SIRT7 has a critical role in bone formation by regulating lysine acylation of SP7/Osterix

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SP7/Osterix (OSX) is a master regulatory transcription factor that activates a variety of genes during differentiation of osteoblasts. However, the influence of post-translational modifications on the regulation of its transactivation activity is largely unknown. Here, we report that sirtuins, which are NAD(+)-dependent deacylases, regulate lysine deacylation-mediated transactivation of OSX. Germline Sirt7 knockout mice develop severe osteopenia characterized by decreased bone formation and an increase of osteoclasts. Similarly, osteoblast-specific Sirt7 knockout mice showed attenuated bone formation. Interaction of SIRT7 with OSX leads to the activation of transactivation by OSX without altering its protein expression. Deacylation of lysine (K) 368 in the C-terminal region of OSX by SIRT7 promote its N-terminal transactivation activity. In addition, SIRT7-mediated deacylation of K368 also facilitates depropionylation of OSX by SIRT1, thereby increasing OSX transactivation activity. In conclusion, our findings suggest that SIRT7 has a critical role in bone formation by regulating acylation of OSX.
Bone is a multifunctional tissue with hematopoietic (stem cell niches), metabolic (mineral and energy metabolism), reproductive (male fertility), and brain (development, cognition, and behavior) functions, in addition to its basic role as a framework for the body. Tight interplay between two types of cells, bone-forming osteoblasts and bone-resorbing osteoclasts, regulates bone remodeling, which is the process of removing older bone and replacing it with a new one. An imbalance between these cells (resorption exceeds formation) causes osteoporosis, which is characterized by impairment of bone strength that increases the risk of fracture. Osteoporosis is the most common bone disease and it is estimated that more than 200 million people suffer from it worldwide. Two transcription factors, Runx-related transcription factor 2 (RUNX2) and zinc finger transcription factor SP7/Osterix (OSX), have previously been shown to be essential for the differentiation of osteoblasts. Endochondral and intramembranous bone formation does not occur in Osx knockout (KO) or Osx KO mice. RUNX2 promotes skeletal development on different levels, including differentiation of mesenchymal progenitors into osteoblasts and differentiation/maturation of chondrocytes and osteoclasts. In contrast, OSX acts at a later step in the process of osteoblast differentiation, i.e., the differentiation of pre-osteoblasts into mature osteoblasts and osteocytes.

Sirtuins (SIRT1-7 in mammals) are nicotinamide adenine dinucleotide (NAD+)-dependent lysine deacylases that regulate a wide variety of biological processes. Although sirtuins were thought to only act as lysine deacylases, recent studies have revealed that these enzymes can also remove other acyl-modifications, including propionylation, succinylation, malonylation, myristoylation, and palmitoylation. SIRT1, SIRT6, and SIRT7 are predominantly located in the nucleus, where they regulate the expression of specific genes by deacylation/deacetylation of histones and transcription factors. Previous studies have demonstrated that Sirt1 haplo-insufficient mice and two lines of osteoblast-specific Sirt1 KO mice exhibit a reduction of bone mass that is related to decreased bone formation. Aged mice with specific knockout of Sirt1 in mesenchymal stem cells (MSCs) show reduction of cortical bone thickness and trabecular bone volume. In addition, Sirt6 KO mice have low-turnover osteopenia caused by impaired bone formation and bone resorption.

The enzymatic activity and functions of SIRT7 were poorly understood, but recent studies have revealed some important biological roles. Barber et al. reported that the acetylated K18 of histone H3 (H3 K18Ac) is a target of SIRT7, and that H3 K18Ac-specific deacetylation by SIRT7 is important for maintaining the fundamental properties of the cancer cell phenotype. SIRT7 also deacetylates PAF53 to promote nuclear transcription of ribosomal RNA. Furthermore, SIRT7 acts as a histone desuccinylase with an important role in the DNA damage response and cell survival. On the other hand, Yoshizawa et al. have found that Sirt7 KO mice show resistance to induction of obesity, glucose intolerance, and fatty liver by a high-fat diet. Other authors have reported on various roles of SIRT7 in the liver, heart, and adipocytes. However, no data exist about the influence of SIRT7 on bone metabolism.

Accordingly, we here investigate the role of SIRT7 in bone metabolism by several approaches using Sirt7 KO mice, osteoblast-specific Sirt7 KO mice, and cell-based studies. Our findings reveal that SIRT7 is essential for bone formation by osteoblasts, and suggest that SIRT7 promotes the N-terminal transactivation activity of OSX by deacylation of lysine 368 in the C-terminal of OSX.
SIRT7 positively regulates osteoblast differentiation. In order to determine the intrinsic role of SIRT7 in osteoblasts, we examined the proliferation and differentiation of calvarial osteoblasts harvested from Sirt7 KO mice and WT mice. Striking reduction of Alizarin Red S-stained mineralized nodules was observed in cultures of Sirt7 KO osteoblasts compared with those of WT osteoblasts (Fig. 3a), although cell proliferation was not significantly different (Fig. 3b). Primary cultures of osteoblasts contain heterogeneous cells at various stages of differentiation. Next, we analyzed the effect of SIRT7 on differentiation from pre-osteoblasts to mature osteoblasts by using an osteoblastic cell line (MC3T3-E1). Sirt7 mRNA was expressed in MC3T3-E1 cells, as well as Sirt1 and Sirt6 mRNAs, but Sirt7 expression was decreased in fully differentiated MC3T3-E1 cells (Fig. 3c), suggesting a role of SIRT7 in the early stage of osteoblast differentiation. When Sirt7 knockdown (KD) MC3T3-E1 cells were cultured for 30 days in differentiation medium, osteoblastic mineralization was markedly impaired (Fig. 3d–f), and expression of osteoblastic marker genes was significantly reduced (Fig. 3g). These results demonstrated that SIRT7 positively regulates osteoblast differentiation and thereby controls bone mineralization via a cell-autonomous mechanism.

