

Monocyte-Specific Knockout of *C/ebpα* Results in Osteopetrosis Phenotype, Blocks Bone Loss in Ovariectomized Mice, and Reveals an Important Function of *C/ebpα* in Osteoclast Differentiation and Function

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ABSTRACT

CCAAT/enhancer-binding protein α (*C/ebpα*) is critical for osteoclastogenesis by regulating osteoclast (OC) lineage commitment and is also important for OC differentiation and function in vitro. However, the role of *C/ebpα* in postnatal skeletal development has not been reported owing to lethality in *C/ebpα*^{-/-} mice from hypoglycemia within 8 hours after birth. Herein, we generated conditional knockout mice by deleting the *C/ebpα* gene in monocyte via *LysM-Cre* to examine its role in OC differentiation and function. *C/ebpα*^{f/f}*LysM-Cre* mice exhibited postnatal osteopetrosis due to impaired osteoclastogenesis, OC lineage priming defects, as well as defective OC differentiation and activity. Furthermore, our ex vivo analysis demonstrated that *C/ebpα* conditional deletion significantly reduced OC differentiation, maturation, and activity while mildly repressing macrophage development. At the molecular level, *C/ebpα* deficiency significantly suppresses the expressions of OC genes associated with early stages of osteoclastogenesis as well as genes associated with OC differentiation and activity. We also identified numerous *C/ebpα* critical cis-regulatory elements on the Cathepsin K promoter that allow *C/ebpα* to significantly upregulate Cathepsin K expression during OC differentiation and activity. In pathologically induced mouse model of osteoporosis, *C/ebpα* deficiency can protect mice against ovariectomy-induced bone loss, uncovering a central role for *C/ebpα* in osteolytic diseases. Collectively, our findings have further established *C/ebpα* as a promising therapeutic target for bone loss by concurrently targeting OC lineage priming, differentiation, and activity. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: GENETIC ANIMAL MODELS; TRANSCRIPTIONAL FACTORS; OSTEOCLAST; OSTEOPETROSIS; OSTEOPOROSIS

Introduction

Osteoclasts (OCs) are multinucleated giant cells that are critical for bone development and homeostasis. OCs originate from monocytic precursors of the hematopoietic cell lineage upon stimulation with the macrophage-colony stimulation factor (M-CSF) and receptor activator of nuclear factor- κ B (RANK) ligand (RANKL).⁽¹⁾ M-CSF maintains the proliferation and survival of OC precursors, and RANKL is required for OC differentiation, survival, and activity. During the early stages of osteoclastogenesis, RANKL strongly upregulates several key transcription factors, such as Finkel-Biskis-Jinkins osteosarcoma oncogene (*c-Fos*), proviral integration 1 (*Pu.1*), and nuclear factor of activated T-cells, C1 to induce OC lineage commitment which precedes cell differentiation.^(1,2) *Nfatc1* is a master regulator of OC differentiation through

its indispensable role in inducing various marker genes, such as tartrate resistant acid phosphatase 5 (TRAP), matrix metalloproteinase 9 (*Mmp 9*), cathepsin K (*Ctsk*), and V-proton pump, H⁺ transporting (vacuolar proton pump) member 1 (*Atp6i*), which are critical for terminal OC differentiation and activity.⁽¹⁾

Excessive bone resorption from enhanced OC formation and activity are mainly responsible for the bone loss associated with many bone diseases, including osteoporosis, rheumatoid arthritis, and periodontal diseases.⁽³⁾ Normal osteoclastic bone resorption involves the removal of the mineral components of bone through extracellular acidification orchestrated by many genes including *Atp6i* and *Mmp9*, which is followed by degradation of the organic constituent of bone by proteases such as *Ctsk*. We previously identified *Atp6i* as a putative OC-specific proton pump subunit and showed that *Atp6i*

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Additional Supporting Information may be found in the online version of this article. For more detailed descriptions of methods, please refer to Supporting Materials and Methods.

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deletion results in a severe osteopetrosis in mice due to impaired extracellular acidification.^(4,5) Similarly, *Ctsk* is induced by RANKL during OC differentiation and activity and is highly expressed in OC.^(6,7) Also, mice deficient in the *Ctsk* gene are osteopetrotic from defective OC activity.

CCAAT/enhancer binding protein α (*C/ebp α*) is a transcription factor of the C/EBP family of transcription factors that carry out critical functions in hematopoiesis and granulopoiesis through their ability to bind on gene promoters to regulate gene expression.⁽⁸⁾ *C/ebp α* can dimerize via its leucine zipper domain at the C terminal to bind DNA and promote gene expression through its N-terminal trans-activation domain.^(9,10) Global deletion of the *C/ebp α* gene in mice leads to death within 8 hours of birth from hypoglycemia stemming from hepatic glycogenesis and impaired myeloid cell differentiation.⁽¹⁰⁾ Notably, we have recently shown that *C/ebp α* is not only critical for OC lineage commitment, but is also important for OC differentiation and activity. Consistently, newborn *C/ebp α ^{-/-}* mice displayed osteopetrosis from impaired OC development. However, the roles of *C/ebp α* in postnatal skeletal OC formation and activity have not been demonstrated because of the early lethality of *C/ebp α ^{-/-}* mice.

Given that OC develops from monocyte precursors, and *C/ebp α* is critical for lineage fate decision during early hematopoietic development,⁽¹¹⁻¹⁷⁾ we generated a *C/ebp α* monocyte-specific conditional knockout model using the *LysM-Cre* cell line to examine its role in OC differentiation during postnatal skeletal development. We showed that the monocyte-specific conditional ablation of *C/ebp α* in mice resulted in osteopetrosis from impaired OC lineage priming, differentiation, and function. Furthermore, we showed that *C/ebp α* functions by regulating the earlier known OC transcription factors, establishing it as the earliest regulator of osteoclastogenesis. Also, we showed that *C/ebp α* can protect mice against ovariectomy-induced bone loss in a mouse model of osteoporosis. Collectively, these results have established *C/ebp α* as a key transcriptional regulator of osteoclastogenesis as well as OC activity during postnatal development.

Materials and Methods

For more detailed descriptions of methods, please refer to Supporting Materials and Methods.

Generation of *C/ebp α ^{ff}*/*LysM-Cre* mice

All animal studies were carried according to the legal requirements of the Association for Assessment and Accreditation of the Laboratory Animal Care International and the University of Alabama at Birmingham Institutional Animal Care and U4-8se Committee as well as the recommendations of the Animal Research: Reporting in Vivo Experiments guidelines. *C/ebp α ^{ff}* mice (The Jackson Laboratory, Bar Harbor, ME, USA; JAX no. 006447)^(17,18) were crossed with *LysM-Cre* transgenic mice (The Jackson Laboratory; JAX no. 004781),⁽¹⁹⁾ and their progeny (*C/ebp α ^{fllox/+}/*LysM-Cre*) were bred to obtain *C/ebp α ^{ff}/*LysM-Cre* mice. Mice were bred in-house and euthanized by CO₂ asphyxiation. Genotyping by PCR was carried out as described.⁽²⁰⁾ To detect floxed and excised bands, the products were amplified under the following conditions: 4 min at 94°C for initial denaturation followed by 35 cycles of 45 s at 94°C, 30 s at 60°C, and 1.5 min at 72°C, with a final round of 10 min at 72°C, in an ABI 2720 thermocycler (Applied Biosystems, Foster City, CA, USA). To detect Cre, the products were amplified under the following conditions: 1.5 min at 95°C for initial denaturation followed by**

35 cycles of 30 s at 94°C, 1 min at 67°C, and 2 min at 72°C, with a final round of 10 min at 72°C, in an ABI 2720 thermocycler. We used a 2% agarose gel for genotyping with ethidium bromide-free dye in the loading buffer. Primers for genotyping have been listed in Supporting Table 1. All mice were maintained under a 12-hour light-dark cycle with *ad libitum* access to regular food and water at the UAB Animal Facility. Both male and female mutant and WT mice were randomly selected into group of five animals each. The investigators were not blinded during allocation, animal handling, and endpoint measurements.

Histological and radiographic procedures

Preparation of tissue samples and histological analyses were performed as described.^(21,22) TRAP staining was performed to examine OC differentiation using a commercial kit (Sigma-Aldrich, St. Louis, MO, USA; 387A-1KT) according to the manufacturer's instructions. TRAP-positive multinucleated (more than three nuclei) cells were counted by light microscopy. Nonspecific esterase (NSE; Sigma; 91A-1KT) staining was performed as described.^(2,23,24) For X-ray analysis, radiography was performed using a high-resolution, soft X-ray system (Faxitron Model MX-20) by the UAB Small Animal Bone Phenotyping Core associated with the Center for Metabolic Bone Disease.

μ CT

Excised mouse humerus and femurs were scanned using the Scanco CT40 desktop cone-beam micro-CT (μ CT) scanner (Scanco Medical AG, Brüttisellen, Switzerland). The trabecular bone scanning was performed from the growth plate (310 slices at 12 μ m per slice) analyzed using the CT Evaluation Program (v5.0A; Scanco Medical). The scanning and analysis of the cortical bone were performed at the midshaft of the femur and consisted of 25 slices (12 μ m per slice).

