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C/EBP Homologous Protein Is Necessary for Normal Osteoblastic Function

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Abstract C/EBP homologous protein (CHOP) suppresses adipogenesis and accelerates osteoblastogenesis in vitro. However, the effects of CHOP in the skeleton in vivo are not known. To investigate the actions of CHOP on bone remodeling, we examined the skeletal phenotype of *chop* null mice from 1 to 12 months of age. *Chop* null mice appeared normal and their growth and serum insulin like growth factor (IGF) I and osteocalcin levels were normal. X-ray analysis of the skeleton revealed no abnormalities and bone mineral density was normal. Static and dynamic histomorphometry revealed that *chop* null mice had decreased bone formation rates, without changes in osteoblast cell number, indicating an osteoblastic functional defect. The number of osteoblasts and osteoclasts and eroded surface were normal. Northern blot analysis revealed decreased type I collagen and osteocalcin mRNA levels in calvariae of *chop* null mice. In conclusion, *chop* null mice exhibit decreased bone formation and impaired osteoblastic function, indicating that CHOP is necessary for the normal expression of the osteoblastic phenotype. J. Cell. Biochem. 97: 633–640, 2006.

Key words: osteoblasts; bone formation; CCAAT enhancer binding proteins; transcriptional factors

Bone marrow stroma contain pluripotent cells with the potential to differentiate into various cells of the mesenchymal lineage including osteoblasts and adipocytes [Bianco and Robey, 2000]. The ultimate cellular phenotype depends on extracellular and intracellular signals. CCAAT-enhancer binding proteins (C/EBP) are a family of transcription factors that regulate cell differentiation [Hanson, 1998; Yamanaka et al., 1998; Ramji and Foka, 2002]. C/EBP proteins contain a highly conserved DNA-binding domain and a leucine zipper dimerization domain, and can form homo- and hetero-dimers that bind to specific sequence motifs. C/EBPs are expressed in multiple cell types, including osteoblasts and adipocytes, and play critical roles in adipocyte differentiation [Lee et al., 1997; Tanaka et al., 1997; Darlington et al., 1998]. C/EBPs also regulate IGF I transcription, and by interacting with runt related protein (Runx)-2, C/EBP β and δ activate osteocalcin transcription [Delany et al., 2001; Gutierrez et al., 2002].

C/EBP homologous protein (CHOP), also termed C/EBPζ, growth arrest and DNA damage-inducible gene (*GADD*)153, or DNA damage inducible transcript (DDIT3), is a member of the C/EBP family of factors and has unique roles in cell differentiation and apoptosis, and in the response to endoplasmic reticulum (ER) stress [Park et al., 1992; Ron and Habener, 1992; Barone et al., 1994; Batchvarova et al., 1995]. CHOP heterodimerizes with other C/EBPs, but the presence of two proline residues in the DNA-binding region disrupts its helical structure and prevents dimer binding to classic C/EBP consensus DNA sequences. A function of CHOP is to act as a transdominant negative inhibitor of classic C/EBPs [Ron and Habener, 1992]. However, CHOP-C/EBP

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dimers bind DNA and activate gene expression, and CHOP target genes have been defined by expression profiling [Ubeda et al., 1996; Wang et al., 1998; Marciniak et al., 2004]. CHOP also has the potential to interact with members of the cAMP responsive element binding protein (CREB)/activating transcription factor (ATF) family and with the Fos/Jun family of nuclear factors [Ubeda et al., 1999; Thiel et al., 2005]. As such, CHOP can modulate the transcriptional activity of genes regulated by factors other than C/EBPs.

