

C/EBP α regulates osteoclast lineage commitment

Wei Chen¹, Guochun Zhu, Liang Hao, Mengrui Wu, Hongliang Ci, and Yi-Ping Li¹

Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294-2182

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Despite recent insights gained from the effects of targeted deletion of the *Finkel-Biskis-Jinkins osteosarcoma oncogene (c-fos)*, *Spleen focus-forming virus (SFFV) proviral integration 1 (PU.1)*, *microphthalmia-associated transcription factor, NF- κ B*, and *nuclear factor of activated cells cytoplasmic 1 (NFATc1)* transcription factor genes, the mechanism underlying transcription factors specifying osteoclast (OC) lineage commitment from monocyte/macrophage remains unclear. To characterize the mechanism by which transcription factors regulate OC lineage commitment, we mapped the critical *cis*-regulatory element in the promoter of *cathepsin K (Ctsk)*, which is expressed specifically in OCs, and found that CCAAT/enhancer binding protein α (C/EBP α) is the critical *cis*-regulatory element binding protein. Our results indicate that C/EBP α is highly expressed in pre-OCs and OCs. The combined presence of macrophage colony-stimulating factor and receptor activator of NF- κ B ligand significantly induces high C/EBP α expression. Furthermore, C/EBP α ^{-/-} newborn mice exhibited impaired osteoclastogenesis, and a severe osteopetrotic phenotype, but unaffected monocyte/macrophage development. Impaired osteoclastogenesis of C/EBP α ^{-/-} mouse bone marrow cells can be rescued by *c-fos* overexpression. Ectopic expression of C/EBP α in mouse bone marrow cells and monocyte/macrophage cells, in the absence of receptor activator of NF- κ B ligand, induces expression of *receptor activator of NF- κ B*, *c-fos*, *Nfatc1*, and *Ctsk*, and it reprograms monocyte/macrophage cells to OC-like cells. Our results demonstrate that C/EBP α directly up-regulates *c-fos* expression. C/EBP α ^{+/-} mice exhibit an increase in bone density compared with C/EBP α ^{+/+} controls. These discoveries establish C/EBP α as the key transcriptional regulator of OC lineage commitment, providing a unique therapeutic target for diseases of excessive bone resorption, such as osteoporosis and arthritis.

cell lineage commitment | gene expression regulation | osteoclast differentiation | bone homeostasis | osteopetrosis phenotype

Osteoclasts (OCs), bone-resorbing cells, play a key role in normal bone remodeling and in many diseases, such as osteopetrosis, osteoporosis, arthritis, periodontal disease, and certain bone metastases. OCs develop from monocytic precursors from the hematopoietic lineage. *Spleen focus-forming virus (SFFV) proviral integration 1 (PU.1)* can induce the expression of the macrophage colony-stimulating factor (M-CSF) receptor and is critical for monocyte/macrophage lineage commitment (1). On receptor activator of NF- κ B ligand (RANKL) stimulation and immunoreceptor tyrosine-based activation motif (ITAM) activation, OC precursors undergo further differentiation to mononuclear OCs (2). RANKL specifically and potently induces nuclear factor of activated T cells cytoplasmic 1 (NFATc1), which is a master regulator of OC differentiation, through both the TNF receptor-associated factor 6 (TRAF6)-NF- κ B (p105n) pathway and the *Finkel-Biskis-Jinkins osteosarcoma oncogene (c-Fos)* pathway (3–7). This robust induction of NFATc1 is based on an autoamplifying mechanism effected through persistent calcium signal-mediated activation of NFATc1 (NFATc1 binds to NFAT binding sites on its own promoter, constituting a positive feedback loop) (7). In the nucleus, NFATc1 cooperates with other transcription factors, such as activator protein-1, PU.1, cAMP-responsive element binding protein, and microphthalmia-associated transcription factor, to induce various OC-specific genes (7, 8). NFATc1, together with other transcription factors, such as *c-fos* and NF- κ B, drives osteoclastogenesis (2, 5). Peroxisome

proliferator-activated receptor γ (PPAR γ) is also reported to be important for OC differentiation (9). Although much has been revealed pertaining to the regulation of terminal differentiation of OCs, the cascade of transcription factors that specifies OC lineage commitment from monocytes/macrophages remains unclear (10). Indeed, a long-standing challenge remaining in the field is to define the mechanism by which M-CSF acts with RANKL to promote OC differentiation, whereas it promotes macrophage differentiation when acting alone (10), and to define the factor that regulates OC lineage commitment.

Cathepsin K (Ctsk) is a lysosomal cysteine protease that is abundantly and selectively expressed in OCs (11). The specific expression of the *Ctsk* in OCs, as well as its enzymatic properties, implies that it has a key role in normal bone remodeling and in pathological processes, such as osteoporosis, osteoarthritis, and pycnodysostosis (12). Because expression of *Ctsk* is OC-specific and induced by RANKL stimulation, we characterized the *Ctsk* critical *cis*-regulatory elements (CCREs) to identify the transcription factor(s) that modulate OC-specific gene (e.g., *Ctsk*) expression and to determine which factor(s) enable monocytes to commit to the OC lineage after RANKL stimulation.

Our research revealed that CCAAT/enhancer binding protein α (C/EBP α) regulates OC cell lineage commitment and differentiation. C/EBP α is a key molecular determinant in myeloid lineage commitment (13, 14), which drives myeloid differentiation through activation of myeloid target genes and through inhibition of the cell cycle through interactions with regulatory proteins (15, 16). We characterized C/EBP α as the binding protein of the OC gene *Ctsk* CCRE. We found that the deletion of C/EBP α in KO mice results in severely blocked osteoclastogenesis and that the overexpression of *c-fos* rescues osteoclastogenesis when C/EBP α is knocked out. Forced expression of C/EBP α , in the absence of RANKL, induces expression of *receptor activator of NF- κ B (RANK)* and OC genes and reprograms the monocyte/macrophage cell line to OC-like cells. These discoveries establish C/EBP α as the key regulator of OC lineage commitment.