Our bone histomorphometric analysis indicated that SIRT7 also regulates osteoclastogenesis in vivo, therefore we investigated the role of SIRT7 in osteoclastogenesis. Osteoclasts are derived from granulocyte/macrophage progenitors (GMP) in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL), which are produced by bone marrow stromal cells, osteoblasts, and osteocytes. First, we performed an osteoblast-free osteoclast differentiation assay to evaluate the intrinsic role of SIRT7 in osteoclasts. Bone marrow-derived monocytes/macrophages were obtained from Sirt7 KO and WT mice for culture with M-CSF and RANKL, after which we assessed the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts. However, there were no significant differences between osteoclasts derived from monocytes/macrophages of Sirt7 KO and WT mice with regard to numbers and expression of marker genes, such as cathepsin K (Ctsk) and T cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 protein A3 (Tcirg1) (Supplementary Fig. 2a–c). Similar results were obtained by using Sirt7 KD RAW264.7 cells, a macrophage cell line (Supplementary Fig. 2d–f). These data suggested that osteoclastic SIRT7 is not essential for in vitro differentiation of
monocytes/macrophages into osteoclasts. To investigate whether SIRT7 in osteoblasts controls osteoclast differentiation, we next performed co-culture of calvarial osteoblasts isolated from Sirt7 KO or WT mice with WT splenocytes. Osteoclast differentiation and the Rankl/osteoprotegerin (Opg) gene expression ratio were similar between Sirt7 KO and WT osteoblasts (Supplementary Fig. 2g–j), suggesting that osteoblastic SIRT7 is not required for osteoclast differentiation in vitro.

Osteoblastic SIRT7 is important for bone formation in vivo. We next generated osteoblast-specific conditional Sirt7 KO mice (Sirt7 osbCKO mice) to further investigate the influence of osteoblastic SIRT7 on bone formation in vivo. µCT analysis of the femur showed that bone mass was reduced in female Sirt7 osbCKO mice aged 14–15 weeks (Fig. 4a–f). Trabecular BV/TV and Tb.Th were significantly reduced in Sirt7 osbCKO mice compared with control mice (Fig. 4a, b), while Tb.Pf was increased in Sirt7 osbCKO mice (Fig. 4c). In the cortical bone, Ct. Ar, Ct.Ar/Tt.Ar, and Ct.Th were all significantly reduced in Sirt7 osbCKO mice (Fig. 4d–f). Static and dynamic bone histomorphometric analysis of the lumbar spine (L4) in Sirt7 osbCKO female mice aged 14–15 weeks confirmed that osteoblastic SIRT7 is important for bone formation (Fig. 4g–j). On the other hand, there were no changes of Oc.S/BS and N.Oc/B.Pm (Fig. 4k, l), indicating that osteoblastic SIRT7 does not control osteoclastogenesis in vivo.

Taken together, these findings demonstrated that osteoblastic SIRT7 positively regulates the differentiation and function of osteoblasts to control ossification both in vitro and in vivo.

SIRT7 interacts with OSX and activates transactivation. To unravel the mechanism by which osteoblastic SIRT7 positively regulates the differentiation of osteoblasts, we investigated the effect of SIRT7 on OSX and RUNX2, which are osteoblastic master regulatory transcription factors. To assess DNA binding-independent transcriptional activity of OSX, Sirt7 KO and WT osteoblasts were transfected with an expression construct containing OSX fused with the GAL4 DNA-binding domain (DBD) and a luciferase reporter plasmid driven by GAL4 binding sites. In Sirt7 KO osteoblasts, the transcriptional activity of OSX was reduced by 80% compared with that in WT osteoblasts (Fig. 5a). In contrast to OSX, there was no significant difference of RUNX2 transcriptional activity between Sirt7 KO and WT osteoblasts (Supplementary Fig. 3a). These results were consistent with our in vitro data that expression of Alp, Coll1a1, and Oxs, which are downstream genes of OSX, was reduced in undifferentiated Sirt7 KD MC3T3-E1 cells (Fig. 3g). In addition, attenuation of Sirt7 expression in MC3T3-E1 cells by using siRNA led to reduced transcription of Oxs and Coll1a1 enhancer/promoter-driven luciferase reporters, which are regulated by OSX (Fig. 5b). These findings demonstrated that SIRT7 increases the transcriptional activity of OSX in osteoblasts in a DNA binding-independent manner. It was recently reported that OSX also acts as a transcriptional coactivator in the Dlx–directed process of osteoblast development. Therefore, we assessed the effect of SIRT7 on transcription of the AT-rich motif-driven luciferase reporter, which is regulated by the Dlx5–OSX complex. As shown in Supplementary Fig. 3b, SIRT7 did not seem to have any influence on the function of OSX as a cofactor for Dlx.

Next, we examined the physical interaction between SIRT7 and OSX. When we performed a HaloTag pull-down assay using lysates of OSX-HA-overexpressing HEK293T cells, OSX strongly interacted with the Halo–SIRT7 fusion protein, but not the Halo protein (Supplementary Fig. 3c). Interaction of SIRT7 and OSX in cultured cells was also detected by the co-immunoprecipitation assay (Fig. 5c, d). Immunocytochemistry revealed that SIRT7 co-localized with OSX in MC3T3-E1 cells (Supplementary Fig. 3d). To identify the domain of SIRT7 that interacted with OSX, Halo-SIRT7 deletion mutants were incubated with lysates of HEK293T cells expressing OSX-HA and interactions were investigated by the pull-down assay. As demonstrated in Fig. 5e, the M2 region of SIRT7, which is part of
a conserved NAD-binding and catalytic domain known as the sirtuin core domain, showed strong binding to OSX.

Next, we performed the pull-down assay with acylated or deacylated OSX. Since binding affinity between an enzyme and its substrate generally decreases after the enzymatic reaction has finished, we considered that acylated OSX would show stronger binding to SIRT7 than deacylated OSX. As expected, endogenous OSX derived from MC3T3-E1 cells treated with nicotinamide (NAM), which inhibits the deacylase activity of sirtuins, was bound to Halo-SIRT7 beads, but endogenous OSX derived from MC3T3-E1 cells treated with NAM showed little binding (Supplementary Fig. 3c). These results suggested that an enzyme-substrate relationship existed between SIRT7 and OSX. We also found an interaction between Halo-SIRT7 beads and OSX in HEK293T cells without NAM treatment, presumably due to insufficient deacylation of OSX by its overexpression (Supplementary Fig. 3c). Furthermore, we studied the effect of NAM on transcriptional activity of OSX. When an osteosarcoma cell line (U2OS), a preosteoblastic cell line (MC3T3-E1), and an embryonic mesenchymal stem cell line (C3H10T1/2) were exposed to NAM for 24 h, GAL4DBD-OSX transcriptional activity was dramatically decreased (Supplementary Fig. 3e). In addition, Sirt7 KD in MC3T3-E1 cells decreased OSX transcriptional activity, and overexpression of SIRT7 restored it (Fig. 5f). This effect on the transcriptional activity of OSX was not seen in an inactive SIRT7 mutant (SIRT7<sup>H188T</sup>) (Fig. 5f), indicating that SIRT7 enzymatic activity was required for the promotion of OSX transcriptional activity.

