Osteocalcin (OCN) is an osteoblast-derived hormone that increases energy expenditure, insulin sensitivity, insulin secretion, and glucose tolerance. The cDNA sequence of OCN predicts that, like many other peptide hormones, OCN is first synthesized as a prohormone (pro-OCN). The importance of pro-OCN maturation in regulating OCN and the identity of the endopeptidase responsible for pro-OCN cleavage in osteoblasts are still unknown. Here, we show that the proprotein convertase furin is responsible for pro-OCN maturation in vitro and in vivo. Using pharmacological and genetic experiments, we also determined that furin-mediated pro-OCN cleavage occurred independently of its γ-carboxylation, a posttranslational modification that is known to hamper OCN endocrine action. However, because pro-OCN is not efficiently decarboxylated and activated during bone resorption, inactivation of furin in osteoblasts in mice resulted in decreased circulating levels of undercarboxylated OCN, impaired glucose tolerance, and reduced energy expenditure. Furthermore, we show that Furin deletion in osteoblasts reduced appetite, a function not modulated by OCN, thus suggesting that osteoblasts may secrete additional hormones that regulate different aspects of energy metabolism. Accordingly, the metabolic defects of the mice lacking furin in osteoblasts became more apparent under pair-feeding conditions. These findings identify furin as an important regulator of bone endocrine function.
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Introduction

It has been recently established that bone acts as an endocrine organ that secretes at least 3 hormones: fibroblast growth factor 23 (FGF23), lipocalin 2 (LCN2), and osteocalcin (OCN). Bone-forming osteoblasts are the cells that produce OCN, a hormone implicated, among other functions, in the regulation of glucose and energy metabolism (1). Accordingly, when fed a normal diet, Ocn-deficient mice (Ocn–/–) exhibit reduced glucose tolerance, insulin sensitivity, and circulating insulin levels, as well as decreased energy expenditure and increased fat mass (2). Mechanistic studies conducted in cell cultures and mice showed that OCN improves glucose handling by promoting insulin secretion from β cells (3, 4), by favoring glucose uptake in myofibers (5), and by increasing energy expenditure (6). Studies in mice demonstrated that OCN function in β cells and myofibers is mediated through its binding to the G protein–coupled receptor family C group 6 member A (GPRC6A) (3, 5). This pathway appears to be conserved in humans, since human OCN can bind and activate human GPRC6A (7), while mutations or polymorphisms in human GPRC6A are associated with insulin resistance (8, 9).

OCN is a small protein (46 aa in mice and 49 aa in humans) that is uniquely produced by osteoblasts and γ-carboxylated on 3 glutamic acid residues (Glu) before it is secreted. γ-Carboxylation of OCN occurs in the ER and is mediated by γ-glutamyl carboxylase (GGCX), which requires reduced vitamin K as an essential cofactor (10). This posttranslational modification increases the affinity of OCN for hydroxyapatite, the mineral component of bone extracellular matrix (ECM). Hence, the vast majority of OCN secreted by osteoblasts is trapped in bone ECM, in which it constitutes the most abundant noncollagenous polypeptide. Although both γ-carboxylated (Gla) and undercarboxylated (ucOCN) forms of OCN are detected in serum, most in vitro and in vivo studies concur to indicate that the endocrine function of OCN is fulfilled by ucOCN in mice and humans (3, 4, 11). This has been recently confirmed genetically through the inactivation of Ggcx specifically in osteoblasts, which resulted in increased circulating levels of ucOCN and improved glucose tolerance in mice (12). Additional studies showed that osteoclasts, the bone-resorbing cells, are responsible for the partial decarboxylation and activation of OCN present in bone ECM (13, 14). Altogether, these observations suggested a model in which hormonally inactive γ-carboxylated OCN is produced by osteoblasts and stored in the bone ECM, before being activated and released as ucOCN by osteoclasts during the process of bone resorption.