Results

Identification of Mouse *Ctsk* CCRE and Characterization of CCRE DNA Binding Protein. To understand the transcription factors that modulate OC gene expression and OC differentiation, we characterized the *Ctsk* CCREs. Our results show that RANKL stimulates *Ctsk* gene promoter activity and indicate the importance of RANKL in combination with the CCRE (Fig. 1A). To analyze the -137 to -31 region identified as containing a potential CCRE further, we examined the effect of internal deletions within the promoter-CAT fusion gene (pCCAT)-137 construct on *Ctsk* activity and found that internal deletions of the sequence between -51 and -34 or between -46 and -41 resulted in the total loss of *Ctsk* promoter activity compared with the complete

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¹To whom correspondence should be addressed. E-mail: ypli@uab.edu or wechen@uab.edu.

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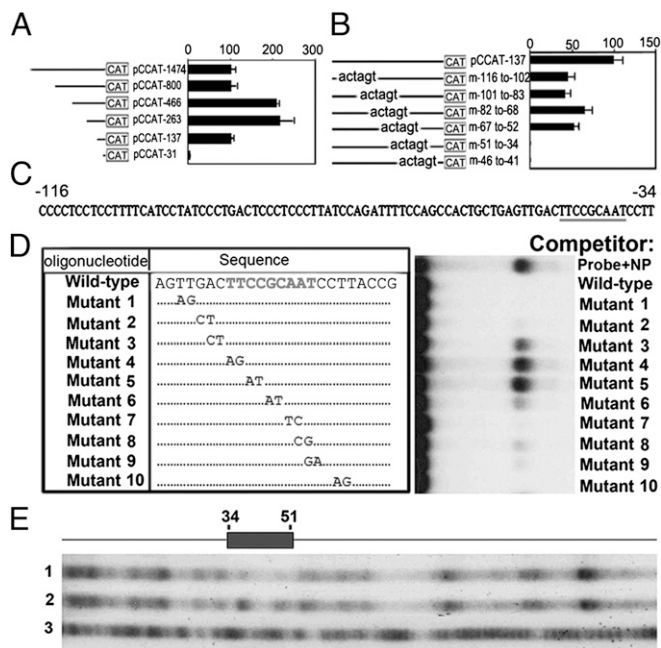


Fig. 1. Identification of *Ctsk* CCRE and CRE DNA binding protein as *C/EBPα*. (A) Activity of deletion mutants of the pCCAT construct was measured in RANKL-induced RAW64.7 cells. Schematic representation of each reporter construct is shown. Values are relative to the activity obtained with the largest promoter fragment (pCCAT-1474). CAT activities are normalized with the cotransfected β-gal activity. Experimental data are reported as mean ± SD of triplicate independent samples ($n = 5$, repeated three times). (B and C) Summary of site-specific mutagenesis of the *Ctsk* promoter binding site from -116 to -34 and characterization of the CCRE as a *C/EBPα* site. (D) Gel mobility shift experiment using a 32 P-labeled -53 to -30 WT *Ctsk* promoter oligonucleotide probe in nuclear extracts prepared from RANKL-induced RAW264.7 cells, with and without 100-fold molar excess of unlabeled WT or mutant oligonucleotides as described on the left. NP, nuclear protein. (E) DNase I footprint assays identify a binding site between -34 to -51 in the -137 to -31 mouse *Ctsk* promoter. The protected region is indicated by a shaded box, which contains a *C/EBPα* site. DNase I digestion of the naked DNA incubated with (lane 1) and without (lane 2) RANKL-induced RAW264.7 cell nuclear extracts is shown. G + A Maxam-Gilbert reaction of the -137 to -31 promoter fragment is shown in lane 3.

pCCAT-137 construct (Fig. 1B). Thus, we determined that the sequence between -51 and -34 contains a *Ctsk* CCRE (Fig. 1C). We performed a gel mobility shift experiment using the 32 P-labeled WT *Ctsk* oligonucleotide probe in nuclear extracts prepared from mouse OC precursor cell line RAW264.7 cells induced with RANKL, with and without a 100-fold molar excess of unlabeled WT or 10 different mutant oligonucleotides. We found that a 100-fold molar excess of mutants 3 and 6 partially competes, whereas mutants 4 and 5 are completely unable to compete, for the DNA-protein complex, indicating that this core sequence of nucleotides is essential for DNA-protein interaction (Fig. 1D). We analyzed this sequence using transcription factor binding site database searches and found that it is a *C/EBPα* binding site (TTCCGCAAT). This indicates that *C/EBPα* may regulate the CCRE. We also performed DNase I footprint analysis and found that the -51 to -34 region, which contains the *C/EBPα* binding site, was protected (Fig. 1E). To confirm that *C/EBPα* binds to this CCRE recognition site, we performed supershift mobility assays using a panel of specific antibodies against different *C/EBP* family members (Fig. S1). The bound proteins only produced supershifted complexes in the presence of *C/EBPα* antibody, indicating that *C/EBPα* is involved in the formation of this DNA-protein complex (Fig. S1). Together, these data indicate that *C/EBPα* may be a major component of the OC-specific complex that regulates the *Ctsk* CCRE.