It has been reported that OSX is modified by phosphorylation, acetylation, and ubiquitination<sup>25–30</sup>. These post-translational modifications regulate its protein stability or DNA binding, but no effect of post-translational modifications on the regulation of its transcription activation in osteoblasts was reported. Therefore, we investigated whether SIRT7-dependent augmentation of the transcriptional activity of OSX was due to the regulation of its protein stability. As shown in Fig. 5g, GAL4DBD-OSX protein levels were not decreased in Sirt7 KD cells, suggesting that regulation of OSX transcription activity by SIRT7 is independent of protein stability and DNA binding.

SIRT7 promotes OSX transcription by deacetylation of K368. Mouse OSX has a transcription domain in the N-terminal region (amino acids 27–192) and a zinc finger domain in the C-terminal region (amino acids 293–428)<sup>9</sup>. To map the domain
involved in the interaction between OSX and SIRT7, Halo and Halo-SIRT7 proteins were incubated with lysates of HEK293T cells expressing OSX deletion mutants fused with GAL4DBD, and interactions were assessed by the pull-down assay. The C-terminal region of OSX (including the zinc finger domain) showed strong binding to SIRT7, while the N-terminal transactivation domain bound weakly to SIRT7 (Fig. 6a). In MC3T3-E1 cells, Sirt7 deficiency significantly decreased the transcriptional activity of GAL4DBD-full length OSX (Fig. 6b). It has been reported that the C-terminal region of OSX attenuates its N-terminal transactivation activity through an unknown mechanism. Consistent with these reports, deletion of the C-terminal region of OSX led to a marked increase of its transactivation activity of OSX lacking the C-terminal region in Sirt7 KD cells, indicating that binding of SIRT7 to the C-terminus of OSX is important for regulating N-terminal transactivation activity (Fig. 6b). Thus, C-terminal deacylation by SIRT7 may promote the activity of OSX.KD MC3T3-E1 cells (Fig. 6e).

The C-terminal region of mouse OSX contains ten lysine residues. To investigate the residues targeted by SIRT7, we generated ten different lysine to arginine replacement (KR) mutants and analyzed the transcriptional activity of each OSX mutant. We found that the OSX (K368R) mutant showed elevation of transcriptional activity after expression of the OSX (K368R) mutant rescued impairment of mineralization in cultures of Sirt7 KD MC3T3-E1 cells (Fig. 6f).

Interestingly, K368 of mouse OSX is conserved in humans, birds, frogs, and zebrafish (Supplementary Fig. 4b). Taken together, these findings support the assumption that SIRT7 promotes N-terminal transactivation activity of OSX by deacylation of C-terminal K368 (Fig. 6f).
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Addition of sodium propionate (Na-prop) to the culture markedly enhanced propionylation of OSX (K368) in primary osteoblasts and MC3T3-E1 cells (in vivo), while acetylation of OSX showed no significant change (Fig. 7b, c). Propionylation of OSX was also enhanced in MC3T3-E1 cells with Sirt7 KO (Supplementary Fig. 5d). While propionylation of OSX was barely detectable in mouse embryonic fibroblasts (MEF), addition of sodium propionate (Na-prop) to the culture medium led to prominent OSX propionylation (Supplementary Fig. 5e).

To identify the sites of propionylation, we performed mass spectrometry (MS) using lysates of HA-OSX-expressing 293T cells treated with NAM and Na-prop. This revealed that SIRT7 interacts with OSX and promotes its transcriptional activity. a Transcriptional activity of OSX in Sirt7 KO and WT osteoblasts. Cells were transfected with the GAL4DBD-OSX expression plasmid and the 5× GAL4-luciferase reporter plasmid, and luciferase activity was determined 24 h after transfection. b Transcription of the Osx (left) and Collα1 (right) reporter assay was performed after 24 h. c Co-immunoprecipitation assay detecting the interaction between HA-SIRT7 and OSX in HEK293T cells (c), and that between endogenous SIRT7 and OSX in primary calvarial osteoblasts differentiated for 6 days (d). e Mapping the site of interaction between SIRT7 and OSX. Pull-down assay of Halo-SIRT7-FLAG deletion mutants was performed with lysate of OSX-HA overexpressing HEK293T cells. f Expression of Halo-FLAG and Halo-SIRT7-FLAG proteins (M1–4 deletion mutants; amino acids 1–402 (M1), 210–332 (M2), 211–332 (M3), and 333–402 (M4)) was determined by WB. f Effect of SIRT7 overexpression on transcriptional activity of OSX. MC3T3-E1 cells were transfected with the indicated siRNA, and 72 h later were transfected with the 5× GAL4-luciferase reporter plasmid, and SIRT7 or SIRT7(K368R) expression plasmid. The reporter assay was performed after 24 h. n = 3 each. g GAL4DBD-OSX protein level analyzed by WB under the conditions in h. WB western blotting; IP, immunoprecipitation. Data are shown as the mean ± SEM. Statistical significance was determined by Student’s t-test. *p < 0.05

Lyase depropionylation of OSX by SIRT7 and SIRT1. Because SIRT7 possesses deacetylation activity, we examined whether OSX is acetylated in primary calvarial osteoblasts and MC3T3-E1 cells. First we confirmed that acetylation of histone H3 (treated with NAM) and OSX (cotransfected with p300) could be clearly detected by our western blotting system (Supplementary Fig. 5a, b). Using the same system, we found that acetylation of OSX was barely detectable in primary osteoblasts (Fig. 7a) or in MC3T3-E1 cells stably overexpressing HA-OSX (Supplementary Fig. 5c), and acetylation of OSX was slightly increased by NAM treatment (Fig. 7a, Supplementary Fig. 5c). Because siRNAs can also remove other acyl-lysine modifications, such as propionylation, we next performed western blotting analysis with an antibody targeting propionylated lyase. In contrast of acetylation, propionylation of OSX was clearly detected in primary cultures of osteoblasts and MC3T3-E1 cells and it was strongly enhanced by treatment of cells with NAM (Fig. 7a, Supplementary Fig. 5c). We next assessed whether acetylation/propionylation of OSX was regulated by SIRT7. Propionylation of endogenous OSX was enhanced in primary cultures of osteoblasts derived from Sirt7 KO mice (in vitro) and in the calvariae of Sirt7 KO mice (in vivo), while acetylation of OSX showed no significant change (Fig. 7b, c). Propionylation of OSX was also enhanced in MC3T3-E1 cells with Sirt7 KD (Supplementary Fig. 5d). While propionylation of OSX was not detected in primary osteoblasts and MC3T3-E1 cells, it was strongly enhanced by treatment with NAM (Fig. 7a, Supplementary Fig. 5c).