In ST-2 murine stromal cells, the levels of CHOP transcripts rise as the cells differentiate toward osteoblasts, and we demonstrated that overexpression of CHOP accelerates osteoblastic cell differentiation and suppresses adipogenesis [Pereira et al., 2002, 2004]. The effect on osteoblastogenesis appeared to be related to an enhancement of bone morphogenetic protein (BMP) activity and signaling. There are additional observations indicating a role for CHOP in the skeleton, and overexpression of the CHOP variant, FUS/TL3-CHOP, causes scoliosis [Perez-Losada et al., 2000]. *Pancreatic endoplasmic reticulum kinase* (*perk*) null mice and humans with *perk* gene mutations develop diabetes and osteoporosis due to increased β cell and osteoblast sensitivity to cellular stress [Harding et al., 2001; Zhang et al., 2002]. PERK induces ATF-4 translation and, as a consequence, CHOP expression [Harding et al., 2001]. ATF-4 is required for normal osteoblast function, and *atf4* null mice exhibit osteopenia [Yang et al., 2004]. Whereas these observations indicate that PERK, ATF-4, and CHOP are novel regulators of skeletal metabolism, the true impact of CHOP on bone physiology is not known.

The purpose of this study was to assess the effects of CHOP on bone remodeling *in vivo*, by examining the skeletal phenotype of *chop* null mice.

MATERIALS AND METHODS

Chop Null Mice

Chop null mice were created by homologous recombination, resulting in the replacement of virtually the entire *chop*-coding region with a LacZ cassette [Marciniak et al., 2004]. The targeted mutation was created in a 129 SVJ/CD1 genetic background and propagated in a 129 SVJ/C57BL/6 background. *Chop* null mice were backcrossed for five generations into the C57BL/6 strain and were compared to wild type mice of the same age, sex, and genetic background, created from former littermate wild type mice of identical genetic background. *Chop* null mice were identified by polymerase chain reaction analysis of tail DNA. For this purpose, a common ATGCCCTTACCTATCGTG-primer, a AACGCCAGGGTTTTCCCAGTCA-primer for the identification of the Lac Z mutant allele, and a GCAGGGTCAAGAGTAGTG primer for the identification of the wild type allele were used to generate a 289 and a 545 base pair product, respectively. The in vivo data presented are from male *chop* null and controls, but there were no substantial differences in the skeletal phenotype of male and female *chop* null mice. All animal experiments were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

Serology

Serum was collected by intracardiac puncture at the time of sacrifice, and immunoreactive osteocalcin levels were measured by radioimmunoassay using a commercial kit, in accordance with manufacturer's instructions (Biomedical Technologies, Stoughton, MA). Serum insulin like growth factor (IGF) I levels were determined by radioimmunoassay using a commercial kit, following extraction of IGF binding proteins using a C18 Sep-Pak column, in accordance with manufacturer's instructions (Nichols Institute Diagnostics, San Clemente, CA).

X-Ray Analysis and Bone Mineral Density

Radiography was performed on mice anesthetized with tribromoethanol (Sigma Chemical Co., St. Louis, MO) on a Faxitron X-ray system (model MX 20, Faxitron X-ray Corp., Wheeling, IL). The X-rays were performed at an intensity of 30 kilowatts for 20 s. Total, vertebral and femoral bone mineral content (BMC) (g), skeletal area (cm^2), and bone mineral density (BMD; gm/cm^2) were measured on anesthetized mice using the PIXImus small animal DEXA system (GE Medical Systems/LUNAR, Madison, WI) [Nagy et al., 2001]. Calibrations were performed with a phantom of a defined value, and quality assurance measurements were performed prior to each use. The coefficient of variation for total BMD is $<1\%$ ($n = 9$ mice).