Histomorphometric analysis

Histomorphometric samples were nondecalcified hard-tissue sections processed as described.^(7,21) Briefly, for quantitative bone volume histomorphometry, 6- μ m sections of *C/ebp α ^{ff}* and *C/ebp α ^{ff}/*LysM-Cre* mice were stained with Goldner's trichrome. For histomorphometric analysis of OC size and number, 6- μ m sections of *C/ebp α ^{ff}* and *C/ebp α ^{ff}/*LysM-Cre* mice were TRAP stained without counterstaining. Histomorphometric analysis of these sections was performed using NIH ImageJ software (NIH, Bethesda, MD, USA; <https://imagej.nih.gov/ij/>). For trichrome stain, analysis was provided by the UAB Center for Metabolic Bone Disease Core Laboratory. In this analysis, we examined the following parameters: bone volume relative to tissue volume, the percentage of OC surface area to total bone surface area of the tibias, the percentage of osteoblast surface area to total bone surface area of the tibias, OC numbers per bone perimeter, osteoblast numbers per bone perimeter, distal hypertrophic growth plate thickness, proximal hypertrophic growth plate thickness, and trabecular thickness. Large multinucleated cells with cytoplasmic vesicles and intimate contact to bone were considered as OCs, and cuboidal mononuclear cells in intimate contact with osteoid or bone were identified as osteoblasts. For bone resorption assays, the concentration of bovine cross-linked C-telopeptide of type I collagen (CTX-1) was measured in the serum of overnight fasted mice using the CrossLaps® for Culture ELISA (CTX-I) kit (Immunodiagnostic Systems, Boldon Business Park, UK) following the manufacturer's instructions. Osteoblast bone formation was measured using the Human Pro-Collagen I alpha DuoSet ELISA**

(P1NP) kit following the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA; catalogue DY6220-05).

Mouse bone marrow cells, OC-like cells, and osteoblast function

Mouse bone marrow (MBM) cells obtained from wild-type (WT) and mutant mice as described^(25,26) were cultured in α -MEM (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Invitrogen) containing M-CSF (20 ng/mL). After 1 day, cells were further cultured in the presence of 10 ng/mL RANKL (PeproTech, Rocky Hill, NJ, USA) and M-CSF (10 ng/mL) to generate OCs. To determine osteoblast function, primary calvarial osteoblasts were isolated from newborn mice and seeded as described.⁽²⁷⁾ Cells were induced using osteogenic medium and BGJb medium (12591; Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 50 μ g/mL L-ascorbic acid (A4544; Sigma-Aldrich), and 5 mM glycerolphosphate (G9891; Sigma-Aldrich). Cells were passaged and osteoblastogenesis was induced using osteogenic medium (α -MEM medium supplemented with 50 μ g/mL L-ascorbic acid, 5 mM β -glycerolphosphate, and dexamethasone). Osteoblastogenesis was analyzed by alkaline phosphatase (ALP) staining according to manufacturer's manual (A2356; Sigma-Aldrich).

Cell staining for F-actin rings

F-actin ring formation was evaluated as previously determined.⁽²⁸⁾ In brief, cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. The cells were then blocked with 1% goat serum and 3% BSA and incubated with 2 U/mL rhodamine phalloidin (Molecular Probes, Eugene, OR, USA) at room temperature for 20 min. The experiment was performed in a 24-well plate in duplicate on four independent occasions. For anti-Ctsk immunofluorescence stain, OCs on bovine bone slides were fixed by 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, blocked with 5% goat serum and 100 μ g/mL unconjugated AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA), incubated with mouse anti-mouse Ctsk primary antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-48353) and then with FITC goat anti-mouse IgG (H+L) secondary antibody. Microscopy and 3D construction by Imaris (Bitplane AG, Zurich, Switzerland) were performed as described.⁽²⁹⁾

Acridine orange staining

Acid production was determined using acridine orange as described.⁽⁵⁾ OCs were incubated in α -MEM containing 5 μ g/mL of acridine orange (Sigma) for 15 min at 37°C, washed, and chased for 10 min in fresh media without acridine orange. The cells were observed under a fluorescence microscope with the Leica Texas Red filter. The experiment was performed in duplicate on four independent occasions in a 12-well or 6-well plate.

In vitro bone resorption assays

Bone resorption activity was assessed as described.^(30,31) For scanning electron microscopy (SEM) analysis, a consistent number of MBM cells were seeded on bovine cortical bone slices in 24-well plates stimulated by RANKL/M-CSF for 6 days. Bone slices were harvested on day 6 using 0.25M ammonium hydroxide and mechanical agitation to remove cells adhering to the bone slices. SEM of bone slices was done using a Philips 515 SEM (Philips, Andover, MA, USA) (at the Department of Materials Science and Engineering, UAB). Bone resorption pits were

analyzed by wheat germ agglutinin (WGA) stain as described⁽³²⁾ with peroxidase-conjugated WGA-lectin (Sigma-Aldrich; L-3892) and DAB Peroxidase (horseradish peroxidase [HRP]) Substrate Kit (Vector Laboratories, Burlingame, CA, USA; SK-4100). The assays were performed in triplicate and presented as a representative observing area from each assay. Data quantification was achieved by measuring the percentage of the areas resorbed in three random resorption sites using ImageJ software from the National Institutes of Health.

Immunostaining

Immunostaining was carried out as described.⁽⁷⁾ Antibodies for C/ebp α , Acp5, Pu.1, c-Fos, and Nfatc1 were purchased from Santa Cruz Biotechnology (1:200 1:1000). Two specific antibodies, F4/80 (BioSource International, Camarillo, CA, USA) and CD11b (Pharmingen, San Diego, CA, USA), were used as markers of monocyte/macrophage precursors as described.^(2,23) For the quantification of immunofluorescence, we used NIH ImageJ to perform counts, and the percent of positive cells represents the number of positive cells expressed the targeted gene divided by TRAP-positive cells.

Real-time quantitative PCR and semiquantitative PCR

RNA samples were obtained from C/ebp α ^{ff} and C/ebp α ^{ff}/LysM-Cre MBM cultured with M-CSF/RANKL, or from transduced pBMN-C/ebp α MBM with TRIzol reagent (Life Technologies, Inc., Grand Island, NY, USA; 15596018). TaqMan probes purchased from Applied Biosystems as listed in Supporting Table 2 were utilized in real-time quantitative PCR (qPCR), which was performed as described.^(33,34) Briefly, cDNA were amplified by Fast SYBR Green Master Mix (5 mL) P/N 4385612 (Applied Biosystems). Step-One real-time PCR system (Applied Biosystems) was used to detect fluorescence from each TaqMan probe using the TaqMan Gene Expression assays (Life Technologies, Applied Biosystems) or SYBR Green reagents (Life Technologies; 4385610). The mRNA expression levels were normalized against the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (Hprt). Primer sequences used for semiquantitative PCR are listed in Supporting Table 2. PCR conditions are available upon request. Experiments were repeated at least three times.

Western blot analysis

Western blotting was performed as described.⁽³⁵⁾ To examine protein expression, gel images were captured, and results were visualized by Fluor-S Multi-Imager, then quantified by Multi-Analyst software (Bio-Rad Laboratories, Hercules, CA, USA). Antibodies for C/ebp α , Pu.1, c-Fos, and Nfatc1 were purchased from Santa Cruz Biotechnology; and anti-cathepsin K antibody was used as described.⁽³⁵⁾ Results were normalized against housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Chromatin immunoprecipitation

The Ctsk (-4000/+301) promoter region was analyzed with PROMO3.0 (Algorithmics and Genetics Group [ALGGEN], Universitat Politècnica de Catalunya, Barcelona, Spain; <http://algen.lsi.upc.es/>) using version 8.3 of the TRANSFAC database for putative C/ebp α binding sites. Chromatin immunoprecipitation (ChIP) was performed using monoclonal anti-C/ebp α antibody (sc-61X; Santa Cruz Biotechnology). Subsequently, DNA was extracted and qPCR was performed as described.^(27,36) The DNA was prepared from MBM induced by RANKL and M-CSF

for 3 days using the primers in the promoter regions of *Ctsk* listed in Supporting Table 3.

Promoter analyses

Ctsk promoter was amplified using PCR from BAC clones provided by the BACPAC Resource Center at Children's Hospital Oakland Research Institute (Oakland, CA, USA). The amplified fragments were cleaved and ligated into the pGL3 vector (Promega, San Luis Obispo, CA, USA). RAW 264.7 cells were cultured in osteogenic medium (no FBS for 5 days), re-seeded on 96-well plates, and cotransfected with 100 ng/well of each construct as well as 50 ng/well of principal singular vector (pSV)- β -galactosidase construct and incubated for 6 to 8 hours. The culture medium was replaced with osteogenic medium and cultured for 2 days. The macrophage RAW264.7 cells were induced by RANKL and M-CSF for 2 days to promote the formation of multinucleated OCs, which was then followed by transfection with the corresponding luciferase reporter vectors. Luciferase activity was measured using a Steady-Glo luciferase assay system (Promega; cat. no. E2510). pSV- β -galactosidase activity was measured using a β -galactosidase Enzyme Assay system (Promega; cat. no. E2000) and normalized against β -galactosidase activity as described.^(7,33)

Ovariectomy procedure

C/ebp α ^{ff}LysM-Cre and *C/ebp α ^{ff}* mice were either dorsal ovariectomized (OVX) or sham-operated (sham) under anesthesia with a mixture of ketamine:xylazine (80:10 mg/kg) as described.⁽³⁷⁾ Mutant and WT mice were compared with sham or OVX mice, respectively. At 4 weeks postoperation, femurs and uteri were harvested for phenotypic and radiological analysis as previously described.⁽³⁴⁾

Statistical analysis

Experimental data are reported as mean \pm SD of triplicate independent samples with male and female mice in each group. Data were analyzed with the Student's *t* test or two-way analysis of variance using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) statistical program. Values of *p* < 0.05 were considered significant; **p* < 0.05, ***p* < 0.01, and ****p* < 0.005 (throughout). Error bars depict SD.