***C/EBPα* Is Expressed in Pre-OCs and OCs and Is Induced by RANKL.** We next examined the tissue and cellular distribution of *C/EBPα* mRNA by performing a Northern hybridization assay. Giant cell tumors (GCTs) of bone contain human stromal cells (hSCs), OC-like precursors, and OC-like giant cells. Because OC-like giant cells from GCTs of bone are effectively the same as OCs in bone, we used GCTs and hSCs obtained as previously described (11). In addition, RANKL-induced mouse bone marrow (MBM), uninduced MBM, rat osteoblast, and a human macrophage cell line (U-937 cells) were used along with *C/EBPα*^{+/+} mouse tissues. The human *C/EBPα* transcript (2.4 kb) and mouse *C/EBPα* transcript (2.7 kb) used as *C/EBPα*-cDNA probes for this Northern blot assay are highly specific to *C/EBPα*. As shown in Fig. 2A, *C/EBPα* (2.7 kb) was highly expressed in RANKL-induced MBM and *C/EBPα*^{+/+} liver tissue. *C/EBPα* (2.4 kb) was prominently expressed in GCTs of bone (which contain hSCs, OC-like precursors, and OC-like giant cells) but was not expressed in hSCs (Fig. 2A). Furthermore, there was very low *C/EBPα* expression in U-937 cells, MBM, and mouse kidney and brain tissue. Expression of *C/EBPα* could not be detected in other tissues in these conditions, but this

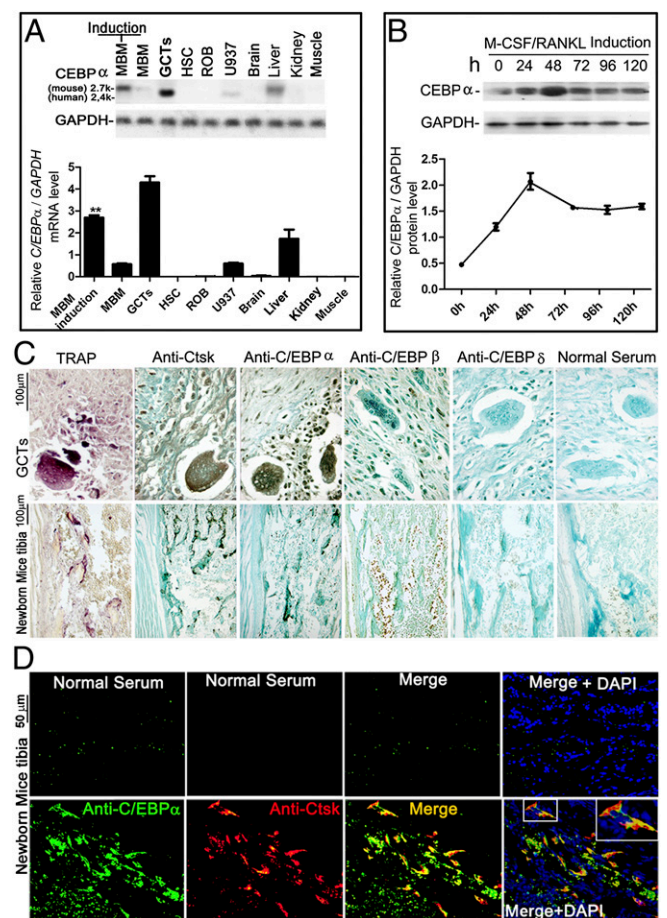


Fig. 2. *C/EBPα* is expressed in pre-OCs and OCs, and it is induced by RANKL. (A) Northern blot hybridization using a *C/EBPα*-specific probe in RANKL-induced and uninduced *C/EBPα*^{+/+} MBM, GCTs of bone, hSCs, rat osteoblast (ROB), a human macrophage cell line (U-937 cells), and *C/EBPα*^{+/+} mouse tissues. (B) Time-course Western blot analysis of *C/EBPα* expression in *C/EBPα*^{+/+} MBM cultured with M-CSF (20 ng/mL) alone for 2 d and then stimulated with M-CSF (10 ng/mL)/RANKL (10 ng/mL) for 0–120 h. (C) TRAP staining and immunostaining for Ctsk, *C/EBPα*, *C/EBPβ*, and *C/EBPδ* in GCTs of bone and *C/EBPα*^{+/+} tibiae sections. Normal serum is shown as a control. (D) Immunofluorescence staining of *C/EBPα* (green) and Ctsk (red) in *C/EBPα*^{+/+} tibiae indicates significant overlap, visualized as a yellow area in the merged image ($n > 9$). Inset is the magnified image of the boxed area.

does not exclude a role for *C/EBPα* in those tissues. In order to characterize *C/EBPα* expression during OC differentiation, we performed Western blot analysis of *C/EBPα* expression in MBM cultured with M-CSF (20 ng/mL) alone for 2 d and then stimulated with M-CSF (10 ng/mL)/RANKL (10 ng/mL) for 0–120 h. We found that *C/EBPα* expression more than doubled after 24 h of stimulation. Interestingly, *C/EBPα* expression peaked at 48 h but retained high expression thereafter (Fig. 2*B*). The dramatic increase in *C/EBPα* expression early during OC differentiation indicates that *C/EBPα* may play a critical role in the regulation of OC commitment and OC differentiation. To determine the expression of *Ctsk* and *C/EBP* family members (i.e., *C/EBPα*, *C/EBPβ*, *C/EBPδ*) in situ, OC cells were subjected to immunohistochemical staining (17). We found strong *C/EBPα* expression in *Ctsk*-positive multinucleated OCs and mononuclear pre-OCs in GCTs and *C/EBPα*^{+/+} tibiae in a similar pattern of expression as *Ctsk* (Fig. 2*C*). In contrast, *C/EBPβ* was weakly expressed in TNF receptor-associated protein 1 (TRAP)-positive mononuclear pre-OCs, and *C/EBPδ* expression was not detected in GCTs or *C/EBPα*^{+/+} tibiae (Fig. 2*C*). Immunofluorescence staining of *C/EBPα*^{+/+} tibiae also revealed that *C/EBPα* colocalized with *Ctsk* (Fig. 2*D*). The pattern of *C/EBPα* expression indicates a possible critical function of *C/EBPα* in OC cell lineage commitment and differentiation.

C/EBPα^{-/-} Newborn Mice Exhibited a Severe Osteopetrotic Phenotype Due to Impaired Osteoclastogenesis.