As is the case for many other peptide hormones, the sequence of Ocn cDNA predicts that it is first synthesized as a pre-prohormone composed of a signal peptide, a propeptide, and a mature hormone (Figure 1A). The propeptide of other γ-carboxylated proteins such as prothrombin and profactor IX are characterized by a recognition signal for the γ-glutamyl carboxylase (15). However, the importance of the propeptide for the γ-carboxylation and secretion of OCN has never been addressed in vivo. Moreover, it is currently unknown whether removal of the OCN propeptide depends on the γ-carbox-
other proteins, either activating or inactivating them, and have been implicated in the regulation of a myriad of biological processes (18). In particular, the PCs of the subtilisin/kexin type include proprotein convertase 1 (PC1), proprotein convertase 2 (PC2), furin, proprotein convertase 4 (PC4), proprotein convertase 5A and B (PC5A and PC5B), paired basic aa–cleaving enzyme 4 (PACE4), and proprotein convertase 7 (PC7) and play critical functions in several endocrine cells by regulating the maturation and secretion of numerous peptide hormones such as insulin, glucagon, adrenocorticotropic hormone (ACTH), glucagon-like peptide 1 (GLP-1), and parathyroid hormone (PTH) (18, 19).

Here, we identified furin, using cell-based and genetic arguments, as the endopeptidase responsible for pro-OCN processing in osteoblasts. We also showed in cell culture and in vivo that γ-carboxylation and processing of OCN are 2 independent processes in osteoblasts. The study of mice lacking furin specifically in osteoblasts revealed that proteolysis of pro-OCN is critical for the activation of this hormone. We also found that furin may modulate energy metabolism through OCN-independent pathway(s) affecting appetite.

### Results

**Pro-OCN is cleaved by an intracellular PC.** The putative mouse OCN propeptide exhibits a C-terminal motif consisting of 3 basic residues: Arg-Leu-Arg-Arg (RLRR), which is highly conserved throughout bony vertebrates (Figure 1A). Because this type of basic residue–containing motif represents a consensus recognition site for PCs that is found in many secreted proteins (18), we hypothesized that pro-OCN may be cleaved by 1 or more PCs in osteoblasts.

In support of this hypothesis, mutation of the RLRR sequence into a colon indicates a strongly conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indicates moderate conservation; a single asterisk indicates a fully conserved residue; a period indica...
results, we next tested the capacity of furin, PC5A, PC7, and PACE4 to cleave pro-OCN in vitro. Recombinant GST–pro-OCN protein produced in bacteria was incubated with the conditioned media of HEK293 cells transfected with either an empty vector or a vector expressing the soluble extracellular enzymatic domains of furin, PC5A, or PC7, or with recombinant soluble PACE4 expressed and purified from insect cells (20, 21). The PC activity of each conditioned media or recombinant PC was measured using an artificial tetrapeptide substrate prior to the assay to ensure that an equal number of enzymatic units was used (see Methods and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI93437DS1). Furin, PC5A, and PACE4, but not PC7, were able to cleave GST–pro-OCN, releasing mature OCN (Figure 2B). We noticed, however, that furin was reproducibly more efficient than were PC5A and PACE4 at cleaving pro-OCN when the incubation was performed for a short period, i.e., 15 minutes (Figure 2B). In addition, further testing revealed that furin could cleave more than 80% of pro-OCN within 30 minutes and in a dose-dependent manner (Figure 2C and D). Since PC5A and PACE4 are known to act primarily on extracellular or plasma membrane–bound substrates, whereas furin acts mainly intracellularly in the secretion pathway (18), these results, combined with the observation that pro-OCN cleavage in osteoblasts was inhibited by Dec-RVKR-CMK, but not D6R (Figure 1E), suggest that pro-OCN is primarily a substrate for furin. Importantly, mutation of the RLRR motif to RLAA completely
Furin fl/fl osteoblasts infected with either Ad-GFP (i.e., control osteoblasts) or Ad-Cre (i.e., Furin−/− osteoblasts) was assessed by qPCR (n = 3). (B) Western blot analysis of secreted OCN from differentiated Furin−/− osteoblasts infected with either Ad-GFP or Ad-Cre. (C) Cre-mediated inactivation of Pcsk5 in Pcsk5fl/fl osteoblasts infected with either Ad-GFP or Ad-Cre was assessed by qPCR (n = 3). (D) Western blot analysis of secreted OCN from differentiated Pcsk5fl/fl osteoblasts infected with either Ad-GFP or Ad-Cre. (E) Western blot analysis of secreted OCN from differentiated Furin−/− mouse calvaria osteoblasts infected with either Ad-GFP or Ad-Cre and treated or not with 50 μM Dec-RVKR-CMK or 20 μM D6R. Results represent the mean ± SEM. **P < 0.01 and ***P < 0.001, by unpaired, 2-tailed Student’s t test.

