CCAAT/Enhancer-binding Protein β Promotes Osteoblast Differentiation by Enhancing Runx2 Activity with ATF4

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Although CCAAT/enhancer-binding protein β (C/EBP β) is involved in osteocalcin gene expression in osteoblast in vitro, the physiological importance of and molecular mechanisms governing C/EBP β in bone formation remain to be elucidated. In particular, it remains unclear whether C/EBP β acts as a homodimer or a heterodimer with other proteins during osteoblast differentiation. Here, deletion of the C/EBP β gene from mice resulted in delayed bone formation with concurrent suppression of chondrocyte maturation and osteoblast differentiation. The expression of type X collagen as well as chondrocyte hypertrophy were suppressed in mutant bone, providing new insight into the possible roles of C/EBP β in chondrocyte maturation. In osteoblasts, luciferase reporter, gel shift, DNAP, and ChIP assays demonstrated that C/EBP β heterodimerized with activating transcription factor 4 (ATF4), another basic leucine zipper transcription factor crucial for osteoblast maturation. This complex interacted and transactivated osteocalcin-specific element 1 (OSE1) of the osteocalcin promoter. C/EBP β also enhanced the synergistic effect of ATF4 and Runx2 on osteocalcin promoter transactivation by enhancing their interaction. Thus, our results provide evidence that C/EBP β is a crucial cofactor in the promotion of osteoblast maturation by Runx2 and ATF4.

INTRODUCTION

Two transcription factors, Runx2/Cbfa1 (Ducy *et al.*, 1997; Komori *et al.*, 1997; Otto *et al.*, 1997) and Osterix (Nakashima *et al.*, 2002), are required for osteoblast commitment and differentiation during the process of bone formation. Expression of Runx2 and Osterix sequentially induce expression of the genes encoding the protein constituents of bone, including alkaline phosphatase, type I collagen, bone sialoprotein, and osteocalcin (Aubin *et al.*, 2006). As the order of expression of these matrix proteins is conserved during bone formation, additional transcription factors may cooperate with Runx2 and/or Osterix to regulate differentiation-specific gene expression in osteoblasts.

Mutations in Runx2, which was originally identified as an osteoblast-specific activator of the osteocalcin-specific element 2 (OSE2) within the osteocalcin gene 2 (OG2) promoter (Ducy *et al.*, 1997), cause cleidocranial dysplasia (CCD; Mundlos *et al.*, 1997). The osteocalcin gene is not induced until late-stage osteoblast differentiation despite being a direct target of Runx2, the initiator of differentiation. The

osteocalcin promoter contains a second *cis*-element, osteocalcin-specific element 1 (OSE1), which is active in osteoblasts specifically (Ducy and Karsenty, 1995). Activating transcription factor 4 (ATF4), a member of the cAMP response element-binding protein (CREB)/ATF family of basic leucine zipper (bZIP) transcription factors, was recently identified as a specific activator of OSE1 (Yang *et al.*, 2004). ATF4 is phosphorylated by RSK2, mutations of that cause Coffin-Lowry syndrome (CLS), with its characteristic skeletal disorders. ATF4 indirectly associates with Runx2 (Xiao *et al.*, 2005) to promote the terminal differentiation of osteoblasts via enhancing osteocalcin expression. Thus, ATF4 is a crucial factor promoting osteoblast maturation. In osteoblasts, activation of the osteocalcin promoter by Runx2 and ATF4 is enhanced by direct interactions with SATB2, a nuclear matrix protein, which recruits the two proteins to a multiprotein complex (Dobrev *et al.*, 2006). A recent study demonstrated that general transcription factor IIA γ (TFIIA γ) interacts both Runx2 and ATF4, preventing the degradation of ATF4, which in turn enhances osteocalcin expression (Yu *et al.*, 2008). In contrast, ATF4 activity in osteoblasts can be suppressed by factor-inhibiting ATF4-mediated transcription (FIAT), a leucine zipper protein (Yu *et al.*, 2005). Thus, accumulating evidence suggests that ATF4 activity during osteoblast differentiation is tightly regulated by a variety of nuclear factors interacting with Runx2 and ATF4. It remains

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unclear, however, whether ATF4 binds OSE1 as a homodimer or a heterodimer with other proteins.

Expression of osteocalcin is also regulated by CCAAT/enhancer-binding proteins (C/EBPs), which form a family of bZIP proteins. C/EBP β can form a heterodimer with ATF4 in vitro (Podust *et al.*, 2001). In addition to an indispensable function in adipogenesis (Wu *et al.*, 1995; Tanaka *et al.*, 1997), C/EBP β is expressed in osteoblastic cells and up-regulated during osteoblast differentiation (Bachner *et al.*, 1998; Ogasawara *et al.*, 2001; Gutierrez *et al.*, 2002; Pereira *et al.*, 2002; Hata *et al.*, 2005). C/EBP β promotes expression from the osteocalcin gene promoter via physical interactions with Runx2 (Gutierrez *et al.*, 2002; Hata *et al.*, 2005; Shirakawa *et al.*, 2006). Mice bearing a bone-targeted transgene of liver-enriched inhibitory protein (LIP), a natural isoform of C/EBP β that acts in a dominant-negative manner against C/EBPs, exhibit osteopenia from reduced bone formation; this phenotype is likely secondary to decreased osteocalcin expression in bone (Harrison *et al.*, 2005). To study the role of C/EBP β in osteoblasts, we previously examined osteoblasts from mice deficient in C/EBP homologous protein (CHOP), a natural universal inhibitor of all C/EBP proteins, (Shirakawa *et al.*, 2006). Endogenous CHOP exhibited two functions in vitro, one suppressing the synergism between Runx2 and C/EBP β and the other role promoting BMP-induced bone formation. Skeletal development of CHOP-deficient mice, however, was not grossly affected. Therefore, the physiological role(s) of C/EBP β in bone formation in vivo have remained unclear. To date, no report has documented the bone phenotype of C/EBP β -deficient mice. Furthermore, it is also unclear if C/EBP β heterodimerizes with ATF4 in osteoblasts to modulate the ATF4 function during osteogenesis.