X-ray analyses revealed that *C/EBPα*^{-/-} mice have dramatically increased bone density compared with *C/EBPα*^{+/+} mice (Fig. 3*A*), indicating that *C/EBPα*^{-/-} mice have an osteopetrotic phenotype. This is supported by microcomputed tomography analysis, which showed significantly increased bone volume and bone mineral density in *C/EBPα*^{-/-} femora compared with *C/EBPα*^{+/+} femora (Fig. 3*B* and *C*). In order to analyze the role of *C/EBPα* in OCs further, we examined whether a null mutation in *C/EBPα* alleles affects the differentiation of OCs (17). Our histochemical stains revealed that *C/EBPα*^{-/-} mice tibiae have long growth plates and a dramatic reduction in TRAP⁺ OCs compared with *C/EBPα*^{+/+} controls (Fig. 3*D*). Furthermore, TRAP stains demonstrated that TRAP⁺ multinucleated OCs were not formed in RANKL-induced MBM culture from *C/EBPα*^{-/-} mice compared with *C/EBPα*^{+/+} controls (Fig. 3*E*). These data confirm that *C/EBPα*^{-/-} mice are osteopetrotic and indicate that this phenotype is mainly due to defective osteoclastogenesis. In order to determine if *C/EBPα*^{-/-} osteoblasts contribute to the defective osteoclastogenesis observed in *C/EBPα*^{-/-} mice, we used a coculture system and TRAP stain as we have previously described (17). We determined that a coculture of calvarial mouse osteoblasts (MOBs) derived from *C/EBPα*^{+/+} mice with MBM from *C/EBPα*^{-/-} mice is unable to rescue osteoclastogenesis (compared with *C/EBPα*^{+/+} MOBs with *C/EBPα*^{+/+} MBM) (Fig. 3*F*). Coculture of *C/EBPα*^{-/-} MOBs with *C/EBPα*^{+/+} MBM revealed that mutant osteoblasts sustain osteoclastogenesis (Fig. 3*F*), indicating that osteoblasts lacking *C/EBPα* do not contribute to the defective osteoclastogenesis in *C/EBPα*^{-/-} mice.

C/EBPα KO Reduces the Expression of OC Differentiation Regulator and Marker Genes.

To determine the mechanism of *C/EBPα* function in OC commitment and differentiation, we performed real-time (RT) quantitative PCR (qPCR) analysis of gene expression in *C/EBPα*^{+/+} and *C/EBPα*^{-/-} MBM cells cultured with M-CSF/RANKL to generate OC-like cells (Table S1) (12, 17, 18). The RT-qPCR results (Fig. S2) suggest that *C/EBPα* is important for the induction of OC-specific genes and that *C/EBPα* may suppress macrophage-specific genes. These results were confirmed by Western blot analysis of *C/EBPα*^{+/+} and *C/EBPα*^{-/-} MBM cells OC-like cells (Fig. 4*A* and *B*). Time-course

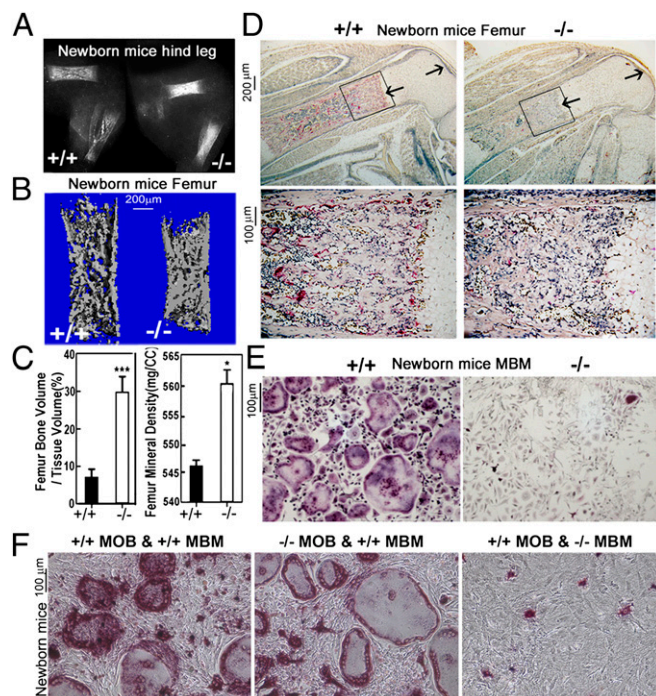


Fig. 3. Increased bone mass and defective osteoclastogenesis in *C/EBPα*^{-/-} mice in vivo and in vitro. (A) Radiographic analysis of femora and tibiae indicates osteopetrosis in *C/EBPα*^{-/-} newborn mice compared with *C/EBPα*^{+/+} newborn mice ($n = 5$, repeated three times). (B) Three-dimensional micro-computed tomography images of *C/EBPα*^{+/+} and *C/EBPα*^{-/-} newborn mice femora ($n = 5$, repeated three times). (C) Quantification of bone volume/tissue volume and bone mineral density of *C/EBPα*^{+/+} and *C/EBPα*^{-/-} newborn mice femora. * $P < 0.05$; *** $P < 0.001$. (D) Histological sections of *C/EBPα*^{-/-} newborn mice tibiae have an extended growth plates (arrows) and a dramatic reduction in TRAP⁺ OCs compared with *C/EBPα*^{+/+} controls ($n > 50$). Boxed areas (Upper) are magnified (Lower). The histological sections of the same area of *C/EBPα*^{-/-} newborn mice also show dramatically decreased TRAP⁺ OCs. (E) TRAP stain of RANKL-induced *C/EBPα*^{+/+} and *C/EBPα*^{-/-} MBM. (F) TRAP stain of cocultures with MOBs and MBM obtained from *C/EBPα*^{-/-} and *C/EBPα*^{+/+} mice.