Furin expression was significantly reduced in osteoblasts infected with Ad-Cre compared with expression levels in the control (Figure 3A). Remarkably, the inactivation of Furin in primary osteoblasts was sufficient to induce a molecular shift in the secreted OCN (Figure 3B). In contrast, the deletion of Pcsk5, the gene encoding PC5A and PC5B, in primary osteoblasts had no impact on the apparent molecular weight of OCN (Figure 3, C and D). Moreover, neither Dec-RVKR-CMK nor D6R further affected the apparent molecular weight of OCN in Furin-deficient osteoblast cultures (Figure 3E), suggesting that secreted OCN retained its propeptide.

To visualize the interaction between furin and pro-OCN, mouse osteoblasts were transfected with a pro-OCN construct in which the propeptide of OCN was tagged with a 3X FLAG epitope at its N-terminus, and the intracellular localization of pro-OCN and furin were assessed by immunofluorescence. As shown in Figure 4A, both the FLAG–pro-OCN and the noncleavable mutant (FLAG–pro-OCNAAA) signals overlapped with the furin signal (middle and bottom panels), suggesting colocalization of pro-OCN with furin. However, the FLAG–pro-OCNAAA was more abundant intracellularly than was the FLAG–pro-OCN construct (Figure 4A, middle and bottom panels). Quantification of the FLAG–pro-OCN immunofluorescence signal intensity and Western blotting confirmed an increase in the intracellular levels of pro-OCN when the furin cleavage site was mutated (Figure 4, B and C). Consistent with the observations made using furin-deficient osteoblasts (Figure 3B), FLAG–pro-OCNAAA was still secreted (Figure 4C), indicating that retention of the propeptide does not prevent secretion of the protein. Altogether, these in vitro and cell biology experiments support the notion that furin is the primary, if not sole, PC responsible for pro-OCN processing in osteoblasts.

Pro-OCN γ-carboxylation and its processing are uncoupled in osteoblasts. In hepatocytes, the cell type in which γ-carboxylation has been the most extensively studied, this posttranslational modification of proteins occurs in the ER (10). Yet furin was shown to be active mainly in the trans-Golgi network (TGN) and beyond in several cell types (25, 26), thus suggesting that pro-OCN γ-carboxylation and cleavage by furin might occur sequentially in osteoblasts. To test whether these 2 processes interfere with each other, differentiated osteoblasts were treated with either warfarin, which inhibits γ-carboxylation to aspartic acid (D), a muta
tion known to prevent this posttranslational modification in prothrombin (27), did not affect the maturation of pro-OCN to OCN.
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into its mature form in vivo, even in the absence of γ-carboxylation (Figure 5D).

Finally, we sought to determine more precisely which form(s) of OCN is/are secreted by osteoblasts under normal conditions and when furin-dependent cleavage or γ-carboxylation is inhibited. For that purpose, OCN from differentiated osteoblasts was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) after treatment with vehicle, Dec-RVKR-CMK, or warfarin. Secreted proteins were collected, concentrated, and subjected to digestion by Arg-C, which cleaves proteins following any arginine residues. Hence, if pro-OCN was present in the medium, Arg-C would release an OCN propeptide lacking its last 3 residues, i.e., KPSGPESDKAFMSKQEGNKVVNR. Interestingly, we did not detect this peptide in the supernatant of vehicle- or warfarin-treated osteoblasts, but only in supernatant from osteoblasts treated with the PC inhibitor, together with several N-terminally truncated fragments of OCN propeptide (Table 1 and Figure 5E). These data further indicate that pro-OCN cleavage is independent of OCN γ-carboxylation and that pro-OCN processing is fully efficient in osteoblasts, as pro-OCN is virtually absent in the medium of vehicle-treated osteoblasts. Last, since OCN propeptide is not detected in the supernatant of osteoblasts in normal conditions, this suggests that the cleaved propeptide is degraded intracellularly and is not secreted. This is consistent with an early study reporting that OCN propeptide was undetectable by radioimmunoassay in human sera or in human osteosarcoma cell supernatant (28).

Inactivation of Furin in osteoblasts results in impaired pro-OCN processing and decarboxylation in vivo. Our cell culture data indicating that furin is responsible for pro-OCN processing in osteoblasts raised the question of whether furin is also required for pro-OCN processing in vivo. To address this question, we generated mice lacking furin specifically in osteoblasts (Furin<sup>fl/fl</sup>OCN-Cre<sup>+</sup> mice; hereafter referred to as Furin<sup>osb–/–</sup> mice), since mice lacking furin in all cell types died in utero before skeletogenesis was initiated (29).