To answer these questions, we characterized osteoblast differentiation in C/EBP β knockout (KO) mice. Bone formation in KO mice was delayed; osteoblast marker genes, such as osteocalcin, exhibited decreased expression both in vivo and in vitro. We determined that C/EBP β heterodimerized with ATF4 at the OSE1 in the osteocalcin promoter to enhance promoter activity. In the presence of C/EBP β , ATF4 could form a complex and synergize with Runx2 to promote osteocalcin expression. In its absence, however, the affinity of ATF4 for OSE1 was diminished in C/EBP β -null osteoblasts. The expression of osteocalcin was dramatically suppressed in C/EBP β -defective osteoblasts treated with a small interfering RNA (siRNA) specific for ATF4. These results suggest that C/EBP β is a crucial DNA-binding partner of ATF4 and mediates the interaction between ATF4 and Runx2 to control osteoblast maturation.

MATERIALS AND METHODS

C/EBP β KO Mice

The generation of C/EBP β -null mice was described previously (Tanaka *et al.*, 1995, 1997). C/EBP β heterozygous mice on the C57BL/6 genetic background were the kind gift of Dr. M. Takiguchi (Chiba University, Chiba, Japan). Genotyping was performed by PCR as described (Bai *et al.*, 2006). Skeletal preparations with alcian blue/alizarin red staining were performed according to a standard protocol. All bone phenotypes were compared between littermates. Animal studies were approved by the Institutional Animal Care and Use Committee of the Japanese Foundation for Cancer Research.

Antibodies

We used an anti-C/EBP β mouse mAb (Santa Cruz Biotechnology, Santa Cruz, CA, clone H-7), an anti-C/EBP β rabbit polyclonal antibody (Santa Cruz, C-19), an anti-ATF4 rabbit polyclonal antibody (Santa Cruz, C-20, H-290), an anti-Runx2 mouse mAb (MBL, Nagoya, Japan, clone 8G5), an anti-FLAG mouse mAb (Sigma, St. Louis, MO, clone M2), an anti-Myc mouse mAb (clone

9E10), an anti-LaminB₁ antibody (Zymed, Carlsbad, CA), and an anti-LaminA/C antibody (BD Biosciences, San Jose, CA).

Histological Analysis

Immunohistochemical staining was performed using a Histomouse Plus Kit (Zymed) according to the manufacturer's protocol. C/EBP β proteins were detected using anti-C/EBP β antibodies (H-7, Santa Cruz). For cell proliferation analysis, pregnant mice were injected once with BrdU (Zymed) 2 h before they were killed, according to the manufacturer's instruction. The incorporated bromodeoxyuridine (BrdU) was detected by BrdU staining Kit (Zymed). Using four embryos per genotype from littermates, four independent sections per embryo were subjected to cell count. RNA in situ hybridization analysis was performed as described (Conlon and Rossant, 1992; Albrecht *et al.*, 1997). Counterstaining was performed by hematoxylin.

Quantitative Real-Time RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using the PrimeScript reverse transcriptase system (TaKaRa, Shiga, Japan). Quantitative real-time RT-PCR was performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) on the ABI Prism 7000 Sequence detection system (Applied Biosystems). The primers used are listed in Supplemental Figure S2A. All samples were measured in duplicate for each experiment. Values were normalized to internal controls of *Hprt1* or *Gapdh*.

Cell Culture, Osteoblast Differentiation, and Transfection

Primary osteoblast isolation and osteoblast differentiation induction were performed as described (Shirakawa *et al.*, 2006). MC3T3-E1 (clone 4) osteoblast cells, obtained from ATCC (Manassas, VA), were cultured under similar conditions as primary calvarial osteoblasts. When specified, cells were treated for 6 h with the proteasome inhibitor MG132 (Peptide Institute, Osaka, Japan) in DMSO at a final concentration of 10 μ M. COS-7 cells, obtained from ATCC, were maintained in DMEM containing 10% FBS, 100 U/ml penicillin G, and 100 μ g/ml streptomycin. Transient DNA transfections were performed using Fugene6 (Roche, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen) reagents according to the manufacturers' instructions.

Plasmid Construction

The plasmid constructs encoding C/EBP β and Runx2 have been described previously (Shirakawa *et al.*, 2006). The expression vector encoding ATF4 was generated by a PCR-based approach using a human ATF4 expression vector (the kind gift of Dr. H. Hayashi, Nagoya City University, Nagoya, Japan) as a template; fragments were then subcloned into pcDEF3-FLAG and pcDEF3-6xMyc. The deletion mutants of C/EBP β were generated by PCR using a wild-type C/EBP β expression vector as a template.

Luciferase Assay

Cells seeded in duplicate in 24-well plates were transiently transfected with the 0.05–0.2 μ g/well osteocalcin reporter constructs and 0.0001 μ g/well pGL4.75hRlucCMV-renilla reporter (Promega, Madison, WI). Luciferase activity was measured using an AutoLumat LB953 luminometer (Berthold Technologies, Bad Wildbad, Germany). The –657 Ocn luc, –657 Ocn OSE1-mut luc, –657 OSE1 + 2-mut luc, and 4xOSE1 wt luc constructs (Ducy and Karsenty, 1995; Xiao *et al.*, 2005) were the kind gifts of Dr. G. Xiao (University of Pittsburgh, Pittsburgh, PA). The –657 Ocn C/EBP-BE-mut luc and –657 Ocn OSE1 + 2+C/EBP-BE-mut luc constructs, which contain a two-base pair substitution mutation in C/EBP-BE (ACGACTGAAC), were generated with a QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX). Osteocalcin reporter activities were normalized to renilla luciferase activity.

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were performed as described (Ebisawa *et al.*, 2001). Samples were analyzed by 7–15% gradient SDS-PAGE. ExactaCruz F (Santa Cruz) was used for immunoprecipitation experiments to detect interactions between endogenous proteins using specific antibodies.

Electrophoretic Mobility Shift Assay

Nuclear extracts were isolated from transfected COS-7 cells using NE-PER (Pierce, Rockford, IL). Electrophoretic mobility shift assay (EMSA) reactions were performed using a LightShift Chemiluminescent EMSA Kit (Pierce). We utilized probes (OSE1^{OG2}) with the following sequences (Ducy and Karsenty, 1995): wild type: 5'-CTCCCCTGCTCTCCTGCTTACATCAGAGAGCACA, and mutant: 5'-CTCCCCTGCTCTTGGAGCATGCATCAGAGAGCACA.