Western blot analysis revealed that there are no major changes in the expression of NF- κ B inhibitor, inhibitor of κ B α (I κ B α), and MAPK 14 (p38) or their phosphorylated forms (p-I κ B α and p-p38) in *C/EBPα*^{-/-} MBM cells cultured with M-CSF/RANKL for 0–60 min, compared with the *C/EBPα*^{+/+} control (Fig. 4*C*). These data indicate that the role of *C/EBPα* in OCs

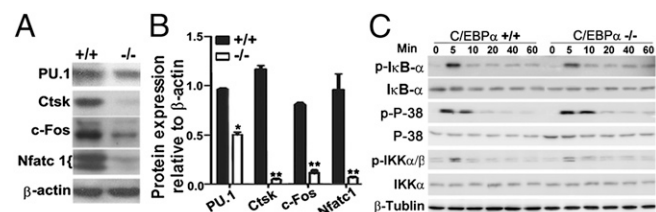


Fig. 4. *C/EBPα* KO reduces the expression of key OC regulators and marker genes. Western blot (A) and quantification of expression (B) of PU.1, *Ctsk*, *c-Fos*, and *Nfatc1* in *C/EBPα*^{+/+} and *C/EBPα*^{-/-} MBM cells cultured with M-CSF (20 ng/mL) alone for 2 d and then stimulated with M-CSF (10 ng/mL)/RANKL (10 ng/mL) for 5 d to generate OC-like cells. * $P < 0.05$; ** $P < 0.01$. (C) Time-course Western blot analysis of I κ B α and p38 and their phosphorylated forms (p-I κ B α and p-p38) in *C/EBPα*^{+/+} and *C/EBPα*^{-/-} MBM cells cultured with M-CSF (20 ng/mL) alone for 2 d and then stimulated with M-CSF (10 ng/mL)/RANKL (10 ng/mL) for 0–60 min.

does not involve the I κ B α or p38 signaling pathways but that it may directly regulate the *c-fos* arm of the RANKL signaling pathways to promote osteoclastogenesis.

Both *C/EBP α* ^{-/-} and *C/EBP α* ^{+/-} Mice Exhibit an Increase in Trabecular Bone Numbers. To analyze the role of *C/EBP α* in bone homeostasis further, we stained *C/EBP α* ^{+/+} and *C/EBP α* ^{-/-} tibiae sections with Goldner's trichrome stain and performed histomorphometric analysis (using Bioquant software; BIOQUANT Image Analysis Corporation). Compared with *C/EBP α* ^{+/+} controls, *C/EBP α* ^{-/-} tibiae have growth plates with an extended zone of growth plate and a dramatic increase in mineralized tissue, which indicates osteopetrosis (Fig. 5A). Histomorphometric analysis revealed that KO of *C/EBP α* results in increased bone volume, distal and proximal hypertrophic growth plate thickness, and trabecular thickness (Fig. 5B). Our analyses also revealed that the ratio of OC surface to bone surface and the number of OCs per bone perimeter dramatically decreased in *C/EBP α* ^{-/-} tibiae compared with *C/EBP α* ^{+/+} tibiae (Fig. 5B), further confirming the important role of *C/EBP α* in osteoclastogenesis. To determine the importance of the *C/EBP α* dose in mature animals, we analyzed the bone phenotype in *C/EBP α* ^{+/-} mature mice.

Femora from heterozygous *C/EBP α* ^{+/-} mice (8 mo of age) exhibit an increase in bone density and an increase in trabecular bone compared with *C/EBP α* ^{+/+} controls (Fig. S3). Goldner's trichrome stain and histomorphometric analysis indicate that *C/EBP α* ^{+/-} femora have an increase in bone volume/tissue volume and trabecular number and a decrease in OC surface/bone surface and OC number/bone perimeter (Figs. S4 and S5).

***C/EBP α* ^{-/-} Impaired Osteoclastogenesis Is Rescued by Ectopic Expression of *c-fos*.** In order to confirm if *C/EBP α* regulates osteoclastogenesis through the *c-fos* arm of the RANKL signaling

pathway, we next investigated whether ectopic expression of *c-fos* using retrovirus-mediated gene transfer could rescue the impaired OC differentiation caused by *C/EBP α* KO. MBM cells were stimulated with M-CSF/RANKL for OC differentiation analysis. These cultured *C/EBP α* ^{-/-} MBM cells were transduced by pBMN-*c-fos* or the control retrovirus pBMN-GFP (Fig. 5C and D). Compared with the control, the number of multinucleated OCs present increased more than 20-fold when *c-fos* was overexpressed in cultured *C/EBP α* ^{-/-} myeloid cells (Fig. 5C and D). This indicates that impaired OC differentiation caused by *C/EBP α* KO can be rescued by ectopic expression of *c-fos*.

***C/EBP α* Induces Osteoclastogenesis in the Absence of RANKL and Activates the *c-fos* Promoter.** To determine the mechanism of *C/EBP α* function in OC differentiation and gene expression further, we forced expression of *C/EBP α* in MBM cells by stimulating MBM with M-CSF alone (Fig. 6). To express *C/EBP α* in MBM ectopically, we constructed a retrovirus vector (pBMN-*C/EBP α*) that was engineered to express both *C/EBP α* and GFP.

Then, MBM was transduced with the *C/EBP α* -expressing virus (pBMN-*C/EBP α*) or with a control virus (pBMN-GFP) as described (3). Western blot analysis demonstrates the effective retrovirus-mediated overexpression of *C/EBP α* in MBM stimulated with M-CSF alone for 96 h (Fig. 6A). The expression of *C/EBP α* increased significantly after 96 h of M-CSF stimulation (Fig. 6A). At the 96-h time point, there is a surprising marked increase in the number of TRAP⁺ multinucleated OCs when *C/EBP α* is overexpressed compared with the control group even in the absence of RANKL stimulation. This underscores the important role of *C/EBP α* in osteoclastogenesis (Fig. 6B). Through qPCR, it was determined that the expression of markers common for macrophages and OCs (e.g., *RANK*, *Traf6*), key OC genes (e.g., *Nfatc1*, *c-fos*, *acid phosphatase 5 (Acp5)*, *matrix metalloproteinase 9 (Mmp9)*, *Ctsk*), and *C/EBP α* increased in the pBMN-*C/EBP α* transfected group (Fig. 6C). These data indicate that *C/EBP α* overexpression in the absence of RANKL stimulation can induce the formation of TRAP⁺ multinucleated cells (Fig. 6B and C). Because our results showed that overexpression of *c-Fos* can rescue the OC defect of *C/EBP α* ^{-/-} cells (Fig. 5C), it is important to determine if the *c-fos* promoter has *C/EBP α* binding sites. Accordingly, we performed ChIP assays with MBM stimulated with RANKL to generate mature OCs and found that *C/EBP α* directly binds regulatory sequences on the *c-fos* promoters (Fig. 6D) and activates *c-fos* expression (Fig. 6E). The truncated *c-fos* promoters were induced by *C/EBP α* , indicating the presence of two *C/EBP α* binding sites (Fig. 6F).