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Since our investigations indicated that SIRT7 increases the transcriptional activity of OSX and reduces its propionylation, we studied the effect of SIRT7 on OSX expression in primary osteoblasts. We transfected the GAL4DBD-OSX expression plasmid and the 5× GAL4-luciferase reporter plasmid, and luciferase activity was determined 24 h after transfection. The reporter assay was performed after 24 h. n = 3 each. g GAL4DBD-OSX protein level analyzed by WB under the conditions in h. WB western blotting; IP, immunoprecipitation. Data are shown as the mean ± SEM. Statistical significance was determined by Student’s t-test. *p < 0.05.

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postulated that lysine propionylation of OSX might inhibit transactivation. We found that treatment of MC3T3-E1 cells with Na-prop inhibited the expression of marker genes for early osteoblastic differentiation, while there was no change in the expression of non-osteoblast related genes, including beta-2 microglobulin (B2m), actin, beta (Actb), and 18S ribosomal RNA (18S) (Supplementary Fig. 7f). Na-prop treatment also decreased GAL4DBD-OSX transcriptional activity in a concentration-dependent manner (Fig. 7g). To obtain more direct evidence that lysine propionylation of OSX attenuates its transactivation activity, we performed a luciferase assay with transfection of OSX (K368R) expression plasmid or empty plasmid. Then cells were cultured for 30 days in differentiation medium. Representative Alizarin Red S-stained images (left) and Alizarin Red S staining area ratio (right). Scale bar, 400 μm

Recent studies have shown that SIRT1 possesses depropionylation activity, while SIRT6 (another nuclear sirtuin) does not. Therefore, we examined whether SIRT1 caused depropionylation of OSX and whether SIRT7-facilitated such depropionylation. We found that SIRT1 interacted with full-length OSX and also with its N-terminal activation domain (Supplementary Fig. 7a, Fig. 8a). SIRT1 reduced lysine propionylation in OSX, and the combination of SIRT1 and SIRT7 nearly abolished it (Fig. 8b). These results suggested that SIRT1 could be involved in the depropionylation of OSX. Consistent with these findings, SIRT1 and SIRT7 synergistically promoted the transcriptional activity of OSX (Fig. 8c). Moreover, SIRT1 more strongly enhanced the transcriptional activity of OSX (K368R) than that of WT OSX, further supporting the cooperative effect of SIRT7 and SIRT1 (Fig. 8d). In conclusion, the present findings suggested that SIRT7-mediated deacetylation of OSX K368 facilitates depropionylation by SIRT1, and thereby
increases the transactivation activity of OSX (Supplementary Fig. 7b).

Discussion

This study provided evidence that Sirt7 KO mice developed severe osteopenia due to decreased bone formation along with an increase of osteoclasts (Figs. 1 and 2). Deficiency of Sirt7 in osteoblasts significantly decreased osteoblastic differentiation and ossification in vitro (Fig. 3), and osteoblast-specific Sirt7 KO mice exhibited the reduction of bone mass and decreased bone formation without a change of osteoclast numbers (Fig. 4). We also identified the underlying molecular mechanism by demonstrating that Sirt7 increased the transactivation activity of OSX through deacylation of lysine 368, as evidenced by MS labeling (Supplementary Fig. 7c, d). Further analysis of lysine residues in OSX demonstrated that Sirt7-mediated deacylation of lysine residues is required for full activation of OSX, allowing these enzymes to regulate osteoblast differentiation and bone formation. To our knowledge, this is the first report concerning a role of SIRT7 in bone turnover and metabolism.

Although we demonstrated that SIRT7 increases the transactivation activity of OSX through deacylation of lysine 368, we could not identify the actual modification of this lysine by MS analysis. When we investigated whether recombinant SIRT7 was able to depropionylate lysine 368 using OSX K368 propionyl peptide, we could not detect depropionylation activity even in the presence of nucleic acid, an activator of SIRT7. While we clearly observed deacylation of H3 K18 acetyl lysine by SIRT7, we could not identify the actual modification of this lysine by MS analysis. Further investigation will be necessary to fully understand the role of SIRT7 in osteoblast differentiation and bone formation.
clarify the actual modification of lysine 368 for a better understanding of the molecular mechanism by which SIRT7 regulates the transactivation activity of OSX.

In the present study, we demonstrated synergistic depropionylation of OSX by SIRT1 and SIRT7 (Fig. 8b), resulting in activation of OSX transactivation (Fig. 8c). SIRT1 has already been reported to modulate bone formation by osteoblasts. In MSC-specific Sirt1 KO mice, it was reported that SIRT1 regulates osteoblastic differentiation of MSCs by deacetylation of β-catenin12. Depletion of SIRT1 in osteoblast progenitors employing Osx-Cre mice led to a decrease in cortical bone thickness associated with decreased bone formation, resulting from increased sequestration of β-catenin by acetylated-FoxOs10. In addition to these mechanisms, our present findings suggest that SIRT1 positively regulates osteoblast differentiation by modulating the depropionylation of OSX.