DNA Affinity Precipitation Assays

Whole cell lysates were isolated from transfected COS-7 cells using lysis buffer (1% Igepal CA-630, 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl). Lysates were incubated with biotin-labeled OSE1^{OG2} probe, and proteins

Figure 1. Expression of C/EBP β protein in developing bone and skeletal preparations from C/EBP β KO mice. (A) Immunohistochemical analysis of C/EBP β in the humerus of an E15.5 wild-type (left) and C/EBP β null (right) fetuses. Double arrows indicate the area in which a positive signal was detected. Scale bar, 200 μ m. (B) Alizarin red/alcian blue skeletal preparations of E14.5 ($n = 2$) littermates. (C and D) Comparison of mineralized areas in the forelimb long bones. The bars of the same color in each panel are of identical lengths. (C) Alizarin red/alcian blue skeletal preparations of E15.5 (left, $n: +/+ = 7, -/- = 8$) and E16.5 (right, $n: +/+ = 8, -/- = 9$) littermates. No ossification could be identified in the metacarpal bones of E16.5 C/EBP β -null mice (arrowhead). (D) Skeletal preparations of forelimbs of newborn ($n: +/+ = 12, -/- = 11$) mice. The alizarin red-positive calcified regions were measured in length. Scale bar, 1 mm. (E) Skeletal preparations of skulls of newborn ($n: +/+ = 11, -/- = 10$) mice. The width of fontanelle (between asterisks) was measured. Scale bar, 1 mm. +/+ : wild-type, +/- : heterozygote, -/- : C/EBP β KO. Statistical significance was assessed by the Student's t test. ** $p < 0.01$.

interacted with probe were collected by streptavidin-agarose (Sigma), and then separated by 8.5% SDS-PAGE, followed by immunoblotting.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described (Kaneshiro *et al.*, 2007) with the following modifications. Briefly, Dynabeads protein A (Invitrogen) was used for immunoprecipitation in combination with anti-C/EBP β (C-19) or anti-ATF4 (C-20) antibodies. The primers used for quantitative PCR are listed in Supplemental Figure S2B.

siRNA

A Stealth RNA interference (RNAi) for *Atf4* (target sequence, 5'-UUUC-UAGCUCCUUACACUCGCCAGU) and a control RNAi were obtained from Invitrogen. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen).

Statistical Analysis

Results of measurements of skeletal elements, histological cell layers, cell size in length or width, quantitative RT-PCR, and luciferase assays are expressed as means and SDs. Statistical significance was determined by the Student's t test.

RESULTS

Bone Formation Is Delayed in C/EBP β Null Mice

We analyzed the protein expression of C/EBP β in developing bone tissues of mouse embryos. In the humerus at embryonic day (E15.5), immunohistochemistry with an anti-C/EBP β antibody detected C/EBP β in mature hypertrophic

chondrocytes and osteoblasts within the primary ossification center, but not in bone collars (Figure 1A). The signals for C/EBP β protein were specific because no staining was detected in C/EBP β KO specimen. The signals detected in the upper part of the growth plate (Figure 1A) were non-specific background staining in the chondrocyte matrix. The expression of C/EBP β in mature chondrocytes and osteoblasts suggested the involvement of C/EBP β in endochondral bone formation. To clarify the physiological roles of C/EBP β in vivo, we analyzed skeletons of mice deficient in C/EBP β (C/EBP β KO; Tanaka *et al.*, 1995, 1997). As few (4.76% of offspring) homozygotes on the C57BL/6 genetic background survived until weaning (Bai *et al.*, 2006), we focused on bone formation during the embryonic and neonatal stages. Skeletal preparations at E14.5 revealed that KO embryos showed delayed mineralization in bones of forelimbs and jaws (Figure 1B, arrowheads). At E15.5 and E16.5, KO embryos exhibited foreshortened limbs (Figure 1C). Mineralized areas of the forelimb long bones in KO mice were smaller than those seen in wild-type littermates (Figure 1C). Mineralized areas were absent from the metacarpal bones of KO littermate (Figure 1C, arrowhead). The delayed endochondral bone formation was still evident at postnatal day (P0) (newborn) in long bones of KO mice (Figure 1D and Supplemental Figure S1A). Moreover, the fontanelle of KO mice was significantly wider than controls, suggesting that

membranous bone formation also was delayed (Figure 1E and Supplemental Figure S1B, distance between asterisks). The results from skeletal preparations of C/EBP β -deficient mice in C57BL/6 background suggested that both endochondral and membranous bone formations were retarded by the loss of C/EBP β gene expression.

Maturation of Chondrocyte Is Delayed in C/EBP β KO Embryos

As C/EBP β was expressed in the terminal hypertrophic chondrocytes of developing bones, we hypothesized that delayed bone formation resulted from altered chondrocyte maturation. Alcian blue staining revealed that the heights of zones of epiphysal (E) and columnar (C) proliferating chondrocytes in humeri of E15.5 KO embryos were identical to that seen in wild-type mice (Figure 2A, top panels). However, the zone of hypertrophic (H) chondrocytes in KO bone was significantly shorter in height. Moreover, hypertrophic chondrocytes in C/EBP β -null embryos were obviously smaller in size than those seen in wild-type mice (Figure 2A, bottom panels). We next assessed the number of proliferating cells in columnar proliferating chondrocytes by BrdU incorporation assay (Figure 2B). However, we could not detect any statistically significant differences in the number of BrdU-positive cells (Figure 2B).

To uncover the stage at which chondrocyte differentiation was affected in C/EBP β KO mice, we examined the expression of chondrogenic marker genes by *in situ* hybridization in humeri of E16.5 embryos. Genes expressed by proliferating chondrocytes, *Sox9*, *Fgfr3*, and *Col2a1*, were unaltered in C/EBP β -null bones (Figure 2C); these results are consistent with our observation of comparable cell proliferation in wild-type and KO littermates (Figure 2B). Prehypertrophic chondrocytes in both wild-type and C/EBP β -null mice expressed similar levels of the parathyroid hormone receptor gene (*Ppr*) and Indian hedgehog gene (*Ihh*; Figure 2D). In contrast, type X collagen gene (*Col10a1*), a marker for mature hypertrophic chondrocytes, exhibited markedly reduced gene expression in KO humeri (Figure 2D), with a result consistent with the shortened hypertrophic chondrocyte zone and reduced cell size in KO bone (Figure 1A). The expression of vascular endothelial growth factor gene (*Vegf*), an angiogenic factor serving as another marker for hypertrophic chondrocytes, was unchanged in KO mice (Supplemental Figure S1C). The terminal hypertrophic chondrocytes expressing matrix metalloproteinase 13 gene (*Mmp13*), and chondroclasts expressing *Mmp9*, still located in the center of bone, whereas these cells already scattered into proximal and distal growth plate areas in wild-type littermate, indicating that the formation of primary spongiosa was delayed in mutant bone (Figure 2D). Quantitated RT-PCR analysis demonstrated the decreased expressions of *Col10a1* and *Mmp13* in newborn KO bone, whereas the levels of *Col2a1* and *Ihh* were not affected (Figure 2E). These results suggest that the loss of C/EBP β lead to attenuated chondrocyte maturation at least in part by reducing the expression of *Col10a1*, whereas the recruitment of blood vessels and osteoclasts/chondroclasts was unaffected.