Results

C/ebp α ^{ff}LysM-Cre mice display an osteopetrotic phenotype

To overcome the early lethality of the *C/ebp α ^{-/-}* mice, a monocyte-specific *C/ebp α* -deficient mouse model was generated by crossing *C/ebp α ^{ff}* mice with *LysM-Cre* mice to generate *C/ebp α ^{ff}LysM-Cre* mice, and the genotypes were confirmed by PCR analysis (Fig. 1A). Compared to WT mice, the *LysM-Cre* mice and *C/ebp α ^{ff}* showed no overt bone phenotype. As such, the WT, *LysM-Cre*, and *C/ebp α ^{ff}* mice were used as controls in the study. Femoral radiograph analysis at 14 and 24 weeks of age showed that the increase in bone density in the *C/ebp α ^{ff}LysM-Cre* mice accentuated as the mice aged (Fig. 1B, C). We noted that both female and male *C/ebp α ^{ff}LysM-Cre* mice displayed similar bone phenotypes as compared to the control mice, indicating that the defect in bone density observed in the mutant mice was not sex-specific. Accordingly, μ CT analysis of the distal femurs of male and female at 14 weeks of age showed

a significant increase in both trabecular and cortical bone density in the *C/ebp α ^{ff}LysM-Cre* mice as compared to WT controls (Fig. 1D). Quantitative μ CT analysis further confirmed that *C/ebp α ^{ff}LysM-Cre* mice showed significant increases in bone volume/trabecular volume, trabecular number, and trabecular thickness, but a significant decrease in trabecular separation, as well as a significant increase in cortical bone density (Fig. 1E). To assess the onset, progression and severity of osteopetrosis in *C/ebp α* -deficient mice, we examined the bone phenotype of both mutant and WT mice at various stages of skeletal development (Supporting Fig. 1). Postnatal *C/ebp α ^{ff}LysM-Cre* mice (ie, 1 to 2 weeks) exhibited enhanced bone radiodensity (Supporting Fig. 1A, B) whereas bone radiographs and quantitative aging data from older *C/ebp α ^{ff}LysM-Cre* mice (ie, 14 and 16 weeks) showed worsening osteopetrosis (Supporting Fig. 1C, D). These results indicated that the conditional deletion of *C/ebp α* in the monocyte cell lineage caused an abnormally dense bone.

C/ebp α ^{ff}LysM-Cre mice display an osteopetrotic phenotype due to impaired OC and macrophage development

Hematoxylin and eosin (H&E) staining of femoral sections from newborn *C/ebp α ^{ff}LysM-Cre* mice showed significantly increased trabecular bone as compared with age-matched *C/ebp α ^{ff}* littermates (Fig. 2A). However, the bone collars lined with osteoblasts were comparable in *C/ebp α ^{ff}LysM-Cre* and *C/ebp α ^{ff}* mice (Fig. 2A, black arrows). Further H&E staining at 14 weeks showed higher bone density in the mutant mice as compared to age-matched *C/ebp α ^{ff}* littermates; further indicating that the bone density of the mutant mice was exacerbated with aging (Fig. 2B). Consistently, Goldner's Trichrome staining confirmed a dramatic increase in mineralized tissue in the *C/ebp α ^{ff}LysM-Cre* mice at 14 week as compared to *C/ebp α ^{ff}* mice (Fig. 2C). Accordingly, *C/ebp α ^{ff}LysM-Cre* mice showed a significant increase in bone volume per trabecular volume, trabecular thickness, and trabecular number, but a decrease in trabecular space as compared to control littermates (Fig. 2D). Although the mutant mice showed no overt defect in osteoblast development, the mutant had a significant decrease in OC number per bone surface as compared to *C/ebp α ^{ff}* (Fig. 2D). Consistently, ALP staining showed that osteoblast differentiation was not affected by *C/ebp α* deletion (Supporting Fig. 2). Additionally, P1NP serum level (bone formation marker) remained unaltered between WT, *C/ebp α ^{ff}LysM-Cre*, and *C/ebp α ^{ff}* mice, indicating that osteoblast function was unaltered in *C/ebp α* conditional knockout (CKO) mice (Fig. 2E). Given that OC originates from the monocyte/macrophage cell lineage, we next examined the effect of *C/ebp α* on the development of OC precursors as well as OC lineage priming (Fig 2F–H). Flow cytometric analysis showed that the *C/ebp α* CKO exhibited decreased macrophage development (ie, CD11b+F4/80+ cells) by almost 30% (Fig. 2F, H). Moreover, conditional *C/ebp α* deletion significantly affected the number of CD11b+CD115+ OCs (decreased by 70%) (Fig. 2G, H), suggesting a block in the OC lineage priming. The data indicated that *C/ebp α* deletion in the monocyte cell lineage caused osteopetrosis by impairing OC development.

LysM-Cre-mediated *C/ebp α* deletion impairs OC differentiation and activity in vitro

Next, we examined the abilities of *C/ebp α* -deficient OC precursors to differentiate into functional OCs through stimulation by M-CSF and RANKL (Fig. 3). Our previous study