Ectopic Expression of *C/EBP α* Reprograms the Monocyte/Macrophage Cell Line to OC-Like Cells in the Absence of RANKL. To question whether ectopic expression of *C/EBP α* can reprogram the monocyte/macrophage cell line RAW264.7 cells to OC-like cells in the absence of RANKL, we ectopically expressed *C/EBP α* in RAW264.7 cells by using pcDNA3.1-*C/EBP α* or the vector control pcDNA3.1 (Fig. 7A-D). Western blot analysis demonstrated the effective overexpression of *C/EBP α* by the plasmid stable transfection approach (Fig. 7A). Similarly, TRAP staining revealed that TRAP⁺ OCs developed when *C/EBP α* was overexpressed in the absence of RANKL stimulation (Fig. 7B). In addition, *Ctsk* immunostaining demonstrated that *C/EBP α* overexpression in the absence of RANKL stimulation still resulted in the expression of the OC marker gene *Ctsk* (Fig. 7B). *C/EBP α* overexpression reduced the expression of F4/80, which is a macrophage marker gene. Semi-qPCR of *c-fos*, *Ctsk*, *Acp5*, and *Nfatc1* also demonstrated that *C/EBP α* overexpression increases the expression of key OC genes even in the absence of RANKL (Fig. 7C). We found that *C/EBP α* overexpression increased the expression of genes important for OC lineage commitment and differentiation (e.g., *Ctsk*, *PU.1*, *c-fos*, *CD115*, *Traf6*, *CD11b*, *Acp5*, *Nfatc1*, *Mmp9*, *Ppar γ* , *CD68*, *RANK*) (9) (Fig. 7D) through

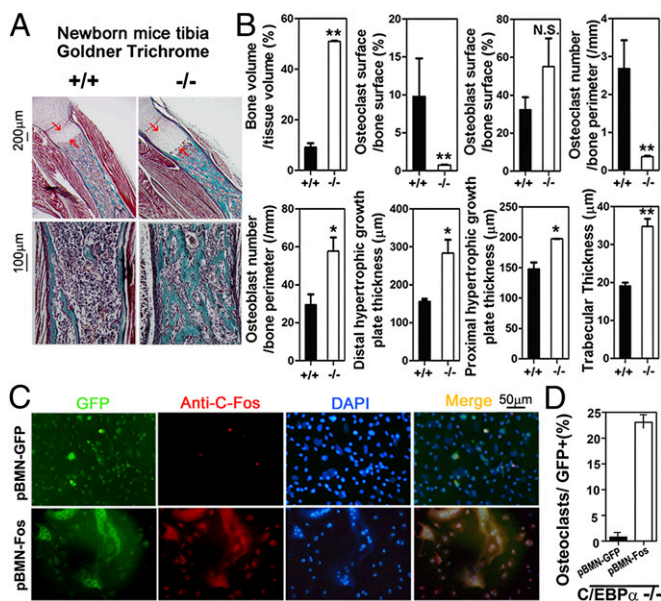


Fig. 5. *C/EBP α* ^{-/-} mice exhibit a dramatic increase in trabecular bone number, and *C/EBP α* ^{-/-} impaired osteoclastogenesis is rescued by ectopic expression of *c-fos*. (A) Compared with *C/EBP α* ^{+/+} controls, *C/EBP α* ^{-/-} tibiae have extended growth plates (arrows) and a dramatic increase in mineralized tissue (bright blue regions), which indicates osteopetrosis ($n = 3$, repeated three times). (B) Histomorphometric analysis of tibiae from *C/EBP α* ^{+/+} and *C/EBP α* ^{-/-} mice. * $P < 0.05$; ** $P < 0.01$. N.S., not significant. (C and D) Impaired OC differentiation in *C/EBP α* ^{-/-} MBM cells cultured with M-CSF/RANKL can be rescued by ectopic expression of *c-fos* using pBMN-*c-fos* compared with control virus (pBMN-GFP). *** $P < 0.001$.