Osteoblast Differentiation Is Delayed in C/EBP β KO Embryos

Next, we investigated the progression of osteoblast differentiation in developing KO humeri from E15.5, because E15.5 is the stage at which ossification begins and is a critical stage in bone development. The mineralization of bone collars was delayed, appearing thin in KO bones upon von Kossa staining at E15.5 (Figure 2A). To investigate the role of

C/EBP β in osteoblast differentiation, we examined the expression profiles of osteoblast marker genes in developing bone by *in situ* hybridization and real-time RT-PCR. We confirmed expression of C/EBP β gene (*Cebpb*) in both terminal hypertrophic chondrocytes and osteoblasts of the primary spongiosa (Figure 3A). No signal could be detected in KO bone. *Runx2* was expressed at high levels in osteoblasts and at low levels in prehypertrophic and hypertrophic chondrocytes in wild-type humeri; wild-type and C/EBP β -null embryos exhibited comparable expression levels of *Runx2* (Figure 3, A and B). The levels of ATF4 gene (*Atf4*) expression were not altered in KO mice (Figure 3B). At E15.5, the expressions of the bone sialoprotein (*Bsp*) and osteopontin (*Opn*) genes were delayed in the primary ossification center in KO bone (Figure 3A), suggesting that the progression of osteoblast differentiation was delayed in C/EBP β -null embryos. At E16.5, the type I collagen gene (*Col1a1*) was down-regulated in the primary spongiosa of mutant mice, whereas *Bsp* and *Opn* were detected at lower levels in both the bone collars and trabecular bone of mutant mice (Figure 3A). Expression levels of *Ppr*, another marker of maturing osteoblasts, were also decreased in spongiosa (Figure 2D). Notably, expression of the osteocalcin gene (*Ocn*), a marker of terminally matured osteoblasts, was barely detectable in primary spongiosa and reduced in bone collars in KO bone (Figure 3, A and B). At birth, bone volume was reduced in C/EBP β -null mice upon von Kossa staining (Figure 3C, top). Indeed, the expressions of *Mepe* and *Phex* genes, the markers for mature osteocytes, were significantly decreased in KO bone, indicating that the terminal differentiation of osteoblasts into osteocytes was delayed (Figure 3C, bottom). These gene expression profiles observed in KO bone suggest that osteoblast differentiation, especially during late maturation, was remarkably delayed in C/EBP β -null mice.

C/EBP β -deficient Osteoblasts Demonstrate Delayed Maturation in Vitro

We then determined if the delayed osteoblast differentiation in KO bones resulted from the delayed maturation of chondrocytes and/or an osteoblast-autonomous event. We studied the cell proliferation of primary calvarial osteoblasts *in vitro* by cell count assay; loss of C/EBP β did not alter cell numbers (Figure 4A). We then monitored *in vitro* bone nodule formation, a hallmark of terminal osteoblast maturation, using von Kossa staining (Figure 4B). Although mineralized bone nodules were barely detectable in 3-wk cultures of KO osteoblasts, wild-type cells produced abundant nodules. We examined several osteoblast marker genes by quantitative RT-PCR to uncover the stage of differentiation suppressed by loss of the C/EBP β gene. Heterozygous osteoblasts served as a control to gain sufficient sample numbers from littermates. *Runx2* and *Atf4* were expressed at normal levels in KO osteoblasts (Figure 4C). Expression of the Osterix gene (*Osx*) and *Bsp* tended to be reduced but not significantly ($p = 0.06$ and 0.07 , respectively) in KO cells (Figure 4, C and D). An early osteoblast differentiation marker gene encoding alkaline phosphatase (*Alp*) was significantly down-regulated in mutant osteoblasts (Figure 4D). Moreover, the expression of *Ocn* was remarkably reduced in KO osteoblasts (Figure 4D). These results indicate that the differentiation and terminal maturation of osteoblasts in knockout mice were significantly delayed in a cell-autonomous manner, whereas the rates of cell proliferation remained normal.

Figure 2. Chondrocyte maturation was delayed in C/EBP β KO mice. (A) The humeri of E15.5 embryos were examined by alcian blue and von Kossa staining. Four embryos per genotype were analyzed. Three histological regions are recognized: E, epiphysis; C, columnar; H, hypertrophic region. Scale bars, 200 μ m. The heights of the different zones were measured. The bottom panels display a higher magnification of hypertrophic chondrocytes. Scale bars, 30 μ m. Four animals per genotype and 10 hypertrophic chondrocytes per section were measured for the cell diameter. (B) Four BrdU-labeled animals per genotype, four sections per animal were subjected to immunohistochemistry for BrdU. The BrdU-positive cells in columnar area were counted. Scale bars, 40 μ m. (C and D) In situ hybridization. Sections of humeri from E16.5 embryos were hybridized with probes specific for *Sox9*, *Fgfr3*, and *Col2a1* (C) and *Ppr*, *Ihh*, *Col10a1*, *Mmp13*, and *Mmp9* (D). Scale bars, 300 μ m. (E) RNA was extracted from femora of newborn mice (n: +/+ = 9, -/- = 5) and subjected to real-time RT-PCR for *Col2a1*, *Ihh*, *Col10a1*, and *Mmp13*. +/+ = wild-type, +/- = heterozygote, -/- = C/EBP β KO. Statistical significance was assessed by the Student's *t* test. * *p* < 0.05, ** *p* < 0.01, ns: not significant.