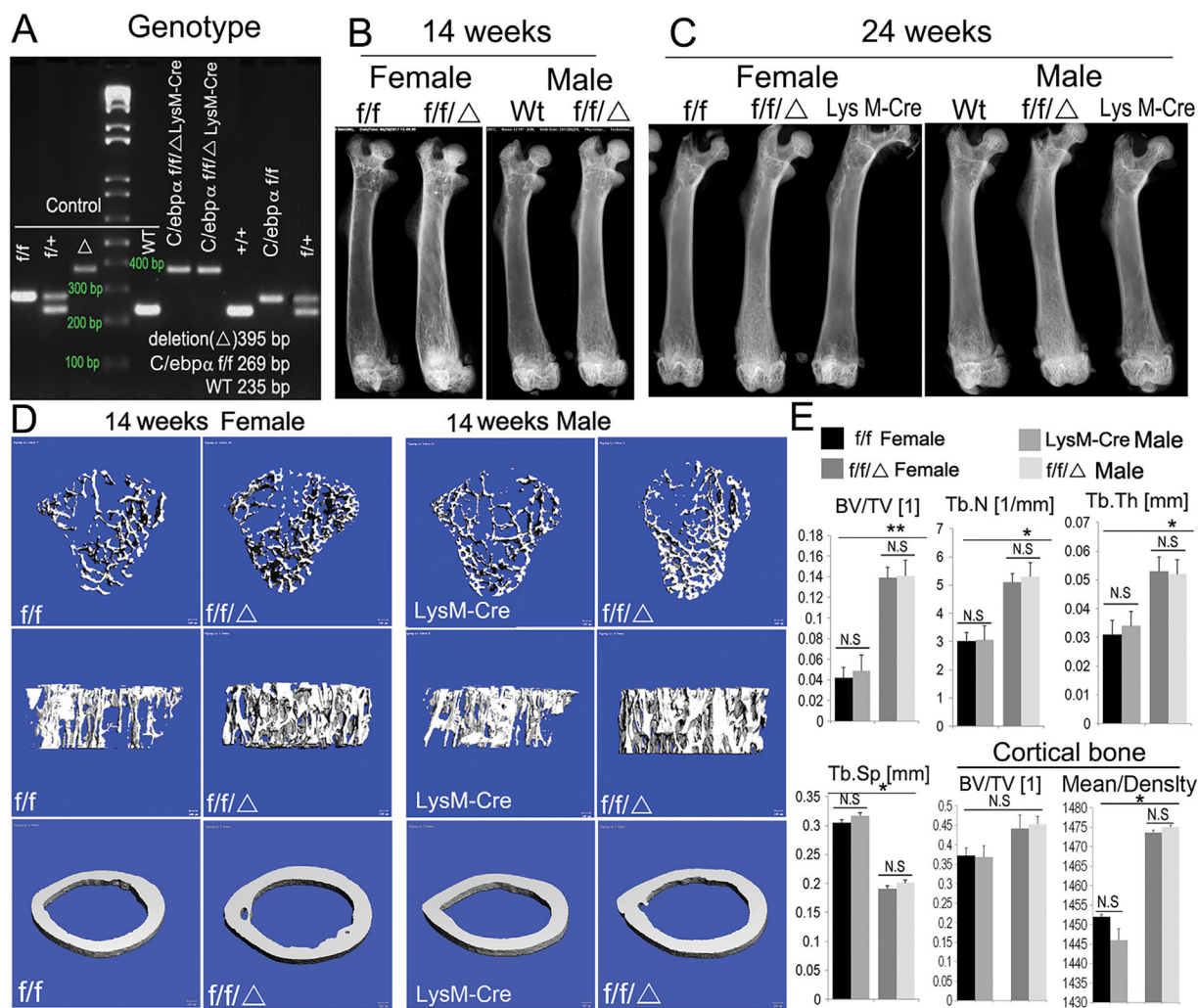


Fig. 1. Mice with *LysM-Cre*-mediated conditional knockout of *C/ebpα* developed osteopetrosis that becomes more severe as mice aged. (A) Genotyping of *C/ebpα^{ff}* and *C/ebpα^{ff}LysM-Cre* mice by PCR. Conditional knockout approach: excision through the endogenous promoter lysosome 2-Cre (*LysM-Cre*). (B, C) Radiographic analysis of male and female mouse femurs of 14-week-old (adult) and 24-week-old (aged) mice ($n = 60$). (D, E) μ CT and bone density analysis for both trabecular and cortical bones of 14-week-old male and female mouse femurs ($n = 8$). *C/ebpα^{ff}* and *LysM-Cre* were used as control. Results are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$. N.S. = not significant; WT = wild-type; (f/+) = heterozygote; (f/f/Δ) = homozygous excision of *C/ebpα*; (f/f) = *C/ebpα* gene surrounded by loxp sites; BV = bone volume; TV = total bone volume; Tb.N = trabecular number; Tb.Sp = trabecular separation; Tb.Th = trabecular thickness.

demonstrated the key role of *C/ebpα* in OC differentiation in vitro through rescue experiments conducted after shRNA-induced gene silencing.⁽³⁸⁾ MBM cells, widely used as primary OC precursors, were isolated from *C/ebpα^{ff}LysM-Cre* and *C/ebpα^{ff}* mice and then treated with M-CSF and RANKL for 5 days to promote OC differentiation (Fig. 3). Whereas WT cells formed numerous OCs as examined by TRAP staining (Fig. 3A, lower panel; Supporting Fig. 3A), the ability of *C/ebpα*-deficient MBM cells to differentiate into OC was reduced by almost 50% (Fig. 3A, F; Supporting Fig. 3C). Notably, nonspecific esterase staining of the induced MBM showed a 30% reduction in the number of positive cells, confirming a mild defect in monocyte/macrophage development (Supporting Fig. 3B, C). This is consistent with previous analysis (Fig. 2F–H). Provided the remaining OCs are functional, a 50% reduction in OC number will be able to maintain bone integrity. But based on the osteopetrosis exhibited by

mutant mice, it is highly possible that *C/ebpα* also plays an indispensable yet independent role in OC function. Therefore, we examined the effect of the *C/ebpα* deletion on actin ring formation, an important feature of mature OCs, and extracellular acidification, critical for OC function. We found that the *C/ebpα* deletion significantly impaired extracellular acidification (Fig. 3A upper panel, E) and also significantly reduced actin ring formation (Fig. 3B, upper panel). For further bone resorption assays, a consistent number of mutant and wild-type OCs were seeded on bovine cortical bone slices in 24-well plates. Unlike the well-polarized F-actin rings in *C/ebpα^{ff}* control, we found significantly impaired actin ring formation on bone slice seeded by *C/ebpα*-deficient OCs (Fig. 3B, lower panel). Consistently with these findings, bone resorption analysis by WGA staining (Fig. 3C, G) and SEM (Fig. 3D, H) showed that the *C/ebpα* deletion inhibited osteoclastic bone resorption. These results indicated that the

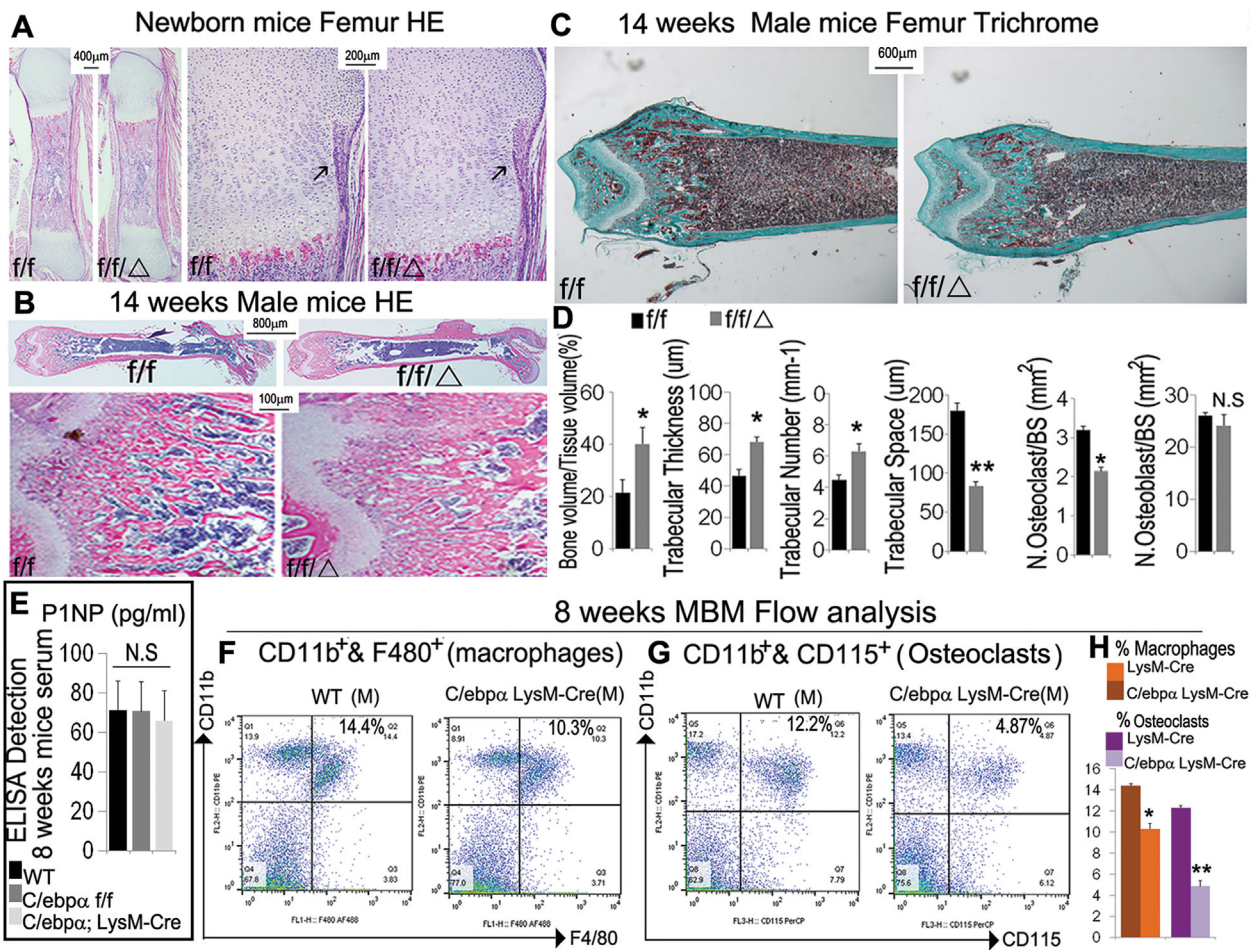


Fig. 2. *C/ebpα^{f/f}LysM-Cre* mice showed significantly impaired OC function, with mild changes in macrophage development and a large reduction in osteoclast number. (A, B) Representative images of H&E staining of (A) newborn and (B) 14-week-old *C/ebpα^{f/f}LysM-Cre* (f/f/Δ) femurs with *C/ebpα^{f/f}* (f/f) and LysM-cre as controls with high magnification of the epiphysis regions ($n = 10$; with 6 males and 6 females in each group). (C, D) Representative images of (C) Goldner's Trichrome analysis and (D) quantification data for male 14-week-old *C/ebpα^{f/f}LysM-Cre* compared to *C/ebpα^{f/f}* mouse femurs ($n = 16$), which reconfirmed the severe osteopetrotic phenotypes in *C/ebpα^{f/f}LysM-Cre* while osteoblast and OC numbers of mutants and wild-type remained almost unchanged. Analyses were repeated for female mouse samples with similar findings. (E) ELISA detection of P1NP (pg/mL) in 8-week-old mouse serum, indicating bone formation is not affected by gene CKO. Bars show means \pm SD. of triplicate independent samples. (F, G) Flow cytometry for (F) immune cell subtyping of CD11b⁺ F4/80⁺ macrophages and (G) CD11b⁺ CD115⁺ OCs from 8-week-old mouse bone marrow. (H) Quantification of F and G. Results show means \pm SD. of triplicate independent samples. * $p < 0.05$, ** $p < 0.01$. N.S = not significant; HE = hematoxylin and eosin.

lack of *C/ebpα* severely affected OC differentiation and function, leading to inhibition of osteoclastic bone resorption.