Our findings suggested that reduced bone formation in Sirt7 KO mice was mainly dependent on OSX. However, some of our data were not in line with published findings about OSX-deficient mice. First, we found that Sirt7 deficiency was associated with a decrease of Runx2 expression in MC3T3-E1 cells (Fig. 3g) and in the femur (Fig. 2c, Supplementary Fig. 1m). It was previously reported that Runx2 expression is normal in Oxs KO mice, indicating that Runx2 is upstream of OSX13. Baek et al. reported an increase of Runx2 expression in the long bones of Osx<sup>lox<sup>-<sup>-<sup>Col1a1-Cre</sup> mice compared with Osx<sup>lox<sup>-<sup>+/+</sup>Col1a1-Cre</sup> mice, although the mechanism involved was unclear35. Sirtuins have multiple substrates and regulate several intracellular signaling pathways, so we cannot exclude the possibility that SIRT7 also regulates osteoblast differentiation by acting on factors other than OSX, e.g., SIRT7 may elevate osteoblast differentiation by acting on factors other than OSX10. In the future, chromatin immunoprecipitation sequencing (ChIP-seq) will provide further information about the role of SIRT7 in the regulation of osteoblast functions. Second, we found that Sirt7 deficiency did not affect the proliferation of primary calvarial osteoblasts (Fig. 3b). Previous studies have shown that calvarial osteoblasts from Oxs KO mice grow faster than WT cells, and calvarial BrdU incorporation was found to be greater in Oxs-null embryos than in WT embryos36. It is generally considered that the canonical OSX pathway involves binding to GC-box DNA elements to regulate the transcription of target genes. Recently, Hojo et al. reported that OSX acts as a transcriptional coactivator in Dlx-containing regulatory complexes bound to AT-rich motifs24. OSX can also form complexes with other transcriptional factors37. Here

**Fig. 8** SIRT7 and SIRT1 regulate OSX transactivation activity through lysine deacetylation. **a** Co-immunoprecipitation assay to assess the interaction between HA-SIRT1 and GAL4DBD-OSX (27–270) in HEK293T cells. **b** Effect of SIRT7 and SIRT1 overexpression on propionylation of OSX. Sirt7 KO MEF were transfected with the 3× HA-OSX expression plasmid, as well as the SIRT7 or SIRT1 expression plasmid, followed by treatment with 50 mM Na-Prop for 16 h. Then propionylation of OSX was assessed by immunoprecipitation and WB. **c** Effect of SIRT7 and SIRT1 overexpression on transactivation activity of OSX. Sirt7 KO MEF were transfected with the 3× HA-OSX expression plasmid, as well as the SIRT7 or SIRT1 expression plasmid, followed by treatment with 10 mM Na-Prop for 24 h. Then the cells were transfected with the Col1a1 enhancer/promoter-driven luciferase reporter plasmid, and the reporter assay was performed after 24 h (n = 6 each). **d** Effect of SIRT1 on transactivation activity of the OSX (K368R) mutant. Sirt7 KO MEF were transfected with the 3× HA-OSX or 3× HA-OSX (K368R) expression plasmid, as well as the SIRT1 expression plasmid, followed by treatment with 30 mM Na-Prop for 24 h. Then the cells were transfected with the Col1a1 enhancer/promoter-driven luciferase reporter plasmid, and the reporter assay was performed after 24 h (n = 6 each). WB, western blotting; IP, immunoprecipitation. Data are shown as the mean ± SEM. Statistical significance was determined by Student’s t-test. *p < 0.05 vs. without SIRT1 and SIRT7 (c)
we demonstrated that SIRT7 regulated OSX transcriptional activity mediated via GC-box DNA elements, but did not seem to affect its Dlx coactivator function. Taken together, it can be suggested that SIRT7 partially activates transactivation by OSX, so that OSX functionality is not completely abolished in Sirt7 KO osteoblasts. Zhang et al. have suggested that disruption of DNA binding by Tcf1, a partner of β-catenin, due to interaction with OSX is at least partly responsible for OSX-mediated inhibition of osteoblast proliferation. Accordingly, the SIRT7-dependent transactivation activity of OSX may not have an important role in osteoblast proliferation.

We found that osteoclast numbers in the lumbar spine were increased in Sirt7 KO mice compared with WT controls (Supplementary Fig. 1A–p), but SIRT7 was not essential for osteoclastogenesis in our cell culture system (Supplementary Fig. 2). What is the reason for this discrepancy? Osteoclasts are tissue-specific multicellular macrophages that differentiate from GMP, which arise from common myeloid progenitors (CMP). The cells used in our osteoclastogenesis assay were monocytes/macrophages, raising the possibility that SIRT7 is not essential for differentiation of macrophages to osteoclasts, but is required for differentiation of hematopoietic stem cells to CMP or differentiation of CMP to GMP. Indeed, Mohrin et al. reported that SIRT7 is involved in the maintenance of hematopoietic stem cells and that myeloid-biased GMP. Indeed, Mohrin et al. reported that SIRT7 is involved in the maintenance of hematopoietic stem cells and that myeloid-biased differentiation was apparent in Sirt7 KO mice. Alternatively, SIRT7 may regulate modulators of osteoclastogenesis derived from sources other than osteoblasts or osteoclasts.

Acetylation of lysine is a well-known post-translational modification involved in a wide variety of cellular processes. In contrast, propionylation of lysine is a modification that was only recently identified in mammalian cells, and has been shown to occur in histones, p53, p300, and CREB-binding protein (CBP). Although propionylation has been characterized in multiple proteins and organisms, the biological effects of lysine propionylation are poorly understood, as are the differences between propionylation and acetylation. In this study, we demonstrated that OSX undergoes lysine propionylation, leading to the reduction of its transactivation activity (Fig. 7). We could not identify differences in transactivation activity between propionylation and acetylation of OSX, because we were unable to prepare acetylated (but not propionylated) recombinant OSX for in vitro experiments. Propionylation of endogenous OSX, but not its acetylation, was increased in primary osteoblasts and calvariae obtained from Sirt7 KO mice (Fig. 7b, c). Further investigations will be needed to define the molecular mechanisms involved. It is possible that SIRT7-facilitated decacylation of lysine residues in OSX affects a limited number of several acetylated lysine residues, so that small changes due to SIRT7 are masked in whole.

Propionic acidemia (PA) is a rare autosomal recessive disorder characterized by the accumulation of propionic acid due to deficiency of propionyl-CoA carboxylase. Adults with PA have an increased risk of osteoporosis or osteopenia, but the reason is unknown. Elevated global propionylation of lysine has been observed in fibroblasts from PA patients. Based on our results, propionylation of OSX may be elevated in PA osteoblasts, and may at least partly contribute to the pathophysiology of osteoporosis in this disease.