For this purpose, Furin<sup>fl/fl</sup> mice (30) were bred with OCN-Cre<sup>+</sup>–transgenic mice, which express the Cre recombinase under the control of the human OCN promoter in differentiated osteoblasts (31). Furin<sup>osb–/–</sup> mice were born at the expected Mendelian ratios and displayed no overt developmental abnormalities. We confirmed by PCR that recombination at the Furin<sup>+</sup> locus occurred only in bone tissues (Figure 6A) and that the expression of furin was appreciably reduced in osteoblast cultures derived from bone marrow mesenchymal cells from Furin<sup>osb–/–</sup> mice compared with control osteoblast cultures, both at the mRNA and protein levels (Figure 6, B and C, and Supplemental Figure 1B). As

**Figure 4. Furin and pro-OCN colocalize in osteoblasts.** (A) Representative images of immunofluorescence analyses of primary osteoblasts transfected with FLAG–pro-OCN or the R46A/R48A/R49A FLAG–pro-OCN mutant (FLAG–pro-OCN<sup>AAA</sup>) expressed from the p3xFLAG-Myc-CMV-23 vector. Furin is shown in green, FLAG (i.e., pro-OCN) in red, and DNA (DAPI) in blue. Scale bar: 10 μm. (B) Quantification of the red signal area in primary osteoblasts transfected with either FLAG–pro-OCN (n = 9 cells) or FLAG–pro-OCN<sup>AAA</sup> (n = 7 cells). Results represent the mean ± SEM. *P < 0.05, by unpaired, 2-tailed Student’s t test. (C) Western blot analysis of cell supernatant and cell extract from primary osteoblasts transfected with either FLAG–pro-OCN or FLAG–pro-OCN<sup>AAA</sup>.
shown in Figure 6D, the inactivation of Furin resulted in accumulation of pro-OCN and an absence of mature OCN in bones. Accordingly, OCN was only present in its proform in the serum of Furin osb–/– mice (Figure 6E). Importantly, and confirming in vivo the observations made in osteoblast cultures, the absence of furin did not affect the level of γ-carboxylation of pro-OCN in the bone ECM, whether it was assessed by Western blotting or ELISA (Figure 6, D and F). The insulin receptor precursor (pro-INSR), another protein cleaved by furin and PACE4 in cell culture (32) and involved in the regulation of energy metabolism by osteoblasts (33), was still processed in the bones of Furinosb–/– mice and in Furin –/– osteoblasts (Supplemental Figure 1, C and D). In addition, OCN was still processed when PC5, another PC known to be expressed in osteoblasts (34) and implicated in skeletal development (35), was inactivated in this cell type (Supplemental Figure 1E).

Interestingly, although total bone OCN content was not reduced in Furin osb–/– mice, circulating levels of total and ucOCN were decreased by almost 2-fold when assessed using ELISAs recognizing both OCN and pro-OCN (Figure 6G). These reductions were not caused by a difference in the specificity of the antibodies used in the ELISAs, since they similarly recognized pro-OCN or mature OCN under nondenaturing conditions (Supplemental Figure 1F). Because carboxylated OCN present in the bone ECM is converted to active ucOCN during the process of bone resorption (14), we hypothesized that in Furinosb–/– mice, pro-OCN was less efficiently decarboxylated and released during bone resorption compared with mature OCN. To test this possibility, RAW 264.7 preosteoclastic cells were cultured on devitalized calvaria bones isolated from control or Furin osb–/– mice in the presence or absence of RANKL to induce osteoclast differentiation. When osteoclasts were differentiated on control bones, we could detect the release of both total OCN and ucOCN, whereas when differentiated on bones isolated from Furin osb–/– mice, osteoclasts released less total OCN and an undetectable quantity of ucOCN (Figure 7A). Importantly, upon tartrate-resistant acid phosphatase (TRAP) staining, osteoclast differentiation did not appear to be impaired on Furinosb–/– calvaria compared with control calvaria (Figure 7, B and C), and the expression levels of the osteoclast-specific markers Acp5 and Clcn7 remained unchanged in the bones of