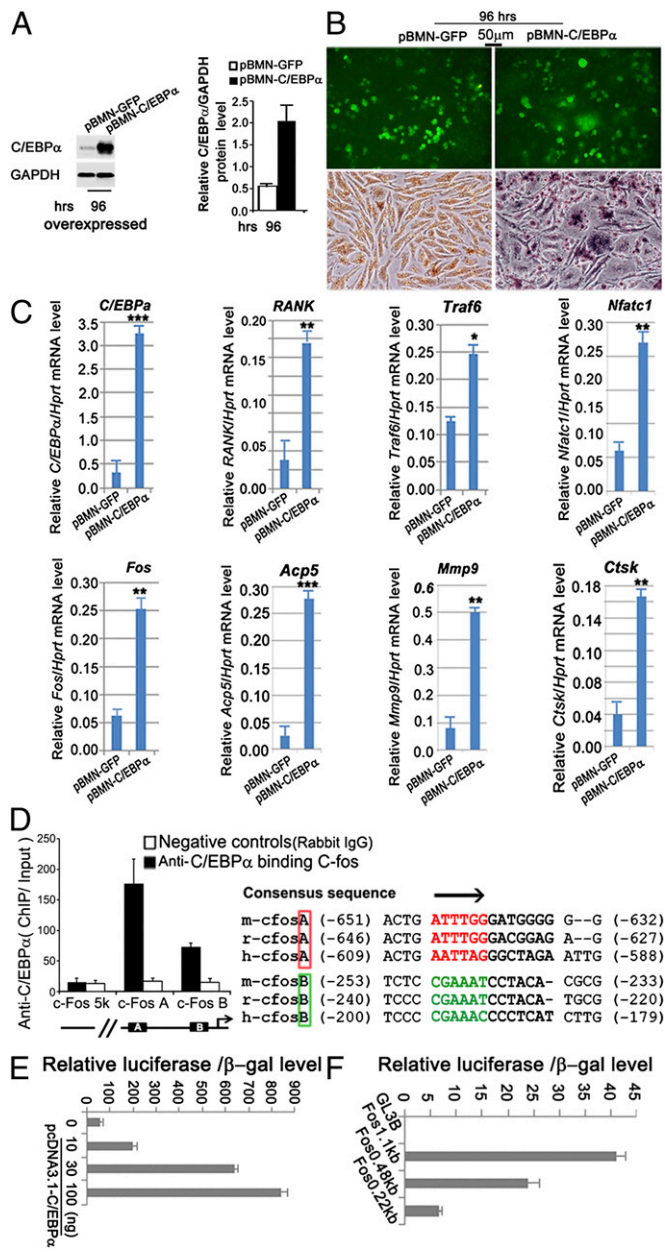


Fig. 6. *C/EBPα* induces osteoclastogenesis in the absence of RANKL and activates the *c-fos* promoter. (A) Western blot and quantification of forced expression of *C/EBPα* in *C/EBPα*^{+/+} MBM stimulated with 20 ng/mL M-CSF for 24 h and then transduced with pBMN-*C/EBPα* for 96 h. Compared with the control group, *C/EBPα* expression increased significantly 96 h after cells were transduced with pBMN-*C/EBPα*. (B) GFP expression and TRAP expression in *C/EBPα*^{+/+} MBM in which *C/EBPα* is overexpressed (pBMN-*C/EBPα*) compared with the control (pBMN-GFP) after 96 h of stimulation with M-CSF (20 ng/mL) alone. (C) qPCR analysis in MBM cultured in M-CSF alone and transduced with pBMN-*C/EBPα* (+) or with the vector control pBMN-GFP for 96 h. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. (D) ChIP analysis of *C/EBPα* binding to the *c-fosA*, *c-fosB*, or upstream promoter region [*c-fos* (-5 Kb) as a negative control] in MBM stimulated with RANKL/M-CSF to generate mature OCs. (E) Consensus sequence alignment of putative *C/EBPα* binding sites of mouse, rat, and human *c-fos* promoter regions. *C/EBPα* activates *c-Fos* expression. (F) Truncated *c-fos* promoters were induced by *C/EBPα*. Luciferase reporter assays with truncated *c-fos* promoters indicated the presence of two *C/EBPα* binding sites (GL3B, control vector).

qPCR analysis of gene expression. Expression of *C/EBPβ* increased slightly when *C/EBPα* was overexpressed in uninduced RAW264.7 cells (Fig. 7D).

Discussion

In this study, we demonstrated that *C/EBPα* expression is essential for OC lineage commitment but that it may not be essential for macrophage differentiation. Importantly, forced expression of *C/EBPα* in MBM and RAW264.7 cells induced the expression of OC marker genes in the absence of RANKL stimulation (Figs. 6 and 7), which demonstrates that high expression of *C/EBPα* is sufficient for OC lineage commitment. This study filled the gap in our understanding of how transcription factor(s) specify OC lineage commitment from monocyte/macrophage cells.

C/EBPα Expression Is Essential for OC Lineage Commitment but May Not Be Essential for Macrophage Differentiation.

We sought insight into the mechanism underlying *C/EBPα*'s role in OC differentiation. Specifically, it is important to clarify if *C/EBPα* expression is important for OC lineage commitment alone or if it is also important for monocyte/macrophage differentiation because OCs are multinucleated cells derived from hematopoietic precursors of the monocyte/macrophage series. Feng et al. (19) discovered that the combined expression of *PU.1* and *C/EBPα* is sufficient to activate a myeloid program in fibroblasts, which are derived from mesenchymal stem cells, and to induce a macrophage-like phenotype. It has also been shown that enforced expression of *C/EBPα* and *C/EBPβ* in B cells leads to their rapid and efficient reprogramming into macrophages (20). Di Tullio et al. (21) recently clarified that this *C/EBPα*-induced transdifferentiation of pre-B cells into macrophages involves no overt retrodifferentiation. These studies indicate that *C/EBPα* is important for monocyte/macrophage differentiation. It has been determined that the loss of *C/EBPα* gene expression in hematopoietic progenitor cells results in a block in differentiation at the common myeloid progenitor and granulocyte/macrophage progenitor stage of development (22, 23), indicating that *C/EBPα* expression is important for the development of macrophage CFUs. There is some controversy regarding the requirement of