C/EBP β Targets *OSE1* in the *Ocn* Promoter as a Heterodimer with ATF4

The expression of C/EBP β overlapped with Runx2 in both hypertrophic chondrocytes and osteoblasts of developing bone (Figures 1A and 3A). The bone phenotype of C/EBP β KO mice, which exhibited delayed chondrocyte maturation and osteoblast differentiation, could be a result of decreased Runx2 activity, as both chondrocyte maturation and osteo-

blast differentiation are arrested in Runx2-deficient mice (Komori *et al.*, 1997). We therefore investigated the functional interactions between C/EBP β and Runx2. To study their cooperation in osteoblast differentiation, we analyzed the transcription machinery governing the promoter of osteocalcin, which gene was the most significantly affected in KO bone. DNA-binding sites for Runx2 and ATF4 have previously been identified within the osteocalcin promoter,

Figure 3. Osteoblast differentiation was delayed in C/EBP β KO mice. (A) In situ hybridization of humeri from E15.5 and E16.5 embryos. Sections of embryo humeri were hybridized with probes specific for *Cebpb*, *Runx2*, *Col1a1*, *Bsp*, *Opn*, and *Ocn*. Scale bars, 300 μ m. (B) RNA was extracted from femora of E15.5 embryos (n: +/+ = 5, -/- = 4) and subjected to real-time RT-PCR for *Runx2*, *Atf4*, and *Ocn*. (C) von Kossa staining of humeri and real-time RT-PCR (*Mepe* and *Phex*) of RNA from femora of newborn mice (n: +/- = 9, -/- = 5). Scale bar, 200 μ m. +/+ : wild-type, +/- : heterozygote, -/- : C/EBP β KO. Statistical significance was assessed by the Student's *t* test. ** *p* < 0.01, ns: not significant.

Figure 4. C/EBP β -deficient osteoblast maturation was delayed in vitro. (A) Primary calvarial osteoblasts were seeded at 10,000 cells per well (12-well plates) in triplicate. Cell numbers were counted on days 1, 4, and 7. (B) Bone nodule formation in primary osteoblast cultures was examined by von Kossa staining on day 21. (C and D) Expression of *Runx2*, *Osx*, and *Atf4* (C) and *Alp*, *Bsp*, and *Ocn* (D) was examined by real-time RT-PCR 14 d after induction (n: +/+ = 6, -/- = 2). Statistical significance was assessed by the Student's *t* test. * *p* < 0.05, ns: not significant. +/+ : wild-type, +/- : heterozygote, -/- : C/EBP β KO.

OSE2 (Ducy and Karsenty, 1995; Ducy *et al.*, 1997) and OSE1 (Ducy and Karsenty, 1995; Yang *et al.*, 2004), respectively (Figure 5A). In addition, a C/EBP-binding element (C/EBP-BE) has been identified in both the rat (Gutierrez *et al.*, 2002) and mouse (*Ocn/OG2*; Hata *et al.*, 2005) osteocalcin gene promoters (Figure 5A), although these sequences are not identical. Previous reports have demonstrated that ATF4 forms a complex with Runx2 to increase the activity of the osteocalcin promoter synergistically (Xiao *et al.*, 2005). ATF4 and C/EBP β can also form a stable heterodimer that binds the CRE (Podust *et al.*, 2001; Vallejo *et al.*, 1993), a sequence that is similar to OSE1. OSE1 can be represented as a hybrid of the complete 5'-half of C/EBP-BE and the complete 3'-half of variant CRE (Figure 5B). We hypothesized that C/EBP β may act as a heterodimer with ATF4 to bind the OSE1, independent of the C/EBP-BE sequence, to enhance the synergistic action of ATF4 and Runx2. To test this hypothesis, we examined the binding of C/EBP β to OSE1 in combination with ATF4 by EMSA (Figure 5C, top). In nuclear extracts purified from COS-7 cells expressing ATF4 alone, we observed a single mild band shift (Figure 5C, lane 4) thought to represent ATF4 homodimers complexed with OSE1. C/EBP β expression resulted in a broad band shift (Figure 5C, lane 5), with an intensity comparable to that of the ATF4 band shift. These results suggest that individual

homodimers of ATF4 or C/EBP β bind OSE1 with moderate affinity. In contrast, combined expression of ATF4 and C/EBP β produced a large band shift (Figure 5C, lane 6), which could be completely eliminated by the addition of an unlabeled OSE1 competitor (Figure 5C, lane 7), but was unaffected by a mutated probe (Figure 5C, lane 8). The addition of antibodies specific for ATF4 (Figure 5C; C-20, lane 9; H-290, lane 10) or C/EBP β (Figure 5C; H-7, lane 11; C-19, lane 12) to nuclear extracts supershifted the band seen in the presence of ATF4 and C/EBP β , indicating that this band represented a complex of ATF4, C/EBP β , and OSE1. A similar result was obtained by DNA affinity precipitation (DNAP) assay; ATF4 could bind OSE1 in the presence of C/EBP β (Figure 5C, bottom). We next investigated the recruitment of endogenous C/EBP β to C/EBP-BE and OSE1 of the osteocalcin promoter in MC3T3-E1 osteoblasts by ChIP assay (Figure 5D) using specific antibodies. The PCR products were confirmed to be specific for each element by agarose gel electrophoresis, in which only a single band of proper size was observed (Supplemental Figure S2C). C/EBP β was recruited to C/EBP-BE at fourfold greater levels than those seen for an unrelated 3-kb upstream or a 5.5 kb downstream sequence. The observed signal was 15-fold stronger than that produced by normal rabbit IgG (Figure 5D). As expected from the EMSA results, endogenous

Figure 5.

C/EBP β could be coimmunoprecipitated with OSE1 with an affinity twofold greater than that seen with C/EBP-BE (Figure 5D). These results demonstrate that physiologically endogenous C/EBP β occupies OSE1 in osteoblasts. We next asked if an endogenous heterodimer consisting of C/EBP β and ATF4 exists in osteoblasts by immunoprecipitation (Figure 5E). In MC3T3-E1 osteoblasts, endogenous C/EBP β could be coimmunoprecipitated with endogenous ATF4, as detected by immunoblotting of precipitated proteins. The specificity of the C/EBP β band was confirmed by peptide blocking. The intensities of the C/EBP β and ATF4 bands were enhanced by treatment with the proteasome inhibitor MG132 (Figure 5E), confirming the high turnover rate of these proteins (Yang and Karsenty, 2004). To determine the functionality of C/EBP β /ATF4 heterodimer binding to OSE1, we examined the capacity of the heterodimer to activate OSE1 in osteoblasts. In MC3T3-E1 osteoblasts, ATF4 up-regulated a 4x-tandem OSE1 reporter ~35-fold, whereas C/EBP β alone induced minimal activity (Figure 5F). The combination of ATF4 and C/EBP β , however, activated OSE1 with striking synergism to levels greater than 160-fold over that of controls (Figure 5F). When a mutant OSE1 reporter construct was used, no activation by ATF4 and/or C/EBP β could be observed (data not shown). Given the role of C/EBP β in supporting the interaction of ATF4 with OSE1, we hypothesized that ATF4 recruitment to OSE1 should be decreased in C/EBP β -deficient osteoblasts. By ChIP assay, we detected significantly reduced association of ATF4 with OSE1 in C/EBP β -null osteoblasts (Figure 5G). Collectively, these results suggest that ATF4 heterodimerized with C/EBP β to increase the binding affinity for OSE1, which increased the ability of the heterodimer to transactivate osteocalcin gene expression from OSE1 from that observed for the ATF4 homodimer alone.