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Table 1. LC-MS/MS analyses of cell supernatant of differentiated mouse osteoblasts treated or not with 50 μM Dec-RVKR-CMK (RVKR) or 50 μM warfarin

<table>
<thead>
<tr>
<th>Peptide sequences</th>
<th>Exclusive spectrum count</th>
<th>Average Mascot ion score</th>
</tr>
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<tr>
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</tr>
<tr>
<td>ESQKAFMSKQEGNKVNR</td>
<td>0</td>
<td>45.2</td>
</tr>
</tbody>
</table>

Figure 5. Pro-OCN processing and γ-carboxylation occur independently of each other in osteoblasts. (A) and (B) Western blot analysis of endogenous total OCN (OCN) and γ-carboxylated OCN (Gla OCN) of cell supernatant and cell extract from differentiated mouse osteoblasts treated or not with 50 μM warfarin (A) or 50 μM Dec-RVKR-CMK (RVKR) (B). (C) Western blot analysis of supernatant from osteoblasts transfected with OCN-V5 or the E13D/E17D/E20D OCN-V5 mutant (OCNΔgco-V5) and treated or not with 50 μM Dec-RVKR-CMK. (D) Western blot analysis of OCN immunoprecipitated from the serum of control mice (Ggcxfl/fl) and mice lacking γ-carboxylase in osteoblasts (Ggcxosb–/–). Total OCN and γ-carboxylated OCN were assessed by Western blotting. (E) LC-MS/MS analyses of cell supernatant of differentiated mouse osteoblasts treated or not with 50 μM Dec-RVKR-CMK or 50 μM warfarin. Quantification of the OCN propeptide area relative to the total peptide area in 3 independent experiments is shown (see also Table 1). Results represent the mean ± SEM.
Furinosb–/– mice compared with levels in their control littermates (Supplemental Figure 1G), suggesting that the absence of furin in osteoblasts does not overtly impact osteoclast differentiation.

Previous studies have shown that the acidic pH generated during the process of bone resorption is responsible for OCN decarboxylation (13). Hence, we compared the decarboxylation of mature OCN and pro-OCN following incubation at a pH comparable to that found in the osteoclast resorption lacuna (i.e., pH 4.3). While the OCN contained in control mouse bone extracts was efficiently decarboxylated at pH 4.3, the pro-OCN contained in Furinosb–/– mouse bone extracts was not (Figure 7D and Supplemental Figure 1H). Importantly, anti–Gla OCN and anti–total OCN antibodies could detect mature OCN in control bone extracts, pro-OCN in the Furinosb–/– mouse bone extracts, and mature OCN generated following the addition of recombinant furin to the latter extracts (Supplemental Figure 1I). Collectively, these results suggest that, in the absence of furin, osteoblasts secrete γ-carboxylated pro-OCN, which, like mature γ-carboxylated OCN, accumulates in the bone ECM. However, pro-OCN is poorly decarboxylated and released during bone resorption, resulting in reduced circulating levels of ucOCN.

Furin in osteoblasts is required to maintain glucose and energy homeostasis. To determine how the absence of pro-OCN cleavage impacts OCN endocrine functions, we next studied glucose metabolism in Furinosb–/– mice. Glucose tolerance and serum insulin levels were normal in Furinosb–/– mice at 3 and 6 months of age (Supplemental Figure 2, A–E). However, at 6 and 9 months of age, there was a significant increase in blood glucose levels under fed conditions in Furinosb–/– mice compared with levels in littermate controls (Supplemental Figure 2F and Figure 8A). In addition, at 9 months of age, Furinosb–/– mice also showed reduced glucose tolerance when challenged with a glucose load (Figure 8B). Furthermore, in agreement with the fact that ucOCN normally favors insulin secretion by β cells, serum insulin levels were significantly reduced in the fed state in Furinosb–/– mice compared with levels in control littermates, while fasting levels were not affected (Figure 8C). These results are consistent with higher blood glucose levels in Furinosb–/– mice only in fed conditions or during a glucose tolerance test (GTT) (Figure 8, A and B). Moreover, and in line with the previously reported positive effect of ucOCN on insulin synthesis by β cells, pancreatic insulin content was also reduced in Furinosb–/– mice compared with that
The conclusion that pro-OCN processing by furin is required for the complete activation of this hormone.

Furin regulates appetite through its expression in osteoblasts. 