In conclusion, we provided evidence that acylation of lysine 368 in OSX is a post-translational modification involved in regulating transactivation. The importance of the role of SIRT7 as an ERASER of lysine acylation in regulating osteoblast differentiation was also clarified. Our findings will hopefully provide a stepping-stone for future studies on the role of lysine non-acetyl acylation in bone metabolism. Further studies on acyltransferases as WRITERS, as well as ERASERS, and the influence on acyl-CoA metabolism will broaden our understanding of the biological significance of lysine acylation. Finally, SIRT7 could be a potential target for the treatment of osteoporosis.

**Methods**

**Mice.** All experimental procedures were approved by the Kumamoto University Ethics Review Committee for Animal Experimentation (Approval ID: A27-024, 20171028). All mice were maintained on a 12-h light:dark cycle and had access to regular chow and water ad libitum, unless otherwise specified. These experiments were conducted according to the guidelines of the Institutional Animal Committee of Kumamoto University. Generation of Sirt7 KO mice was accomplished as reported previously. Sirt7 KO mice were backcrossed for five generations with C57/Bl6J mice (CLEA Japan Inc.). Only the heterozygotes (Sirt7+/−; Mouse Flp/Frt) were bred and littermates (WT and Sirt7 KO mice) were used for these studies. There was no apparent increase of embryonic lethality, postnatal death, or growth retardation in Sirt7 KO mice, as shown in two previous independent studies. Sirt7−/−;CKO mice were generated by intercrossing the progeny of crosses between Sirt7−/−;CAG-FLP (a gift from Dr. Y. Sonoda) and Sirt7−/−;CAG-cre (a gift from Dr. K. Davies). These mice were bred onto a C57/Bl6J background and genotypically analyzed by PCR.

**Cell culture.** A murine preosteoblast cell line (MC3T3-E1; RIKEN BRC, Japan) was cultured in α-MEM with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml β-mercaptoethanol. The cells were subcultured with a 1:3 split every 3 days. The pGFp-LX construct was a gift from Dr. B. Plesnila (Baylor College of Medicine, TX). These cells were cultured for 4 days in differentiation medium supplemented with 10 nM β-glycerophosphate, 50 μg/ml ascorbic acid, and 10 μM recombinant human BMP-2 (GIBCO, NY).
gift from Osteopharma Inc., Osaka, Japan), Primary MEF were isolated as follows. Dissected mouse embryos of WT and Sirt7 KO mice (E13.5) were minced and incubated for 1 h at 37 °C in a 5% CO2 incubator using 10× MEF medium (DMEM with 25 mM glucose, 0.1 mM pyruvate, 10% (v/v) FBS, and 0.1% (v/v) penicillin/streptomycin). To study mineralization, confluent cells were cultured for 3 days in a α-MEM with 5% FBS, followed by incubation in differentiation medium supplemented with 10 ng/ml β-glycerophosphate and 50 μM ascorbic acid. The medium was changed every 3 days. After culture for 10 days (for primary osteoblasts) or 30 days (for MC3T3-E1 cells), the cells were subjected to Alizarin red S staining. The osteoblast proliferation assay was performed using WST-1 (Roche) according to the manufacturer’s instructions, while the osteoblast-free osteocalcin differentiation assay was done as described previously. Briefly, bone marrow cells (3 × 10^5 cells per cm²) harvested from the femurs of mice aged 6–8 weeks were cultured for 2 days in α-MEM with 10% FBS and 10 ng/ml human M-CSF (R&D Systems), and then differentiation into osteoblasts was achieved by incubation with 50 ng/ml human RANKL (Peprotech) and M-CSF for 4 days. Subsequently, osteoblast differentiation was evaluated by TRAP staining. For differentiation, RAW264.7 cells (5 × 10^5 cells per cm²) were cultured in DMEM with 10% FBS and 50 ng/ml RANKL for 5 days. The medium was changed daily. The co-culture osteo-crest-forming assay was performed as previously described. Briefly, primary osteoblasts (5 × 10^5 cells per cm²) were cultured alone for 1 day and then were co-cultured for 15 days with sponges (1 × 10^5 cells per cm²) obtained by mincing sponges in α-MEM with 10% FBS and 1 × 10⁻⁸ M 1,25-dihydroxyvitamin D₃.

μCT and bone histomorphometric analyses. Mice were injected with Calcein (Sigma) twice (days 1 and 4) and then were sacrificed on day 6. The femur was harvested, fixed, and scanned using a SkyScan 1076 μCT scanner (Bruker Corporation, Massachusetts, USA) following the guidelines of the American College of Veterinary Research. Structural bone parameters were determined with CTAn analysis software (SkyScan). For bone histomorphometric analysis, undecalcified vertebralia were embedded in methylmethacrylate. Serial sections were stained using von Kossa, Toluidine blue, and TRAP, after which static and dynamic bone parameters were measured using the OsteoMeasure Analysis System (Osteometrics) as described previously.

Gene expression analysis. After total RNA was extracted by using Sepasol RNA I super reagent (Nacalai Tesque, Japan), qRT-PCR was performed with SYBR Premix Ex Taq II (RR820A, TaKaRa) and an ABI 7300 thermal cyclers (Applied Biosystems, CA). For extraction of total RNA from femur, soft tissues and epiphyses were removed, and diaphyses were flushed with PBS to remove bone marrow. The relative expression of each gene was normalized to that of OATaA box binding protein (Tbp). Primer sequences are listed in Supplementary Table 1.

RNA interference experiments. For transient knockdown of Sirt7, transfection of Sirt7 shRNA (FlexiTube siRNA Mm_Sirt7_5; Qiagen) was performed with HiPerfect transfection reagent (Polyplus, NY). Indicated time after transfection, cells were harvested for 3 days in α-MEM with 5% FBS and 10 ng/ml recombinant propionylated or underpropionylated OSX protein was purified from OXs-transfected Sirt7 KO MEF treated or untreated with 50 mM Na-propionate, respectively. Protein (2 μg well⁻¹ (24-well plate)) was transfected with Xpect Protein Transfection Reagent (Clontech Laboratories, Inc.) according to the manufacturer’s protocol.