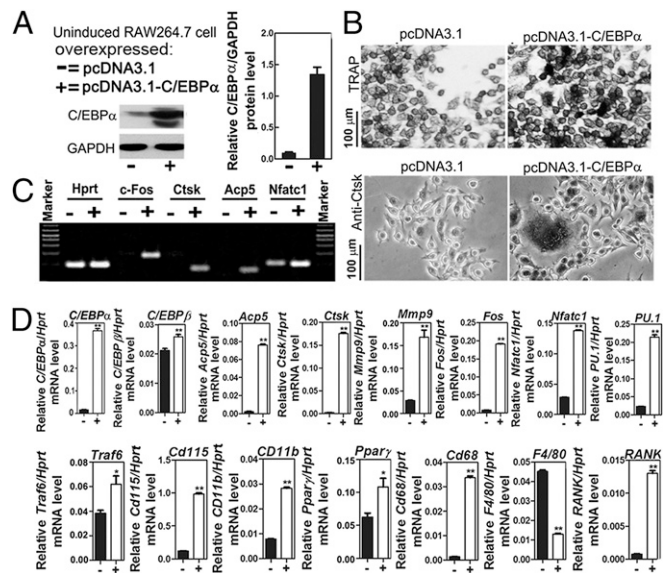


Fig. 7. Ectopic expression of *C/EBPα* reprograms the monocyte/macrophage cell line to OC-like cells in the absence of RANKL. (A) Western blot of *C/EBPα* expression in RAW264.7 cells (not stimulated with M-CSF/RANKL) transfected with pcDNA3.1-*C/EBPα* (+) or with the vector control pcDNA3.1 (-). (B) TRAP and Ctsk immunostaining in uninduced RAW264.7 cells in which *C/EBPα* is overexpressed [pcDNA3.1-*C/EBPα* (+)] compared with the control [pcDNA3.1 (-)]. (C) Semi-qPCR (C) and qPCR (D) analysis (25 cycles, *Hprt* served as a loading control) of genes important for monocytes/macrophages and OCs in uninduced RAW264.7 cells transfected with pcDNA3.1-*C/EBPα* (+) or with the vector control pcDNA3.1 (-). **P* < 0.05; ***P* < 0.01.

C/EBP α for macrophage development (22, 23). Heath et al. (23) demonstrated that there was a defect in macrophage development in mice that received transplants of *C/EBP α ^{-/-}* fetal liver cells and that *C/EBP α ^{-/-}* fetal liver cells had decreased M-CSF receptor (Cd115). In contrast, Zhang et al. (22) determined that circulating blood and hematopoietic organs from *C/EBP α ^{-/-}* mice did not lack monocytes or macrophages and that there was no defect in the M-CSF receptor. Consistent with the study by Zhang et al. (22), our analysis of *C/EBP α ^{-/-}* fetal liver cells, *C/EBP α ^{-/-}* tibiae, and MBM-derived monocyte/macrophage cells indicates that *C/EBP α* is not essential for macrophage differentiation (Figs. S6–S8). These contrasting results are likely due to differences in the experimental systems utilized, such as *C/EBP α ^{-/-}* mice and mice transplanted with *C/EBP α ^{-/-}* fetal liver cells.

***C/EBP α* Is a Major Regulator of OC Lineage Commitment but Is Not Sufficient for Terminal OC Differentiation.** *C-fos* functions downstream of RANKL signaling and plays an essential function in the developmental stage from monocyte/macrophage precursors to mature OCs (5). *C-fos* is also important for the expression of *Nfatc1*, which is a regulator of terminal differentiation of OCs (3). Importantly, forced expression of *C/EBP α* , even in the absence of RANKL stimulation, induced the expression of these key OC transcription factors (e.g., *c-fos*, *Nfatc1*) and *PU.1* (24) (Fig. 6). Our data indicate that *C/EBP α* may directly regulate *c-fos* to promote osteoclastogenesis. We determined that the *c-fos* promoter has two *C/EBP α* binding sites (Fig. 6D) and that *C/EBP α* may activate the *c-fos* promoter through the two *C/EBP α* binding sites (Fig. 6E and F). Notably, our results indicate that *C/EBP α* modulates both OC lineage commitment and terminal OC differentiation. Indeed, *C/EBP α* binding sites are present at both the *c-fos* promoter and the *Ctsk* promoter, the latter of which is a gene expressed by committed OCs. In addition, ectopic expression of *C/EBP α* induces upregulation of both early and late OC markers. Thus, these findings demonstrate that high expression of *C/EBP α* alone is sufficient for OC lineage commitment and that *C/EBP α* also modulates terminal OC differentiation.

Potential Mechanism Underlying *C/EBP α* Is Induced by RANKL. How *C/EBP α* is induced by RANKL is unclear. However, NF- κ B, which is induced by RANKL, regulates *C/EBP α* expression in myeloid cells. It is thus possible that RANKL induces NF- κ B expression, which, in turn, activates *C/EBP α* expression. It was noted that *C/EBP α* overexpression largely increased RANK (RANKL receptor) expression (Figs. 6C and 7D). Thus, another way that *C/EBP α* may be induced by RANKL (ligand) is through a self-amplification pathway. We suspect that *C/EBP α* upregulates the

expression of receptor RANK, which allows for increased binding of RANKL and triggers the cascade of transcription factors specifying OC lineage commitment.

Importantly, our research indicates that a threshold level of *C/EBP α* expression is an important component in the mechanism by which M-CSF acts with RANKL to promote OC differentiation, whereas it promotes macrophage differentiation when it acts alone. Thus, this study provides essential insight into the long-standing question of how RANKL promotes OC differentiation. These findings expand the existing view of the role of *C/EBP α* in bone homeostasis beyond its role in lineage allocation of mesenchymal stem cells and macrophage cells to include a direct and specific role in OC lineage commitment from monocyte/macrophage cells. *C/EBP α* may be an ideal target for treating osteolytic diseases.

Materials and Methods

***Ctsk* Promoter CAT Constructs.** A *Ctsk* pCAT containing a 1,505-bp XbaI/EcoRI fragment (–1,474 to –31, numbered relative to the cap site) of the mouse *Ctsk* 5'-flanking region was used as a starting point for deletion analysis. The sizes of series of 5' deletions generated were approximated by electrophoresis in a 3:1 NuSieve-agarose gel (Lonza) or, in selected cases, by DNA sequence analysis with the dideoxy chain termination method.

Forced Expression. We used a retrovirus system to express *c-fos* ectopically, as described previously (17), in *C/EBP α ^{-/-}* myeloid cells cultured with M-CSF/RANKL to induce osteoclastogenesis. We similarly used a retrovirus system to express *C/EBP α* ectopically in RANKL-induced *C/EBP α ^{+/+}* MBM as described previously (17). *C/EBP α* was also overexpressed in uninduced RAW264.7 cells by stable transfection.

Statistical Analysis and Data Quantification Analysis. Experimental data are reported as mean \pm SD of triplicate independent samples. Data were analyzed with the two-tailed Student's *t* test. *P* values <0.05 were considered significant. Data quantification analyses were performed using the National Institutes of Health ImageJ program as described (12, 17) (**P* < 0.05 and ***P* < 0.01 throughout the paper). Additional details are provided in *SI Materials and Methods*.

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