Figure 5 (cont). C/EBP β binds OSE1 in the osteocalcin promoter to form a heterodimer with ATF4. (A) The positions of OSE2, C/EBP-BE, and OSE1 in the mouse osteocalcin gene (OG2) promoter are represented schematically. (B) Alignment of the ATF-binding element, C/EBP-binding element, and OSE1^{OG2}. Bold letters indicate mismatches of sequences with that of OSE1^{OG2}. (C) EMSA (top) and DNA affinity precipitation (DNAP) assay (bottom) using the OSE1^{OG2} oligonucleotide probe. COS-7 cells were transfected with either empty vector (ϕ) or vectors encoding FLAG-tagged ATF4 (A4) and/or FLAG-tagged C/EBP β (C β). Competition assays (comp.) utilized the addition of unlabeled wild-type (wt) or mutant (mt) OSE1^{OG2} probes. Antibodies (Ab) used for supershift assays were as follows: 1) anti-ATF4 (C-20), 2) anti-ATF4 (H-290), 3) anti-C/EBP β (H-7), and 4) anti-C/EBP β (C-19) antibodies. TF, transfected plasmids. (D) ChIP was performed from MC3T3-E1 osteoblast cell lysates using anti-C/EBP β (C-19) antibody (C β) or non-specific rabbit IgG (G). Quantitative PCR of C/EBP-BE, OSE1, and sequences -3 kb upstream (-3 kb) and +5.5 kb downstream (+5.5 kb) of the osteocalcin promoter was performed. (E) MC3T3-E1 osteoblasts were cultured in vehicle alone (DMSO, ϕ) or 10 μ M MG132 (MG) 6 h before harvest. Proteins were immunoprecipitated from nuclear extracts using anti-ATF4 (C-20) antibodies, followed by SDS-PAGE and immunoblotting with an anti-C/EBP β (C-19) antibody. Peptide blocking of anti-C/EBP β (C-19) antibody was performed using C/EBP β peptide (C-19) before immunoblotting. LaminB₁ served for loading control. (F) 4x tandem OSE1^{OG2} luc activity was examined in MC3T3-E1 osteoblasts transfected with ATF4 (A4) and/or C/EBP β (C β) plasmids. (G) Cell lysates from wild-type and KO osteoblasts were subjected to ChIP using anti-ATF4 (C-20) antibody (A4) or nonspecific rabbit IgG (G). We performed quantitative PCR for OSE1. g-type: genotype of osteoblasts.

C/EBP β Potentiates the Synergistic Transactivation of the Osteocalcin Promoter by Runx2 and ATF4

Runx2 cooperates with ATF4, as well as with C/EBP β , in the transactivation of the osteocalcin promoter. As C/EBP β and ATF4 exhibit synergistic action on OSE1, we speculated that the C/EBP β /ATF4 heterodimer may be able to interact with Runx2 more efficiently than ATF4 homodimer to produce maximal activity at the osteocalcin promoter. To test this hypothesis and determine the contributions of both the C/EBP-BE and OSE1, we examined the effect of C/EBP β on the osteocalcin gene reporter in the absence or presence of generated mutations in these C/EBP β -responsive elements. In COS-7 cells transfected with a wild-type osteocalcin 657-bp promoter construct, Runx2 augmented the activity from this promoter 2.4-fold, whereas ATF4 exhibited only 1.2-fold induction. Runx2 and ATF4 together displayed a synergistic fourfold induction (Figure 6A). C/EBP β exhibited twofold augmentation of reporter activity when expressed alone, but also demonstrated synergism with Runx2 (22-fold) and ATF4 (5.4-fold; Figure 6A). Surprisingly, the combination of C/EBP β , Runx2, and ATF4 activated the osteocalcin promoter greater than 55-fold over control levels (Figure 6A). When C/EBP-BE was deleted with the upstream OSE2, the synergistic inductions of activity by C/EBP β and Runx2 or ATF4 declined to 12- and 5.4-fold, respectively (Figure 6B). Similar reductions were seen with mutation of the C/EBP-BE alone (Figure 6C). Although the activity produced by the combination of all three proteins decreased to approximately half that seen for the 657-bp wild-type reporter, it remained maximal of all conditions (Figure 6, B and C). The C/EBP β -induced optimization of the interaction between Runx2 and ATF4 was abrogated when OSE1 was mutated, while the synergy between Runx2 and C/EBP β was retained (Figure 6D). These results indicate that maximal activity, induced by combination of Runx2, ATF4, and C/EBP β , was OSE1-dependent; C/EBP β does not require C/EBP-BE to potentiate the synergistic interaction between Runx2 and ATF4. When all elements except C/EBP-BE were mutated, the promoter could no longer be activated by Runx2, ATF4, and C/EBP β (Figure 6E), similar to the results seen in the presence of mutations in all four elements (Figure 6F), suggesting that C/EBP β alone is unable to activate the osteocalcin promoter. These results indicate that C/EBP β activates the osteocalcin promoter in at least two ways, through synergism with Runx2 by interacting at C/EBP-BE and by heterodimerization with ATF4 on OSE1 to produce maximal activity with Runx2.

