/+}* mice) and *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice; $n = 7$ *Tnfrsf11a-Cre;Slc7a5^{fl/fl};Tsc1^{fl/+}* mice. Data were analyzed by the two-way ANOVA with Bonferroni and/or Dunnett post hoc test (B, C, and E) or the one-way ANOVA with Bonferroni and/or Dunnett post hoc test (G). * $P < 0.05$ and ** $P < 0.01$, significantly different from control cells (B, C, and E) or control mice (G). # $P < 0.05$ and ### $P < 0.01$, significantly different from the value obtained for *Slc7a5*-deficient cells infected with control vector (E) or *Tnfrsf11a-Cre;Slc7a5^{fl/fl}* mice (G). Scale bars, 50 μm (D) and 1 mm (F).

Fig. 7. Nuclear accumulation of NFATc1 increases in *Slc7a5*-deficient osteoclasts. BMs from *Slc7a5*^{fl/fl} mice were retrovirally infected with Cre recombinase and subsequently stimulated with RANKL, followed by immunoblotting and densitometric analysis of (A to C) NFATc1 abundance in the nucleus and whole-cell extracts and (D to I) phosphorylation of Erk1/2, JNK1/2, and p38. *n* = 4 mice per genotype. All data were analyzed by the two-way ANOVA with Bonferroni and/or Dunnett post hoc test. ***P* < 0.01, significantly different from the value obtained in control cells.

Fig. 8. The Akt-GSK3β and canonical NF-κB pathways are activated in *Slc7a5*-deficient osteoclasts.

BMMs from *Slc7a5*^{fl/fl} mice were retrovirally infected with Cre recombinase and subsequently stimulated with RANKL, followed by immunoblotting and densitometric quantification of (A to C) phosphorylation of Akt and GSK3β, (D to F) phosphorylation of IKKα/β and IκBα, and (G and H) abundance of p65. *n* = 4 mice per genotype. (I and J) BMMs from *Slc7a5*^{fl/fl} mice were retrovirally infected with Cre recombinase and subsequently stimulated with RANKL in the presence or absence of Akt inhibitor X (I) or the IKK inhibitor BMS-345541 (J), followed by TRAP staining. *n* = 4 mice per genotype. All data were analyzed by the two-way ANOVA with Bonferroni and/or Dunnett post hoc test. ***P* < 0.01, significantly different from the value obtained in control cells. ##*P* < 0.01,

significantly different from the value obtained in *Slc7a5*-deficient cells without inhibitors.

(K) Model for LAT1-mediated control of osteoclast activation. The LAT1-mTORC1 axis (red dashed square) inhibits the activation of Akt, thus preventing GSK3 β from stimulating the translocation of NFATc1 from the nucleus to the cytoplasm. LAT1-mTORC1 signaling also inhibits the activation of IKK and I κ B α in osteoclasts, thus preventing the degradation of I κ B and the nuclear translocation of the p65 and p50 subunits that constitute NF- κ B. These actions repress the nuclear accumulation of NFATc1 and *Nfatc1* expression, respectively, leading to the inhibition of NFATc1-dependent osteoclastic gene expression, thereby impeding osteoclastogenesis.