C/ebpα deletion represses gene associated with OC differentiation and function in vivo

In investigating the molecular basis of *C/ebpα* deletion on early and late states of OC development, we examined the expressions of OC marker genes by immunostaining of paraffin sections of newborn *C/ebpα^{f/f}LysM-Cre* mice as compared to WT controls, counting only TRAP-positive cells expressing the protein of interest (Fig. 4). Our immunostaining confirmed that *C/ebpα* was effectively ablated (Fig. 4A, G) and OC number was significantly reduced, through analysis of TRAP staining, in the *C/ebpα^{f/f}LysM-Cre* mice as compared to *C/ebpα^{f/f}* mice (Fig. 4F, G). Interestingly, the *Nfatc1* target gene *Ctsk* was repressed in *C/ebpα^{f/f}LysM-Cre* mice (Fig. 4B, G). Consistent with

the decreased OC development, *C/ebpα* deletion significantly repressed *c-Fos* (Fig. 4C, G) and *Pu.1* (Fig. 4D, G), critical for the commitment of bone marrow macrophages to the OC lineage⁽¹⁾ as compared to *C/ebpα^{f/f}* mice. Moreover, the *C/ebpα^{f/f}LysM-Cre* mice showed significantly reduced expression of *Nfatc1* (Fig. 4E, G), which is critical for OC lineage priming as well as terminal OC differentiation. The results indicate that the conditional deletion of the *C/ebpα* in the monocyte cell lineage impaired OC development by repressing genes associated with OC differentiation and function.

Monocyte-specific *C/ebpα* deletion inhibited the expression of genes associated with OC differentiation and function in vitro

We further confirmed these findings by examining the expressions of the OC marker genes from the mutant and WT

8 weeks *C/ebpα^{ff}* LysM-Cre MBM induced by M-CSF/RANKL

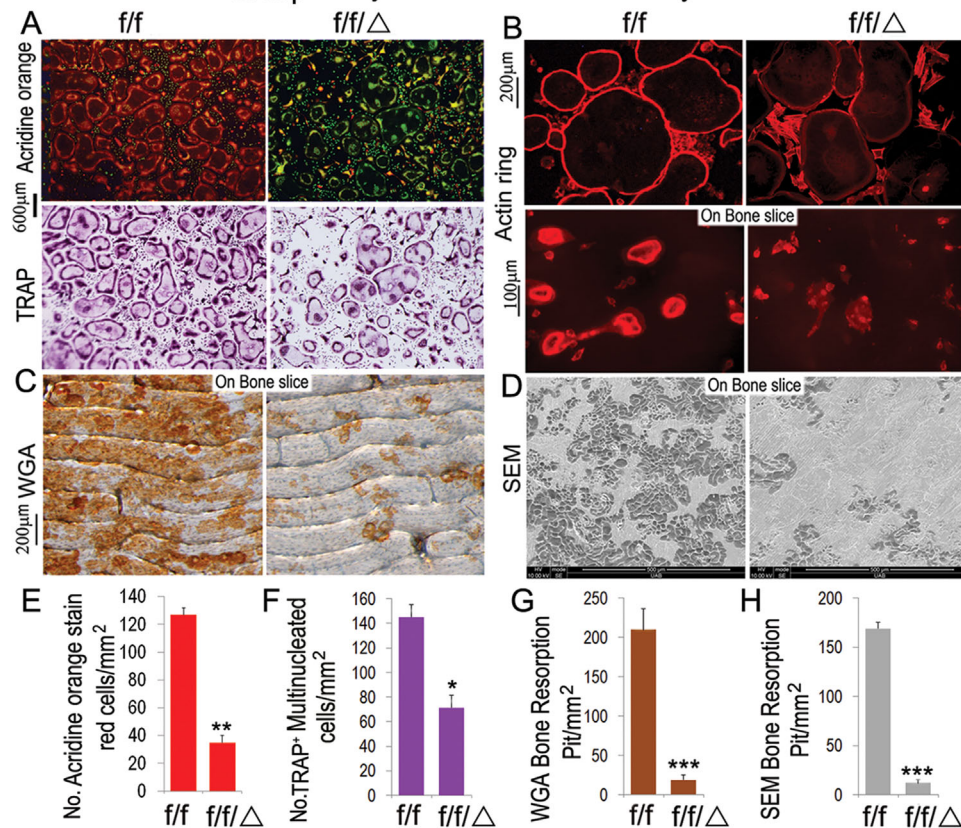


Fig. 3. Conditional knockout of *C/ebpα* resulted in severely defective OC activation and function, leading to diminishing osteoclastic activity in vitro. Eight-week-old *C/ebpα^{ff}*LysM-Cre (*f/f/Δ*) and *C/ebpα^{ff}* (*f/f*) MBM were stimulated by M-CSF (10 ng/mL)/RANKL(10 ng/mL) to promote formation of multinucleated OCs. Assessment of OC differentiation and function was carried out at the end of day 5 post-osteoclastogenic induction. (A) Acridine Orange staining and TRAP staining of *C/ebpα^{ff}*LysM-Cre OCs with *C/ebpα^{ff}* OCs as control. Acidity (red-orange) and number of multinucleated OCs were significantly reduced in *C/ebpα^{ff}*LysM-Cre OCs compared to *C/ebpα^{ff}* control. (B) F-actin ring formation assay on bone plates and bone slices shows disrupted or absent ringed structures of F-actin dots (actin rings) in M-CSF/RANKL-induced *C/ebpα^{ff}*LysM-Cre OCs compared to *C/ebpα^{ff}* control. (C) WGA staining to detect bone resorption pits. (D) SEM analysis of bone slices to detect bone resorption pits. (E–H) Quantification of A–D analyses. Number of positive cells were quantified per mm² plate area NIH image software (ImageJ). Results are presented as mean ± SD, *n* = 70. **p* < 0.05, ***p* < 0.01, ****p* < 0.005. WGA = wheat germ agglutinin; SEM = scanning electron microscopy.

mice by both qPCR and Western blotting (Fig. 5). Consistently, our Western blotting analysis confirmed that *C/ebpα* protein levels were significantly reduced in the *C/ebpα^{ff}*LysM-Cre mice. Moreover, the c-Fos, Nfatc1, Ctsk, and Atp6i protein levels were significantly repressed in the *C/ebpα^{ff}*LysM-Cre mice as compared to the *C/ebpα^{ff}* mice (Fig. 5A, B). We confirmed that the *C/ebpα* mRNA level was significantly repressed in the *C/ebpα^{ff}*LysM-Cre mice as compared to control *C/ebpα^{ff}* mice (Fig. 5C). Consistently, we showed that the mRNA levels of c-Fos, Nfatc1, and Ctsk as well as selected OC genes critical for cell function (*Mmp9*, *Atp6i*, and *Calcr*) were significantly repressed in the *C/ebpα^{ff}*LysM-Cre mice (Fig. 5C). However, although our Western blotting analysis showed that the *C/ebpα* deletion led to a threefold decrease in Pu.1 protein levels (Fig. 5A, B), our data also showed that *C/ebpα* deletion showed no effect on Pu.1 mRNA levels (Fig. 5C). We noted that the *C/ebpα* conditional deletion decreased Ctsk, c-Fos, Atp6i, and Nfatc1 protein levels by 2.5-fold, 2.5-fold, 5.0-fold, and 2.6-fold, respectively (Fig. 5B), which was associated with reduced mRNA levels by 2.5-fold, 1.5-fold, 2.5-fold, and 3.0-fold, respectively (Fig. 5C). This analysis

indicated that *C/ebpα* deficiency significantly repressed the expression of OC genes at the transcriptional level, which translated into decreased protein levels. The results indicate that *C/ebpα* deletion impairs OC differentiation and function by repressing gene expression at the transcriptional level.

C/ebpα silencing reduced OC differentiation as well as OC function in vitro

C/ebpα was excised in *C/ebpα^{ff}* MBM through Adenovirus-Cre vector for in vitro OC differentiation analysis along with a Scramble control, most efficiently in early stages (within day 1) of osteoclastogenic induction (Supporting Fig. 4A,B). TRAP staining showed a significant decrease in osteoclastogenesis and OC fusion in the *C/ebpα* deficient cells induced by RANKL (Supporting Fig. 4C). In addition, we analyzed actin ring formation by immunostaining, which showed that the formation of F-actin rings was severely defective in the *C/ebpα*-deficient cells at 24 and 48 hours (Supporting Fig. 4D, E). Interestingly, at 72 hours, when MBM had already differentiated into multinucleated