C/EBP β Facilitates Physical Interaction between Runx2 and ATF4

ATF4 and Runx2 interact indirectly to produce synergistic activation of the osteocalcin promoter (Xiao *et al.*, 2005). As C/EBP β promoted this synergistic activation (Figure 6A) and interacts with ATF4 (Figure 5E), C/EBP β may function as a bridge between Runx2 and ATF4. We therefore confirmed the interaction between endogenous Runx2 and C/EBP β in osteoblasts by immunoprecipitation using specific antibodies (Figure 7A). As both C/EBP β and Runx2 are unstable proteins (Zhao *et al.*, 2003), we used the proteasome inhibitor MG132 to increase cellular levels of both proteins. Immunoprecipitation demonstrated that endogenous C/EBP β formed a complex with endogenous Runx2 in primary calvarial osteoblasts (Figure 7A). We next assessed the interaction between ATF4 and Runx2 in the presence of C/EBP β , using an immunoprecipitation-immunoblot assay (Figure 7B). ATF4 was not coimmunoprecipitated by Runx2

Figure 6.

in the absence of C/EBP β (lane 3). However, the addition of C/EBP β produced the interaction between ATF4 and Runx2 in a dose-dependent manner (lanes 4–6), indicating that C/EBP β serves as a physical bridge between ATF4 and Runx2 to potentiate complex formation. To determine the domain(s) of C/EBP β responsible for the bridging, we generated two truncated mutants of C/EBP β (Figure 7C) and performed immunoprecipitation-immunoblot assays. We confirmed that leucine zipper domain of C/EBP β is essential in interaction with ATF4 (Figure 7C, left). Also, we found that both leucine zipper and basic domains of C/EBP β were important for binding with Runx2 (Figure 7C, right). The band representing Runx2-associated ATF4 in the presence of wild-type C/EBP β (asterisk), was completely eliminated when the C/EBP β deletion mutants were transfected instead of wild-type C/EBP β (Figure 7D). Therefore, these results demonstrate that the leucine zipper domain enables C/EBP β to facilitate the physical interaction between ATF4 and Runx2.

Given the role of C/EBP β in supporting the promotion of osteocalcin expression by ATF4, we hypothesized a genetic interaction between C/EBP β and ATF4. We treated wild-type or C/EBP β -null primary osteoblasts with an *Atf4*-specific siRNA (Figure 7E), which decreased *Atf4* expression in osteoblasts by ~80% without affecting the levels of *Hprt1* and *Gapdh* (Supplemental Figure S3), genes commonly used as normalizing controls. *Atf4* silencing decreased the expression of *Ocn* in wild-type osteoblasts (Figure 7E). Baseline *Ocn* levels were reduced in C/EBP β -deficient cells, which was further suppressed by *Atf4* siRNA treatment (Figure 7E). Together with the results of the *Ocn* reporter and immunoprecipitation assays, it is clear that C/EBP β plays critical roles in complex formation by ATF4 and Runx2 and in ATF4-mediated osteocalcin gene expression.

DISCUSSION

Here, we provide the first description of the bone phenotype of C/EBP β -null fetuses, which demonstrated delayed bone formation. The phenotype was not drastic, which might result from possible compensation by other C/EBPs like C/EBP δ which was reported to up-regulate osteocalcin gene expression by overexpression assays (Gutierrez *et al.*, 2002; Shin *et al.*, 2006). We previously demonstrated, however, that relatively weak expression of C/EBP δ compared with C/EBP β was detected in bone as well as in cultured osteoblastic cells (Shirakawa *et al.*, 2006). Therefore we considered the contribution of endogenous C/EBP δ in osteoblast differentiation not to be a major one, although loss of C/EBP δ may enhance the bone phenotype of C/EBP β KO mice. Unexpectedly, we also observed the delayed maturation of chondrocytes, which was accompanied by decreased expression of *Col10a1*. The height of hypertrophic zone in the growth plate of KO bone was reduced, which we considered

to be due to, at least in part, the reduced cell size of hypertrophic chondrocytes. As we detected C/EBP β expression in hypertrophic chondrocytes, the observed delay in maturation may be a direct effect of C/EBP β . We speculate that C/EBP β and Runx2 cooperate in the induction of *Col10a1*, as the gene is downstream of Runx2 activation (Takeda *et al.*, 2001; Ueta *et al.*, 2001), and Runx2 directly activates *Col10a1* promoter (Zheng *et al.*, 2003). We have no evidence, however, to implicate *Col10a1* promoter to be a direct target of C/EBP β . Therefore, the mechanism has yet to be elucidated.

The expression of the osteoblast markers, *Col1a1*, *Bsp*, *Opn*, and *Ocn* were down-regulated in C/EBP β KO bone, whereas expressions of *Runx2* and *Atf4* were unaffected. In wild-type mice, C/EBP β mRNA and protein were detected in osteoblasts of primary spongiosa, in which *Runx2* and *Atf4* were coexpressed. In Runx2-deficient mice, the expression of all osteoblast marker genes was absent (Komori *et al.*, 1997). Although *Runx2*, *Osx*, and *Col1a1* levels were unchanged in ATF4-deficient mice, *Bsp* and *Ocn* levels were decreased in both bone collars and spongiosa (Yang *et al.*, 2004). In C/EBP β KO mice, *Atf4* was not grossly affected, whereas *Col1a1* was suppressed in primary spongiosa, and *Bsp*, *Opn*, and *Ocn* were repressed throughout all osteoblastic cells (Figure 3A). Although C/EBP β KO mice display lymphoproliferative dysregulation (Screpanti *et al.*, 1995), chondrocyte and osteoblast cell proliferation was not altered in KO bone (Figures 2B and 4A), indicating that the delayed differentiation of bone cells was not due to problems of cell proliferation.

The cooperation of C/EBP β and Runx2 in *Ocn* expression has previously been reported in vitro; C/EBP β physically interacts with Runx2 and binds C/EBP-BE within the *Ocn* promoter (Gutierrez *et al.*, 2002; Hata *et al.*, 2005; Shirakawa *et al.*, 2006). However, an interaction between endogenous Runx2 and C/EBP β has not been described. Here, we demonstrated complex formation by endogenous C/EBP β and Runx2 in osteoblasts (Figure 7A). The C/EBP-BE sequence was important for C/EBP β synergism with Runx2, as shown by deletion and point mutagenesis of C/EBP-BE in the *Ocn* reporter (Figure 6, B and C). Mutations in the *Ocn* promoter, however, were not sufficient to eliminate the synergistic action of C/EBP β and Runx2, suggesting the involvement of another sequence, which we determined to be OSE1. This result differed from that reported by Hata *et al.* (2005), which revealed the abrogation of synergism between Runx2 and C/EBP β upon deletion of C/EBP-BE from the *Ocn* reporter. This difference may stem from the different lengths of promoters used; they used a 1.3-kb promoter, whereas we used a 657-bp construct. Other differences between these studies were in the doses of Runx2 and C/EBP β transfected and the cell types used, although the observed synergistic action was similar.