Bone Histomorphometric Analysis

Static and dynamic histomorphometry was carried out on *chop* null mice and wild type controls at 1, 3, 5, and 12 months of age. Mice were injected with calcein, 20 mg/kg, and demeclocycline, 30 mg/kg, at an interval of 2 days for 1 month old and of 5–7 days for 3- to 12-month-old mice, and sacrificed by CO_2 asphyxiation 2 days after the demeclocycline injection. Femurs were dissected, fixed in 70% ethanol, dehydrated and embedded undecalcified in methyl methacrylate. Longitudinal sections, 5 μm thick, were cut on a Microm microtome (Microm, Richard-Allan Scientific, Kalamazoo, MI) and stained with 0.1% toluidine blue, pH 6.4. Static parameters of bone formation and resorption were measured in a defined area between 181 and 725 μm from the growth plate, using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA) [Devlin et al., 2002]. For dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured in unstained 10 μm sections under ultraviolet light, using a B-2A set long pass filter consisting of an excitation filter ranging from 450 to 490 nm, a barrier filter at 515 nm, and a dichromatic mirror at 500 nm. Bone formation rate (BFR) was calculated. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [Parfitt et al., 1987].

Northern Blot Analysis

Total RNA was isolated from calvariae using the Trizol reagent solution as per manufacturer's instructions (Invitrogen, Carlsbad, CA). The RNA was resolved by electrophoresis on a formaldehyde agarose gel following denaturation, blotted onto GeneScreen Plus charged nylon and hybridized with a 1.6 kb rat type I collagen cDNA (B. Kream, Farmington, CT), a 0.5 kb murine osteocalcin cDNA, and a ribosomal 18S RNA cDNA, both from the American Type Culture Collection (ATCC, Manassas, VA), as described [Genovese et al., 1984; Lian et al., 1989]. DNAs were labeled with Ready-To-Go DNA Labeling Beads (-dCTP) Kit (Amersham Pharmacia Biotech, Piscataway, NJ), in accordance with manufacturer's instructions. Hybridizations were carried out at 42°C for 16–72 h, followed by two post-hybridization washes at room temperature for 15 min in 1× saline sodium citrate (SSC), and a wash at 65°C for 20–30 min at 0.1× to 1× SSC. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film, employing Cronex Lightning Plus or Biomax MS intensifying screens. Northern analysis are representative of five samples.

Statistical Analysis

Results are expressed as means ± SEM. Statistical significance was determined by *t* test.

RESULTS

Chop null mice appeared normal, and their weight and serum IGF I and osteocalcin levels were not different from wild type controls (Fig. 1). Contact radiography of *chop* null mice revealed no obvious abnormalities or fractures at 1–12 months of age, and in accordance with

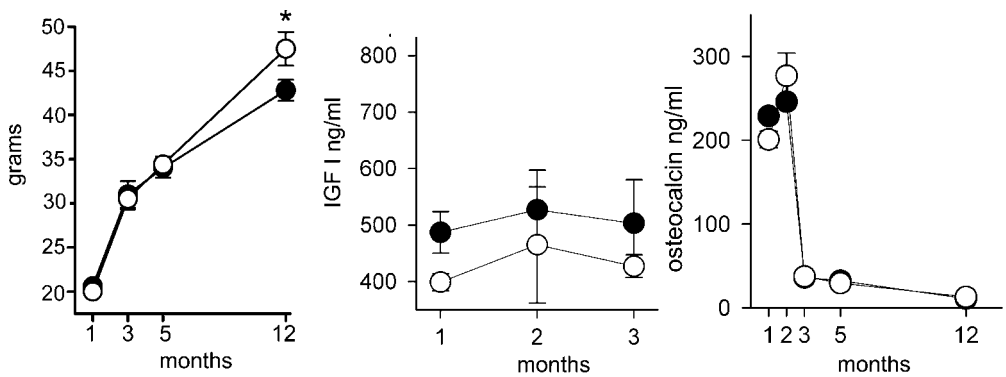

Fig. 1. Total body weight, serum osteocalcin, and serum IGF I in male *chop* null (black circles) mice and wild type (WT; white circles) controls from 1 to 12 months of age. IGF I was measured in 1- and 3-month-old mice. Data points represent means, lines SEM; n = 5 to 9. White dots only represent overlapping WT and *chop* null data. *Significantly different from control, $P < 0.05$.