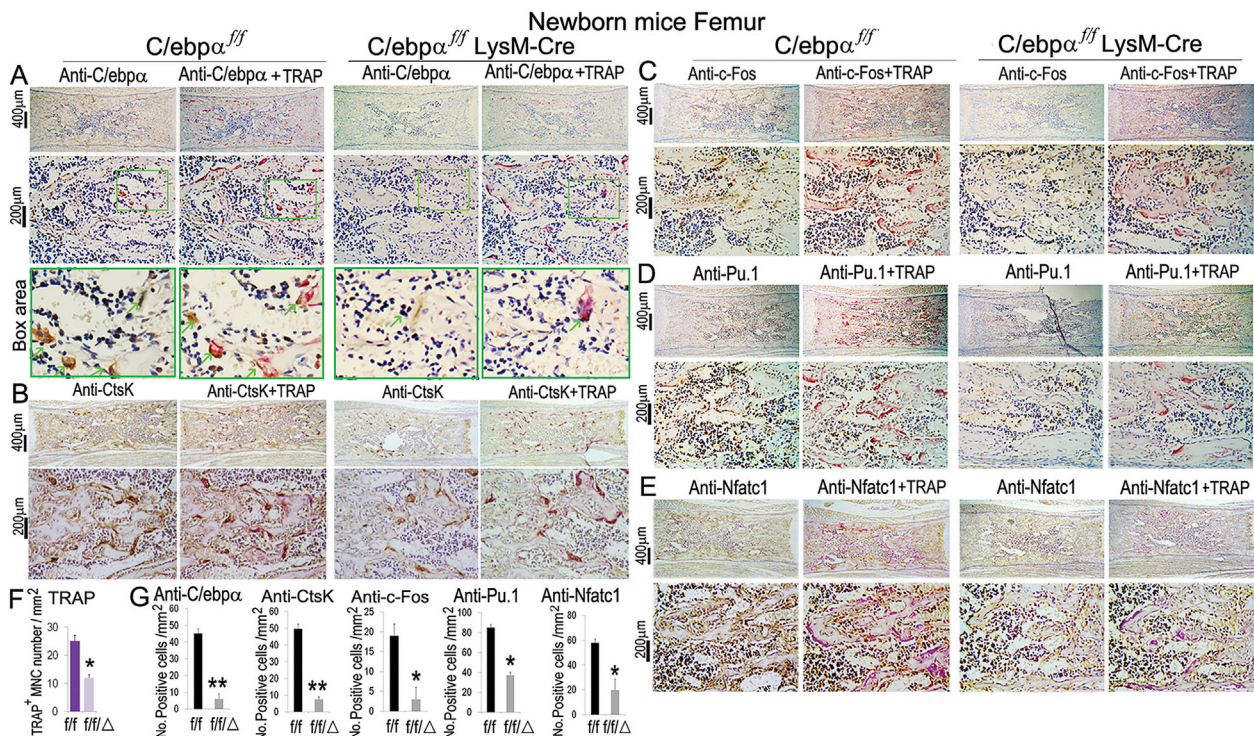


Fig. 4. Conditional knockout of *C/ebpα* significantly reduced the expression of OC regulator genes (ie, *Nfatc1*, *c-fos*) and OC function genes (ie, *Ctsk*, *Atp6i*). (A–E) Immunostaining using antibodies against (A) *C/ebpα*, (B) *Ctsk*, (C) *c-Fos*, (D) *Pu.1*, and (E) *Nfatc1* and their corresponding TRAP⁺ co-stained images for newborn *C/ebpα*^{ff/f}LysM-Cre (*f/f/Δ*) and *C/ebpα*^{ff/f} mouse femurs. IHC analyses were also carried out using LysM-Cre mice showing similar results to *C/ebpα*^{ff/f} group. Lower panels in A–E show high magnification images of immunostaining results. (F) Quantification data for TRAP⁺ cells. (G) Quantification data of staining results in A–E. Number of positive cells were quantified per mm² plate area using NIH image software (ImageJ). Bars show means ± SD with *n* = 30. **p* < 0.05, ***p* < 0.01. IHC = immunohistochemistry.

OCs before being transfected with a Adenovirus-Cre vector, the TRAP activity of Adenovirus-Cre–transfected OCs was significantly reduced, and F-actin rings were also disrupted and significantly reduced compared to the scramble control, indicating impaired OC polarization and function (Supporting Fig. 4D, E). The results further establish a critical role for *C/ebpα* in OC differentiation and function.

C/ebpα directly upregulates *Ctsk* expression by associating with its promoter

We previously reported that the sequence between –51 and –34 within the pCCAT-137 construct contains a *Ctsk* CCRE in which internal deletion induced a total loss of *Ctsk* activity, which was found to be a *C/ebpα* binding site (TTCCGCAAT).⁽³³⁾ Nevertheless, it has been suspected that there might be more binding sites for *C/ebpα* on the *Ctsk* promoter. In order to further examine whether *C/ebpα* has additional binding sites on the *Ctsk* promoter (–4000/+301), ChIP assay was performed using DNA prepared from MBM cells induced by M-CSF and RANKL for 4 days as described.^(27,36) DNA was pulled down, amplified, and analyzed using primers shown in Fig. 6A. The ChIP input value using each primer represents the binding efficiency of an adjacent region around the location of the primer pair. We found several *C/ebpα* binding sites on the *Ctsk* promoter region (–4000/+301) (Fig. 6B). We discovered that *C/ebpα*/*Ctsk* primer pair 5 resulted in the highest amplification followed by significant amplification resulting from primer pair 4, indicating

that these *C/ebpα* binding sites on these *Ctsk* promoter regions are the most efficient (Fig. 6B). Our subsequent luciferase assay showed highest activity in the longest *Ctsk* promoter fragments (–2445/+301), which still remained with the (–2232/+301), (–1825/+301), and (–1630/+301) fragments, suggesting critical *C/ebpα* binding sites at –1141 and –1030 in addition to the known binding site at –47⁽³³⁾ (Fig. 6C). Notably, the (–1630/+301) fragment retained approximately 80% of the luciferase activity of the longest fragment, which is a significantly higher percentage than that of the (–459/+301) and (–717/+301) fragments, thereby suggesting the presence of an important enhancer in addition to the previously reported CCRE at –47⁽³³⁾ (Fig. 6C). The results demonstrated that *C/ebpα* directly associated with the *Ctsk* promoter to upregulate *Ctsk*, further establishing it as a key transcriptional regulator of OC differentiation, activation, and function through direct transcriptional gene regulation.

C/ebpα^{ff/f}LysM-Cre mice exhibit protection against OVX-induced bone loss

In order to examine how *C/ebpα* regulates bone remodeling under pathological states, we performed sham surgery and OVX on *C/ebpα*^{ff/f} and *C/ebpα*^{ff/f}LysM-Cre mice. The OVX mouse model mimics estrogen-depletion-induced bone loss in postmenopausal women, and provides an important mouse model to study the role of *C/ebpα* in bone loss–induced estrogen deprivation. Four weeks following the operations, mouse body

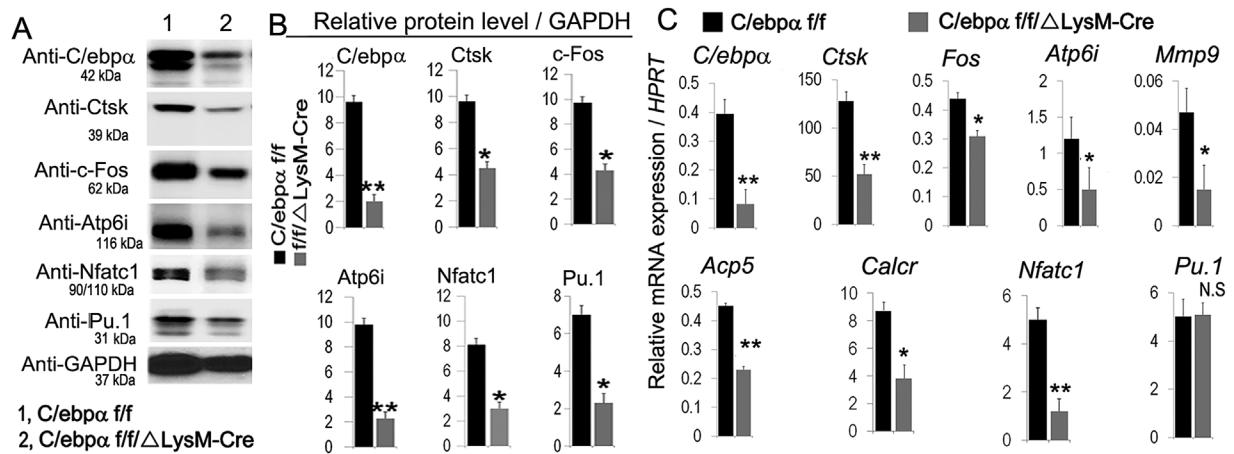


Fig. 5. *C/ebpα* upregulated the expression of OC regulator genes and OC function genes at both protein and mRNA levels. (A, B) Western blot and quantification data for *C/ebpα^{ff}/LysM-Cre* (*f/f/Δ*) and *C/ebpα^{ff}* (*f/f*) mice showing expressions of *C/ebpα*, *Ctsk*, *C-Fos*, *Atp6i*, *Nfatc1*, and *Pu.1* at the translational level. GAPDH is shown as loading control. (C) qRT-PCR of genes important for osteoclastogenesis and OC-specific function genes and a gene that is common to macrophages and OCs (ie, *Pu.1*) in RANKL-induced MBM *C/ebpα^{ff}* and *C/ebpα^{ff}/LysM-Cre* mice. *Hprt* served as a loading control. Bars show means ± SD with *n* = 16. **p* < 0.05, ***p* < 0.01. N.S = not significant.

nd uterine sizes were examined to confirm the effects of OVX in *C/ebpα^{ff}/LysM-Cre* and *C/ebpα^{ff}* mice (Fig. 7A, B). Uteri in OVX-*C/ebpα^{ff}* and OVX-*C/ebpα^{ff}/LysM-Cre* mice had dramatically decreased in size compared to that in post-sham *C/ebpα^{ff}* mice (Fig. 7B). Radiographic images revealed that, although OVX-*C/ebpα^{ff}* mice exhibited severe OVX-induced bone loss,