Luciferase assays. Cells were transfected with various plasmid DNAs using jetPRIME transfection reagent (Polyplus). Indicated time after transfection, cells were lysed and assayed using Firefly luciferase and Renilla luciferase substrates in the Dual-Luciferase Reporter Assay (Promega). Firefly activity was normalized to Renilla luciferase activity in the same cell extract to correct for variation in transfection efficiency. The total amount of transfected DNA was kept constant by the addition of empty vectors. The pRL-TK Renilla luciferase plasmid was used as an internal control, except pBRL-CD. The pBRL plasmid not expressing RNA polymerase II and pCD were used as a negative control under the control of the SV40 promoter, which allows the user to normalize for differences in transfection efficiency. For transfection of recombinant protein, recombinant propionylated or underpropionylated OSX protein was purified from OXs-transfected Sirt7 KO MEF treated or untreated with 50 mM Na-propionate, respectively. Protein (2 μg well⁻¹ (24-well plate)) was transfected with Xpect Protein Transfection Reagent (Clontech Laboratories, Inc.) according to the manufacturer’s protocol.

HaloTag pull-down assay. Either pFN18A-Halo-FLAG, pFN18A-Halo-SIRT7-FLAG, or pFN18A-Halo-SIRT7 (M1/M2/M3/M4)-FLAG was transfected into E. coli K12 (Single Step (KRX) Competent Cells, Promega). E. coli was pre-cultured overnight in 5 ml of LB medium with ampicillin. Then the pre-culture was diluted into 1:1000 using the same LB medium and culture was continued at 37 °C until OD600 reached 0.5. After further culture at 20 °C to 1000 mL reached 0.7, 20% rhomnase was added to a 1:2000 dilution and incubation was done overnight. Following centrifugation at 1400g, the pellet was resuspended in Halo purification buffer (50 mM Hepes-KOH (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM PMSF, and protease inhibitor cocktail (Nacalai Tesque)). Following centrifugation by three freeze–thaw cycles, followed by sonication twice for 20 s each at level 2 (Sonifier-150, Branson). After centrifugation at 6000g for 10 min, the cleared lysate was incubated with HaloLink Resin (Promega) overnight at 4 °C. After binding, the resin was washed five times with the same Halo purification buffer. HA antibodies (30 μg) affinity purified on Halo Link Resin was incubated with 500 μg of cell lysate in pull-down buffer (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 10 mM NaF, 10 mM Na3PO4, 1 mM PMSF, and protease inhibitor cocktail (Nacalai Tesque)). After incubation overnight at 4 °C for binding, the resin were washed five times with the same pull-down buffer and the bound proteins were separated by SDS-PAGE.

Proteomic identification of proteins. IP samples were used in solution and in gel for higher accuracy and precision MS analyses. To prepare the sample in solution for MS, 100 μL of 0.1 M glycine HCl (pH 2.0) was added to the washed resin beads, and the mixture was incubated at room temperature for 5 min with gentle shaking. The supernatant was obtained and neutralized with 10 μL of 10× TBS (0.5 M Tris–HCl, 1.5 mM NaCl, pH 7.4). These samples were frozen at −80 °C until use. After cysteine reduction and alkylation by DTT and iodoacetamide, respectively. The samples were digested with Trypsin/Lys-C Mix (Promega) in the solution containing 50 mM Tris–HCl (pH 8.5) at 37 °C overnight. To stop the reaction, trifluoroacetic acid (TFA) was added to the sample at final concentration of 1%. The samples were desalted using ZipTip C18 pipette tips (Millipore) or stir-endorivinylbenzene (SDB)-StageTip™, and dissolved with 0.1% TFA in 2% acetonitrile (ACN) for LC-MS analyses. To identify proteins detected in SDS-PAGE, in-gel digestion of proteins was performed according to the previous report. The samples were separated onto SDS-PAGE gels. The gels were fixed with 30% methanol and 7.5% acetic acid and then stained with LavaPurple Total Protein Stain (FLUOROChemics) according to the manufacturer’s instructions. After scanning the gel image with a Amersham Typhoon scanner RGB system (GE Healthcare), protein bands of interests were selected with Progenesis software (Nonlinear Dynamics) and marked by an Etchant spot picker (GE Healthcare). The gel pieces manually cut out were washed three times with 50 mM ammonium acetate in 100% (v/v) ACN, and vacuum-dried. For cysteine reduction, 100 μL of 10 mM of DTT in 100 mM ammonium bicarbonate was added to the gel pieces which were incubated at 56 °C for 1 h, and was removed. Following cysteine alkylation, 100 μL of iodoacetamide in 100 mM ammonium bicarbonate was added, and the gel pieces were incubated at 24 °C for 45 min, and was removed. The gel pieces were washed once with 100 mM ammonium bicarbonate, dehydrated in 100% (v/v) ACN, and vacuum-dried. The sequencing grade modified trypsin (Promega) was added to the gel pieces at a concentration of 50 ng mg⁻¹ in 10% (v/v) ACN including 50 mM ammonium bicarbonate, and the mixture was incubated on ice for 30 min followed by the trypsinization peptides were sequentially extracted from the gels with 0.1% (v/v) TFA in 30% (v/v) ACN, 0.1% (v/v) TFA in 50% (v/v) ACN, and 0.1% (v/v) TFA in 80% (v/v) ACN, for 5 min each. The extracted
peptides were vacuum-dried and dissolved in 20 μL of 0.1% (v/v) TFA in 2% (v/v) ACN. These samples were desalted with a strendiyvinylbenzene (SDS)-StageTip, dissolved with 0.1% TFA in 2% ACN, and subjected to LC-MS analysis. The peptide samples were subjected to a nano-flow reversed-phase MS/MS system (EASY-nLC 1200 System) coupled to an Orbitrap Fusion Tribrid Mass Spectrometer; Thermo Fisher Scientific, San Jose, CA) with a nanospray ion source in positive mode. Samples were separated with a nano-HPLC C18 capillary column (0.075 x 150 mm, 3 mm) (Nikkol Technos, Tokyo, Japan). A 60-nm gradient was used at a flow rate of 300 nL/min. The spray voltage was 2.2 kV with ion transfer tube temperature 250 °C. MS and MS/MS scan properties were as follows; Orbitrap MS resolution 120,000, scan range 350–1500, isolation window m/z 1.6, and MS/MS detection type was ion trap with a rapid scan rate. The peptide and fragment mass tolerances were 10 ppm and 0.6 Da, respectively.