Fig. 2. Representative radiograph of a 3-month-old *chop* null mouse and wild type (WT) control.

the X-ray findings, *chop* null mice had normal BMC and BMD when compared to wild type controls (Fig. 2 and Table I). Total (Table I) as well as femoral and vertebral BMC and BMD (not shown) were not significantly different between *chop* null and wild type mice.

Bone histomorphometric analysis of femurs from *chop* null mice revealed a consistent 50% to 70% decrease in bone formation rate and a consistent 40% to 60% decrease in mineralizing surface (Fig. 3). The number of osteoblasts was not decreased, indicating that the suppressed bone formation rate was secondary to impaired osteoblastic function. Unlike bone formation rate, trabecular bone volume was not altered consistently, and a 25% decrease was observed at 3 months ($P < 0.05$) and 12 months ($P < 0.055$) of age (Figs. 4 and 5). Osteoclast number was not changed, and eroded surface was not consistently increased in *chop* null mice, therefore showing no evidence of altered bone resorption. The skeletal phenotype was not substantially different between male

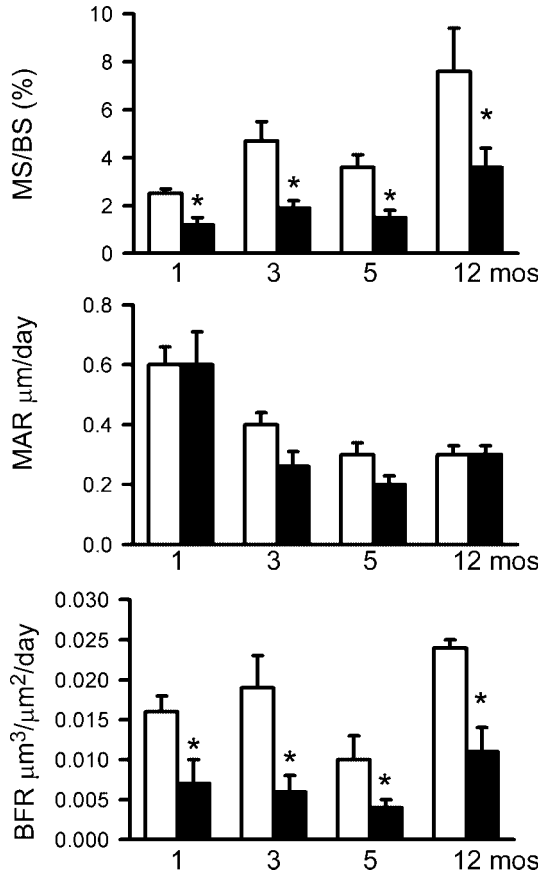

Fig. 3. Femoral trabecular bone dynamic histomorphometry in 1-, 3-, 5-, and 12-month-old male *chop* null (black bars) mice and wild type (WT; white bars) controls. Unstained sections were analyzed by fluorescence microscopy to determine mineralizing surface per bone surface (MS/BS%), mineral apposition rate (MAR/μm/day), and calculate bone formation rate (BFR/$\mu m^3/\mu m^2$/day). Values are means ± SEM (n = 5–11). *Significantly different from wild type controls, $P < 0.05$.

(Figs. 3–5) and female (not shown) *chop* null mice, except for variations in the temporal expression of the trabecular bone volume phenotype.

To confirm the impairment in osteoblastic function, osteoblastic gene marker expression

TABLE I. Total Bone Mineral Content (BMC, g) Skeletal Area (cm^2), and Bone Mineral Density (BMD, g/cm^2) Were Obtained From 1-, 3-, 5-, and 12-Month-Old Male *Chop* Null Mice and Wild Type (WT) Controls