It has been unclear whether ATF4 acts as a homodimer or a heterodimer on OSE1 when cooperating with Runx2. The colocalization of C/EBP β and ATF4 in osteoblasts and the a similar delay in *Bsp* and *Ocn* expression in C/EBP β -null mice as seen in ATF4 KO mice prompted us to examine the cooperation of C/EBP β and ATF4 during osteoblast maturation. In this report, we revealed that endogenous C/EBP β formed a heterodimer with endogenous ATF4 to bind OSE1 in osteoblasts; both proteins cooperated in the activation of OSE1 (Figure 5). In the absence of C/EBP β , the affinity of ATF4 for the OSE1 was decreased, suggesting that C/EBP β is a crucial adaptor allowing ATF4 to act on OSE1. ATF4 is reported to be monomeric in the absence of the DNA target, but forms low levels of a homodimer in the presence of the DNA target. Even in the absence of the DNA target, how-

Figure 6 (cont). C/EBP β potentiates the functional interactions between Runx2 and ATF4 through OSE1. (A) Synergism between Runx2 and ATF4 for the -657 *Ocn* luciferase reporter was tested in the presence or absence of C/EBP β in COS-7 cells. (B–F) We tested the relative potency of C/EBP-BE and OSE1 as C/EBP β -responsive elements using mutant reporters: (B) the -242-bp *Ocn* luc reporter, which lacked the C/EBP-BE and upstream OSE2, (C) the -657-bp *Ocn* luc reporter with a mutated C/EBP-BE, (D) the -657-bp *Ocn* luc reporter with a mutated OSE1, (E) the -657-bp *Ocn* luc reporter with mutations in both OSE2s and OSE1, and (F) the -657-bp *Ocn* luc reporter with mutations in all of the indicated elements.

Figure 7. C/EBPβ facilitates complex formation between Runx2 and ATF4. (A) Primary calvarial osteoblasts were incubated with MG132. Proteins were immunoprecipitated from nuclear extracts with an anti-C/EBPβ antibody, followed by immunoblotting with either anti-Runx2 or anti-C/EBPβ antibodies. LaminA/C served for loading control. (B) Proteins were immunoprecipitated from lysates of COS-7 cells transfected with the indicated plasmids using anti-FLAG antibodies and then subjected to SDS-PAGE and immunoblotting with anti-Myc or anti-FLAG antibodies. (C) Truncated mutants of C/EBPβ, one lacking leucine zipper domain (ΔLZ) and another lacking both basic and leucine zipper domains (ΔbLZ), were generated. aa, number of amino acids. Interactions between C/EBPβ mutants and ATF4 (left panel) and Runx2 (right panel) were tested by immunoprecipitation assay. (D) The complex formation by ATF4 and Runx2 in the presence of wild-type or truncated mutant C/EBPβ was examined by immunoprecipitation assay. (E) The genetic interaction between C/EBPβ and ATF4 in osteoblasts. We performed *Atf4* gene silencing by transfecting wild-type and C/EBPβ-null osteoblasts with siRNA (Ctr, control siRNA, A4, *Atf4* siRNA). g-type, genotype of osteoblasts. Real-time RT-PCR for *Atf4*, *Ocn*, and *Hprt1* was performed on day 4. +/+ : wild-type, -/- : C/EBPβ KO.

ever, ATF4 forms a stable heterodimer with C/EBP β (Podust *et al.*, 2001). This heterodimer binds to CRE, but not to the C/EBP site, with high affinity. OSE1 has only one base pair mismatch with variant CRE (Figure 5B). Therefore, the heterodimer of C/EBP β and ATF4 should have a stronger affinity for OSE1 than the ATF4 homodimer.

Heterodimerization with C/EBP β also provides another advantage to ATF4 by facilitating cooperation with Runx2. As C/EBP β physically interacts with Runx2 and the interaction between Runx2 and ATF4 is known to be indirect (Xiao *et al.*, 2005), it is likely that another protein(s) facilitates the formation of a complex including ATF4 and Runx2. Two proteins have been reported to interact with both Runx2 and ATF4 to promote *Ocn* expression. SATB2, which interacts with both Runx2 and ATF4, has been suggested to recruit both proteins to a multiprotein complex that enhances transcription from the osteocalcin promoter (Dobrev *et al.*, 2006). SATB2 enhanced both Runx2 activity and the synergistic action of Runx2 and ATF4, but had no effect on ATF4 alone. Recently, general transcription factor TFIIA γ (TFIIA γ) was demonstrated to interact with Runx2 and ATF4; TFIIA γ also enhanced *Ocn* expression (Yu *et al.*, 2008). In contrast to SATB2, TFIIA γ did not promote Runx2 activity, instead enhancing ATF4-mediated activation of the *Ocn* promoter by preventing the proteasomal degradation of ATF4. The involvement of either protein as a bridge between Runx2 and ATF4 has remained elusive, because the effects of a gain or loss of SATB2 or TFIIA γ on the interaction between Runx2 and ATF4 has not been tested. We demonstrate here that a gain of C/EBP β promoted the formation of the Runx2/ATF4 complex without affecting ATF4 protein levels (Figure 7B). C/EBP β enhanced the ability of both Runx2 and ATF4 to activate the *Ocn* promoter individually and promoted the maximal synergistic action of Runx2 and ATF4, an effect that required an intact OSE1 (Figure 6A). These data clearly demonstrate a crucial role for C/EBP β mediating Runx2 and ATF4 cooperation in osteocalcin expression. Combinations of Runx2, ATF4, and C/EBP β , possibly with SATB2 and TFIIA γ , contribute to the physiological regulation of osteocalcin expression in a context-dependent manner.

In conclusion, our examination of the skeletons of C/EBP β -null mice demonstrated a delay in chondrocyte maturation and osteoblast differentiation. We identified C/EBP β as a bridging protein mediating interactions between Runx2 and ATF4, which lead to maximal synergy between the two transcription factors on the osteocalcin promoter. C/EBP β and ATF4 generated a heterodimer with a higher affinity for and more potent transcriptional activity at OSE1 than that seen for each homodimer. Thus, C/EBP β is a key partner of ATF4 in binding to the osteocalcin promoter and forming an active transcription factor complex with Runx2.

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