The m/z values of propionyl-peptides identified by data-dependent MS analysis were listed into inclusion list for targeted mass mode analysis (m/z = 529.8029 VYGKRK10Prop, 560.7973 DSTLTK12PropGGTK, 572.9647 287Prop, 512.7151 K369PropPHSCGCK, 520.7145 K369PropPHSCGCK, 620.8126 FTCCa1L1c529.8029Prop, 703.6864 THGEPGP705.6864PropGLEGR, 744.9087 GGT396PropKYDALSPAK). MS and MS/MS scan properties for targeted mass analysis were performed as follows; Orbitrap MS resolution 120,000, MAS scan range 550–1500, isolation window m/z 6.1, and MS/MS detection type was Orbitrap with resolution 15,000. The peptide and fragment mass tolerances were 10 ppm and 0.02 Da, respectively. K propionylation (KProp), K acetylation (KAc), C carboxymethylation (CCam).

All MS/MS spectral data were searched against entries for mice in the Swiss-Prot database (v2016-10-05) using the SEQUEST database search program using Proteome Discoverer 2.2. For variable peptide modifications, propionylation and oxidation of methionine in addition to carboxymethylation of cysteine for a fixed modification, were taken into account. Database search results were filtered by setting the peptide confidence value as high (FDR < 1%) for data dependent mass analysis data, and high correlation (Xcorr > 1.9) for targeted mass analysis data, respectively.

**Co-immunoprecipitation assays.** HEK293T cells were transfected with the indicated expression plasmids for 24 h using JetPRIME transfection reagent (Polyplus, NY). Primary cultures of calvarial osteoblasts were differentiated for 6 days. Cells were lysed in IP buffer (20 mM Tris–HCl (pH 7.4), 200 mM NaCl, 2.5 mM MgCl2, 0.05% NP-40, 1 mM PMSF, and protease inhibitor cocktail (Nacalai Tesque)) and then were incubated on ice for 30 min. Next, the lysed cells were passed through a 29G needle (Terumo) and centrifuged at 14,000 × g; the cleared lysates were subjected to immunoprecipitation overnight at 4 °C with anti-HA antibody beads (clone 4B2, Wako Pure Chemical Industries, Ltd.) or anti-OSX-conjugated magnetic beads, which were prepared using 10 μg of OSX antibody (ab22552, Abcam) and 1 mg of beads according to the protocol of the Dynabeads Antibody Coupling Kit (Invitrogen). Then the beads were washed five times with IP buffer, after which proteins were eluted using 2× SDS sample buffer (100 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, and 0.2% bromophenol blue) and were examined by western blotting with the indicated antibodies.

**Immunochemistry.** Both the 3x HA-OSX and FLAG-SIRT7 expression plasmids were transfected into MCT3-E1 cells with JetPRIME transfection reagent (Polyplus). After 24 h, cells were fixed in 10% neutralized formalin and permeabilized with 0.1% Triton X-100/3% BSA/PBS. Monoclonal rat anti-HA antibody (clone 4B2, Wako) or anti-OSX-conjugated magnetic beads, which were prepared using 10 μg of OSX antibody (ab22552, Abcam) and 1 mg of beads according to the protocol of the Dynabeads Antibody Coupling Kit (Invitrogen). Then the beads were washed five times with IP buffer, after which proteins were eluted using 2× SDS sample buffer (100 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, and 0.2% bromophenol blue) and were examined by western blotting with the indicated antibodies.

**Preparation of recombinant SIRT7.** pT7N18A-Halo-SIRT7 plasmid was transfomed into E. coli K12 (RRX) cell. A single colony was inoculated with LB medium and cultured in LB medium and further cultured and expressed in LB medium. The Halo-SIRT7 protein was purified from the resulting E. coli lysate using HaloLink resin (Promega, G9114). ProTeV Plus protease (Promega, V6104) was used for the separation of the SIRT7 protein from Halo-tag.

**In vitro deacetylation and depropionylation assay.** H3 K18Ac (KSTGKAPR-K18Ac) (RFRTRDHSKQRTHGEPGP), OSX K369Prop (RFRTRDHSKQRTHGEPGP) and OSX K369Prop (RFRTRDHSKQRTHGEPGP) were used for the in vitro deacetylation/depropionylation assay. 8 μM of peptides were incubated in 60 μL reaction volume, with or without 3 μg of recombinant SIRT7 with 1 μg nucleic acid containing 1.0 mM NAD in 20 mM Tris–HCl buffer (pH 7.5) and 1 mM DTT for 2 h at 37 °C. The reactions were stopped with 0.1% formic acid and analyzed by LC-MS/MS.

Peptides were analyzed by using Agilent 6460 Triple Quadrupole LC/MS (Agilent Technologies, Santa Clara, USA) to perform LC-ESI-MS/MS. LC conditions employed were as follows; column; Agilent Zorbelex Eclipse Plus C18 (2.1 × 50 mm) (Agilent Technologies); column temperature, 45 °C; injection volume, 3 μL; mobile phase, A, 0.1% formic acid, B, acetonitrile; gradient (B concentration), 0 min—3%, 10 min—8%, 10.1 min—3%, 15 min—3%; flow rate, 0.2 μL/min. Multiple reaction monitoring parameters were determined as follows; precursor ion (m/z), 695.8, product ion (m/z), 84.1, fragmentor voltage (V), 210, collision energy (V), 55, polarity, positive for H3 K18Ac, precursor ion (m/z), 709.8, product ion (m/z), 84.1, fragmentor voltage (V), 170, collision energy (V), 55, polarity, positive for acetylated H3 K18 (H3 K18Ac), precursor ion (m/z), 469.7, product ion (m/z), 70.2, fragmentor voltage (V), 90, collision energy (V), 55, polarity, positive for propylated OSX K368 (OSX K368Prop).

**Statistical analysis.** No statistical methods were used to determine sample size, but the sample sizes were similar to those of previous reports. No exclusion/ inclusion criteria were applied to the mice used in this study. Group allocation and outcome assessment were performed in a blinded manner. In vitro experiments were repeated at least three times. All results are expressed as the mean ± standard error of the mean. Statistical significance was determined by using the two-tailed Student’s t-test and p < 0.05 was considered to indicate a significant difference.

**Data availability.** The authors declare that all the data supporting the findings of this study are available within the article and its Supplementary Information Files. All MS raw data were stored in JPOST repo1 [https://repository.jpostdb.org/]. The project ID containing these data was JPOST00398/PXD009147.

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