	BMC (g)		Skeletal area (cm^2)		BMD (g/cm^2)	
Months	WT	$Chop^{-/-}$	WT	$Chop^{-/-}$	WT	$Chop^{-/-}$
1	0.250 ± 0.002	0.258 ± 0.009	6.42 ± 0.052	6.34 ± 0.134	0.0390 ± 0.001	0.0407 ± 0.001
3	0.454 ± 0.017	0.421 ± 0.007	8.82 ± 0.166	8.19 ± 0.176	0.0507 ± 0.001	0.0507 ± 0.001
5	0.380 ± 0.015	0.392 ± 0.007	7.65 ± 0.135	7.80 ± 0.116	0.0496 ± 0.001	0.0503 ± 0.001
12	0.460 ± 0.019	0.413 ± 0.017	9.12 ± 0.332	8.53 ± 0.263	0.050 ± 0.001	0.048 ± 0.001

Values are means ± SEM (n = 4–8).

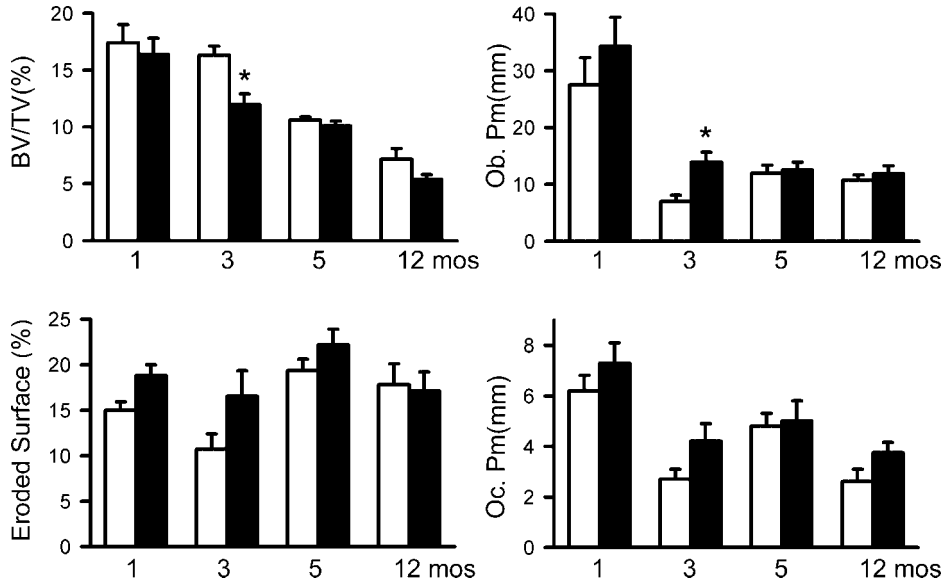

Fig. 4. Femoral trabecular bone static histomorphometry in 1-, 3-, 5-, and 12-month-old male *chop* null (black bars) mice and wild type (WT; white bars) controls. Sections were stained with toluidine blue, and trabecular bone volume/total volume (BV/TV), number of osteoblasts (Ob.Pm) and osteoclasts (Oc.Pm) per perimeter (mm), and eroded surface per bone surface (%) were determined. Values are means ± SEM (n = 5–11). *Significantly different from wild type controls, $P < 0.05$.

was determined in calvariae from *chop* null mice and wild type controls. *Chop* null mice exhibited decreased osteocalcin and type I collagen mRNA levels in calvarial extracts (Fig. 6).

DISCUSSION

The present studies demonstrate that *chop* null mice have impaired osteoblastic cell function. This is manifested by a consistent decrease in bone formation rates observed from 1 to 12 months of age and decreased expression of osteoblastic gene markers in skeletal tissue. The decreased bone formation observed by dynamic bone histomorphometry was mostly secondary to a decrease in mineralizing surface per bone surface, indicating that the areas of active bone formation were reduced. The consequence of the impaired bone formation was a decrease in trabecular bone volume, observed at 3 months, although the effect was not sustained. One-year-old *chop* null male mice exhibited a nearly significant decrease in trabecular bone volume. The reason for the modest trabecular bone volume phenotype observed is not clear. However, the findings would suggest either low levels of basal expression of CHOP in skeletal cells, or a modest impact of CHOP on bone cell function so that the null mutation is sufficiently compensated by alternate signals necessary for the maintenance of skeletal homeostasis [Margolis et al., 1996; Canalis et al., 2003].