OVX-*C/ebpα^{ff}/LysM-Cre* femurs conversely showed an enhanced bone density compared to the control femurs (Fig. 7C, D). To further explore the bone phenotypes of OVX-mutant mice, μ CT analysis was performed on mouse femurs at 4 weeks post-operation (Fig. 7E). Sham-*C/ebpα^{ff}/LysM-Cre* mice retained significantly higher bone density compare to sham-*C/ebpα^{ff}*

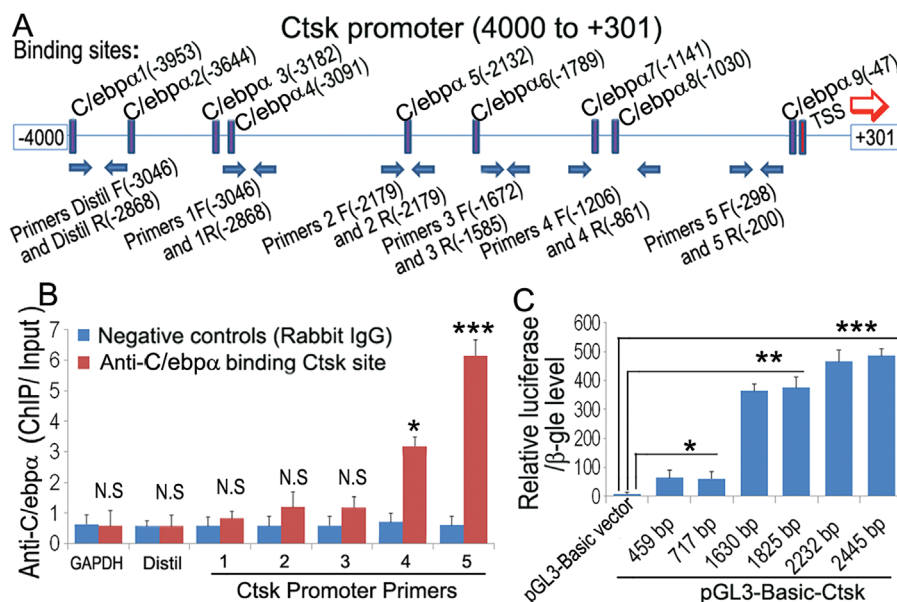


Fig. 6. *C/ebpα* directly upregulates *Ctsk* expression through interactions with its promoter region. (A) Schematic display of *Ctsk* (−4000/+301) promoter region: TSS, predicted *C/ebpα*-binding sites, and ChIP primers positions. (B) ChIP analysis of *C/ebpα* binding to the *Ctsk* promoter in RANKL-induced MBM using primers as indicated on the x-axis. Results are presented as ChIP/Input. (C) *C/ebpα* promoter fragments were inserted into pGL3-basic vector. RAW 264.7 cells were transfected with pGL3-*C/ebpα* −459 bp, −717 bp, −1630 bp, −2232 bp, and −2445 bp. Luciferase was detected at 48 hours posttransfection and normalized to β -gal activity. Results are presented as mean ± SD with *n* = 10. **p* < 0.05, ***p* < 0.01, ****p* < 0.005. N.S = not significant; TSS = transcriptional start site; F = forward; R = reverse; MBM = mouse bone marrow.

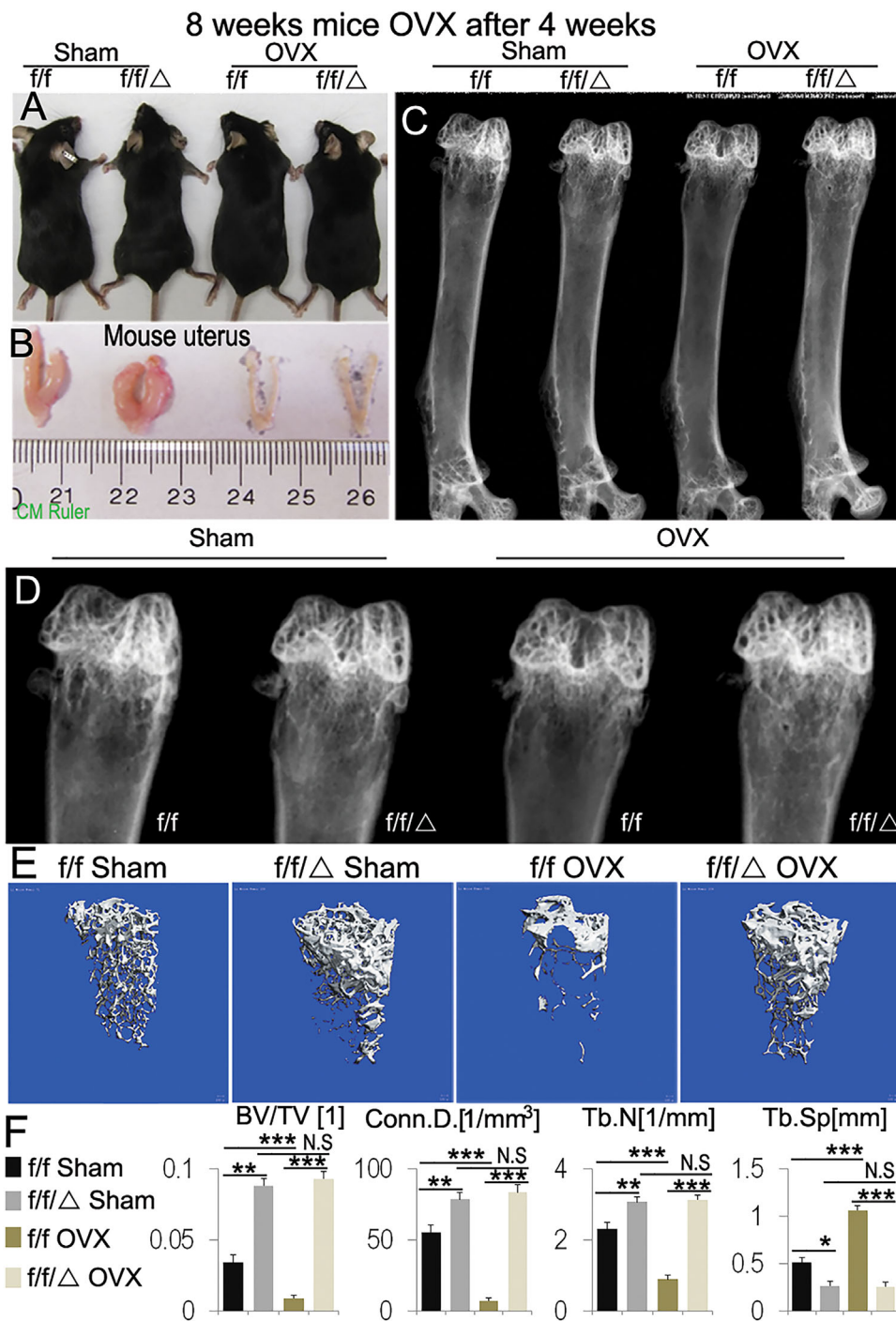


Fig. 7. The deficiency of *C/ebpα* in OCs blocks OVX-induced bone loss in osteoporosis mouse model. *C/ebpα*'s roles in OVX-induced bone loss analysis were examined in 8-week-old *C/ebpα^{ff}* (*f/f*) and *C/ebpα^{ff}*LysM-Cre (*f/f/Δ*) mice at 4 weeks after sham surgery and OVX ($n = 15$). (A) Mouse photographic images and (B) assessment of uterine size to confirm the effects of OVX. (C) Representative images of radiographic analysis of mouse femurs. (D) High-power image of the epiphysis regions from C data. No significant changes were found between sham-*C/ebpα^{ff}*LysM-Cre and OVX-*C/ebpα^{ff}*LysM-Cre. Compared to OVX-*C/ebpα^{ff}*, which displayed significant bone loss, *C/ebpα*-deficient mice retained normal bone mineral density similar to that of sham-*C/ebpα^{ff}* control. (E) μ CT analysis of *C/ebpα^{ff}* and *C/ebpα^{ff}*LysM-Cre mice femurs from C and D ($n = 4$). (F) Quantification of E revealed that femurs from OVX-*C/ebpα^{ff}*LysM-Cre mice retained significantly higher ratios of bone volume/tissue volume and increased trabecular numbers compared to OVX-*C/ebpα^{ff}* mice at 4 weeks postoperation. Bars show means \pm SD. of triplicate independent samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. N.S = not significant; OVX = ovariectomy; BV = bone volume; TV = total bone volume; Conn.D = connectivity density; Tb.N = trabecular number; Tb.Sp = trabecular separation; Tb.Th = trabecular thickness.

mice. Furthermore, when compared to the OVX-*C/ebpα*^{ff} control group, quantification analysis of the distal femurs showed increased bone volume/tissue volume and trabecular number in OVX-*C/ebpα*^{ff}LysM-Cre mice, which was comparable to that of the sham-*C/ebpα*^{ff}LysM-Cre mice (Fig. 7F). The results indicate that *C/ebpα* deficiency protects mice from OVX-induced bone loss.