Perk and *atf-4* null mice exhibit decreased bone formation and osteopenia [Zhang et al., 2002; Yang et al., 2004]. Although PERK and ATF-4 can induce CHOP expression, the skeletal phenotype of *perk* and *atf-4* null mice is more pronounced, arguing for direct, and not CHOP-mediated, effects of PERK and ATF-4 on skeletal homeostasis. Although CHOP deficiency might contribute to the phenotype, the present studies would suggest that the contribution would be partial, and not explain the *perk* and *atf-4* null phenotype in its entirety. Recent observations have confirmed the role of ATF-4 on skeletal function, and mice overexpressing factor inhibiting ATF-4-mediated transcription (FIAT) in the bone environment exhibit osteopenia because of dimerization of FIAT with ATF-4 [Yu et al., 2005]. Consequently, the PERK/ATF-4/CHOP axis may play a significant role in osteoblastic function and skeletal homeostasis.

CHOP's target genes include *GADD34*, a phosphatase that dephosphorylates translation initiation factor 2a and thereby promotes recovery of protein synthesis during the ER

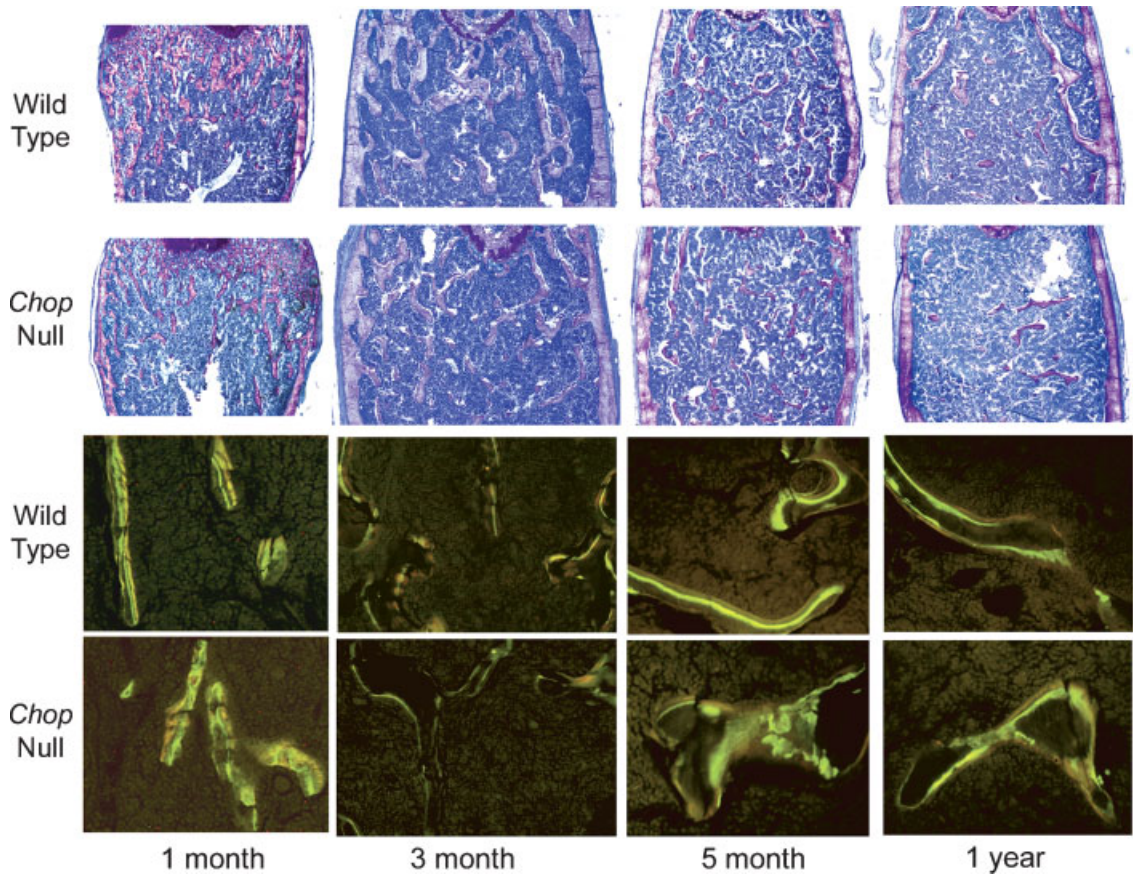

Fig. 5. Representative bone histomorphometry in 1-, 3-, 5-, and 12-month-old *chop* null and wild type (WT) controls. **Upper panels** show sections stained with toluidine blue at a final magnification of 40×. **Lower panels** show unstained sections from *chop* null and WT mice following sequential injections of calcein and demeclocycline, which were visualized by fluorescence microscopy at a final magnification of 100× for 1 and 3 month(s) old, and 200× for 5- and 12-month-old mice. Note clear sharp labels in wild type and the absence of two labels and presence of diffuse bands in *chop* null mice.

stress response and ERO1a, a protein involved in SS bond formation in the ER [Marciniak et al., 2004]. Thus, biosynthesis of bone matrix proteins could be impaired in CHOP null osteoblasts, compounding the observed defect in collagen I gene expression.

The current studies do not address the mechanism of CHOP action on osteoblastic differentiation. In vitro overexpression of CHOP accelerates osteoblastogenesis and enhances BMP-2 signaling and the transactivation of BMP-2/Signaling mothers against decapentaplegic (Smad) dependent constructs [Pereira et al., 2004]. But, the mechanisms involved have not been elucidated completely, and overexpression of CHOP in vivo may or may not result in enhanced osteoblastogenesis. Interactions between C/EBPs and CREB/ATF-4 family of transcription factors have been described and are operational in osteoblasts and may explain

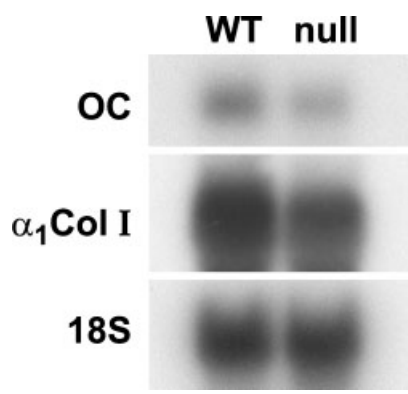

Fig. 6. Expression of osteocalcin (OC) and type I collagen (α_1ColI) in calvariae from 3-month-old *chop* null and wild type (WT) mice. Total RNA was extracted, subjected to Northern blot analysis, and hybridized sequentially with [α^{32}P]-labeled osteocalcin, type I collagen and 18S cDNAs. Northern blots are representative of five calvariae.

some of the stimulatory effects of CHOP overexpression on osteoblastic activity in vitro (18 and Pereira et al., unpublished observations). Activation of G protein associated receptors and cyclic AMP signaling play a central role in the differentiated function of the osteoblast and in the expression of IGF I [Canalis et al., 1989; Gazzerro et al., 2005]. Therefore, it is not surprising that the CREB/ATF-4 family of nuclear factors plays a role in osteoblastic function, and possibly in osteoblastogenesis. It is of interest that Wnt, a critical regulator of osteoblastic differentiation, can activate adenylyl cyclase signaling via protein kinase A and its target CREB during myogenesis [Westendorf et al., 2004; Chen et al., 2005]. It is not known whether Wnt or other factors can use this signaling pathway during osteoblastogenesis.

In conclusion, the CHOP null state results in decreased bone formation and osteoblastic activity, indicating that CHOP is necessary for selected aspects of osteoblastic function.

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