Discussion

The role of *C/ebpα* in postnatal skeletal development and bone homeostasis has not been studied because newborn *C/ebpα* null mice die within several hours after birth. Hence, this study provides significant insights into the role of *C/ebpα* as a key regulator of OC differentiation and function, under physiological and pathological conditions, beyond its known functions in regulating cell lineage commitment. We revealed that *C/ebpα* directly regulates *Ctsk* gene expression thereby resulting in OC differentiation and function and mildly defective macrophage development.

The molecular basis underlying *C/ebpα* control of the cascade of transcription factors that specify OC terminal differentiation, including cell activation and function

C/ebpα is an important transcriptional regulator of hematopoiesis, which acts through its ability to bind DNA and influence transcriptional activities of various genes. Recent studies thus far have exclusively argued for the importance of *C/ebpα* in monocyte/macrophage and OC lineage allocation.^(15,39) Notably, our in vitro gain-of-function and loss-of-function studies have indicated that *C/ebpα* functions throughout osteoclastogenesis, and potentially, in OC function.⁽³⁹⁾ In this study, we found that in vivo OC terminal differentiation in *C/ebpα* CKO mice was affected along with severely impaired osteoclastic bone resorption, which suggests that *C/ebpα* might play a critical yet independent role in OC terminal differentiation. However, the mechanisms underlying the role of *C/ebpα* in postnatal physiological and pathological skeletal development remain elusive. Because *C/ebpα* can be removed through an ubiquitin-proteasome system-mediated mechanism,⁽⁴⁰⁾ it is possible that RANKL protects *C/ebpα* from being degraded, thereby leading to its high expression in the cytoplasm during osteoclastogenic induction, leading to upregulated expressions of *Nfatc1* and *c-fos* to induce OC lineage commitment, which precedes OC differentiation and function. Notably, *C/ebpα* also directly upregulates OC genes (eg, *Ctsk* and *Atp6i*), which contributes to OC-mediated bone resorption involving actin ring formation, extracellular acidification, and matrix protein degradation. Hence, deficiency of *C/ebpα* can suppress the expression of these regulating factors and function genes, resulting in osteopetrosis. Interestingly, Pu.1 protein levels, but not its mRNA level, were downregulated in *C/ebpα*^{ff}LysM-Cre. We have shown that constitutive *C/ebpα* ablation significantly reduced Pu.1 expression at both protein and mRNA levels, indicating *C/ebpα* could play major roles in early OC differentiation and terminal OC differentiation and that there might be a posttranslational mechanism which accounts for the differential Pu.1 gene expression profile in OCs.⁽³³⁾ It is also possible that in OCs, *C/ebpα* interacts and forms multiprotein complexes with other coactivators to induce gene expression and posttranslational changes which are specific to OC function. On the other hand, c-Fos (a key regulator of OC-macrophage lineage determination) is significantly downregulated in *C/ebpα*^{ff}LysM-Cre, suggesting an

important role for c-Fos at different stages throughout OC development due to direct interactions with two *C/ebpα* binding sites in the promoter region of c-Fos.⁽³³⁾

C/ebpα plays a key role in monocyte/macrophage development and OC differentiation while also regulating OC function and thereby mediating bone resorption

In differentiating from their hematopoietic precursors, OC undergoes a robust process of lineage determination and cell maturation before committing to terminal differentiation in which OC activation and function take place, allowing for further investigation of later differentiation stages.⁽⁴¹⁾ In this study, we examined the effects that conditional loss of *C/ebpα* using LysM-Cre in myeloid precursor cells using a variety of parameters. The gene knockout strategies using the macrophage-specific LysM-Cre might result in incomplete *C/ebpα* knockout in mouse mature OCs,⁽⁴²⁾ potentially affecting the presentation of osteopetrosis in mutant mice. To overcome this challenge, we examined a large number of both male and female mice, as well as utilized the Ad-Cre lentiviral approach to knock out the *C/ebpα* gene in vitro for further OC differentiation and function assays. We revealed that *C/ebpα* CKO impaired actin ring formation, inhibited extracellular acidification, and decreased osteoclastic bone resorption, and that monocyte/macrophage development was repressed (Fig. 3; Supporting Fig. 4). Moreover, our flow cytometry data showed that *C/ebpα* deletion in monocyte/macrophages significantly altered the abundance of OCs (reduced by more than 70%) while reducing the number of the myeloid precursors (ie, macrophages) by only 30% (Fig. 2F–H), suggesting either that *C/ebpα* is directly involved in regulating the proliferation and function of macrophages and/or that its regulation also occurs at a much later stage in OC lineage commitment. Provided the remaining OCs are functional, a 50% reduction in OC number will be able to maintain bone integrity (Fig. 3F). But based on the osteopetrosis exhibited by mutant mice, it is highly possible that *C/ebpα* also plays an indispensable yet independent role in OC function. Furthermore, when comparing the bone slices seeded with the same number of WT and mutant OCs, the numbers of induced cells were in fact similar despite the disrupted actin ring indicating nonfunctional OCs (Fig. 3B). The bone resorption defects were further confirmed by subsequent WGA and electron microscopy analyses. Pertaining to the lack of TRAP+ cells in *C/ebpα* CKO mice, because *C/ebpα* also plays a role in early osteoclastogenesis and other cells of the hematopoietic origin, the loss of *C/ebpα* in monocytes through LysM-Cre CKO impaired monocyte and early OC differentiation, potentially leading to the decrease in TRAP+ cells. It would be interesting in future studies to determine the exact mechanisms by which *C/ebpα* regulate monocyte/macrophage development because macrophages have major functions in many inflammatory conditions.

C/ebpα may be able to exert its regulatory effects on critical OC regulator genes and OC function genes to selectively alter the activation and function stages in normal OC development

Direct interaction with *C/ebpα* binding sites as a CCRE in the *Ctsk* promoter region could also be the mechanism underlying the effects of *C/ebpα* on OC function.⁽³³⁾ This interaction, despite being predominant in early stages of osteoclastogenesis, might

still retain significant influences throughout OC differentiation maturation, activation, and function in postnatal bone homeostasis. Furthermore, it is likely that the critical *C/ebp α* binding sites at -1141 and -1030 in addition to the known binding site at -47 in the *Ctsk* promoter region could also play important regulatory roles in OC activity (Fig. 6). This pathway underscores another possibility pertaining to *C/ebp α* 's control of the cascade of transcription factors that specifies postnatal OC activation and function. *C/ebp α* might directly and potently bind to the promoter regions of *c-Fos* and *Ctsk* to exert its regulatory effects and thereby promote OC function. Through directly upregulating the expressions of OC transcription factors and key OC function genes, *C/ebp α* may play an indispensable role in upregulating the expression of important OC-specific genes, which further promotes postnatal OC activation and function. Taken together, our findings strongly suggest that *C/ebp α* plays a major regulatory role in OC activation and function beyond its known functions in OC lineage commitment and differentiation.

C/ebp α may be a new target for the development of future novel and effective treatments for osteolytic diseases

Osteoporosis is a chronic condition characterized by abnormally high bone resorption along with diminishing bone formation, which potentially results in decreased bone strength and increased risk of bone fracture. Bone fragility associated with osteoporosis can lead to severe disability and even early mortality. Notably, bone loss in women rapidly increases after menopause due to lower levels of estrogen. Despite the significant effort that has been invested in past several decades, current treatment options are constrained by lower response rates and adverse side effects. This study not only improves our understanding of the role of transcription factors in OC differentiation, activation, and function, but it may also facilitate the development of novel treatments for osteolytic diseases, including osteoporosis. *C/ebp α* is an endogenous regulator that plays a key role in OC activation and function. *C/ebp α* deficiency in OCs completely protected mice against OVX-induced bone loss (Fig. 7). Thus, *C/ebp α* is an effective bone disease target and *C/ebp α* inhibitors may lead to desirable therapeutics and further optimize the current treatment options for local bone loss in periodontal disease and osteoarthritis. However, screening for effective *C/ebp α* inhibitors can be problematic in systemic osteolytic disorders, such as osteoporosis, because *C/ebp α* is ubiquitously expressed and involved in proliferation arrest and the differentiation of multiple cell types. In order to overcome these limitations, it is important to further explore the mechanisms underlying how *C/ebp α* specifically regulates osteoclastogenesis OC differentiation, activation, and function and to reveal effective upstream *C/ebp α* regulators that are specific to OCs. Although our work may not result in new osteoporosis therapies currently or directly, the characterization of the mechanism underlying how *C/ebp α* regulates OC differentiation, activation, and function will provide important mechanistic insights into OC biology that can greatly benefit future research in the investigation of potential *C/ebp α* regulators, as well as pointing to a new target for the development of novel and effective treatments for osteolytic diseases.

Disclosures

All authors state that they have no conflicts of interest.

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Authors' roles: Study design: YPL and WC. Study conduct: WC, GZ, and YPL. Data collection and analysis: WC, GZ, DN, and YPL. Drafting manuscript: YPL, WC, GZ, JJ, and DN. Revising manuscript: YPL, WC, GZ, JJ, and DN. All authors approved the final version of the manuscript for submission. YPL and WC take responsibility for the integrity of the data analysis.

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