Furinosb–/– mice are characterized by normal glucose tolerance at 3 and 6 months of age when fed a regular chow diet (Supplemental Figure 2, A and D). Moreover, insulin sensitivity, as assessed by insulin tolerance tests (ITTs), was normal in Furinosb–/– mice in all the age groups tested (Supplemental Figure 2, G–I). This contrasts with Ocn inactivation that results in both glucose intolerance and insulin resistance as early as 3 months of age in mice fed a chow diet (2). Likewise, on a chow diet, Furinosb–/– mice had lower fat accumulation than did Ocn–/– mice (Figure 9F and ref. 2). Hence, the metabolic phenotypes are delayed or milder in Furinosb–/– mice compared with Ocn–/– mice.

Although several possibilities could explain these differences, one of them is that furin negatively modulates other aspects of energy metabolism independently of OCN. In support of this hypothesis, we observed a significant reduction of approximately 30% in food intake by 6-month-old Furinosb–/– mice during the dark phase (Figure 9G). Accordingly, cumulative food intake was reduced by approximately 20% over a 3-day period (Figure 9H). We found that cumulative food intake was also reduced by approximately 15% in 3-month-old Furinosb–/– mice compared with their WT littermates, although their body weight was not significantly changed compared with that of their littermate controls, whether the mice were fed a normal chow diet or a high-fat diet (Figure 9I).

Since OCN also increases energy expenditure, we assessed the energy balance in control and Furinosb–/– mice through indirect calorimetry. At 3 and 6 months of age, chow-fed Furinosb–/– mice displayed reduced O2 consumption and CO2 production in the dark phase, i.e., when the mice were more active (Figure 9, A and B, and Supplemental Figure 2, J and K), which resulted in a significant decrease in global energy expenditure (Figure 9C and Supplemental Figure 2L). We observed no decrease in physical activity in Furinosb–/– mice during the dark and light phases (Figure 9, D and E), suggesting that the reduced energy expenditure of the Furinosb–/– mice was not caused by a diminution in the voluntary movements of these animals. The lower energy expenditure in Furinosb–/– mice was associated with an increased epididymal fat pad weight and whole-body fat tissue percentage at 12 months of age compared with control mice, although their body weight was not significantly changed compared with that of their littermate controls, whether the mice were fed a normal chow diet or a high-fat diet (Figure 9F and Supplemental Figure 3, A–D). Overall, the phenotypic characterization of Furinosb–/– mice revealed that furin, through its expression in osteoblasts, plays a role in regulating glucose and energy metabolism. Our findings also support the conclusion that pro-OCN processing by furin is required for the complete activation of this hormone.

Furin regulates appetite through its expression in osteoblasts. Furinosb–/– mice are characterized by normal glucose tolerance at 3 and 6 months of age when fed a regular chow diet (Supplemental Figure 2, A and D). Moreover, insulin sensitivity, as assessed by insulin tolerance tests (ITTs), was normal in Furinosb–/– mice in all the age groups tested (Supplemental Figure 2, G–I). This contrasts with Ocn inactivation that results in both glucose intolerance and insulin resistance as early as 3 months of age in mice fed a chow diet (2). Likewise, on a chow diet, Furinosb–/– mice had lower fat accumulation than did Ocn–/– mice (Figure 9F and ref. 2). Hence, the metabolic phenotypes are delayed or milder in Furinosb–/– mice compared with Ocn–/– mice.

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Figure 7. Pro-OCN is not efficiently decarboxylated by osteoclasts. (A and B) In vitro resorption assay of devitalized calvaria from Furinfl/fl mice (n = 3) and Furinosb–/– mice (n = 3) in the presence of osteoclast-like RAW 246.7 cells, with or without 10 ng/ml RANKL. (A) Total OCN and ucOCN ELISA measurements in the cell culture supernatant. ND, not detected. (B) Representative image of TRAP staining of calvaria. Scale bar: 500 μm. (C) Percentage of area with TRAP-positive staining (n = 3). (D) Western blot analysis of bone extracts from Furinfl/fl and Furinosb–/– mice incubated in phosphate-buffered solution at pH 7.5 or pH 4.3 for 14 days at 37°C. Graph shows quantification of the Gla OCN/OCN ratio (representative result from 3 independent experiments). The ELISAs used in A quantify both pro-OCN and mature OCN. Results represent the mean ± SEM. **P < 0.01 and ***P < 0.001, by 1-way ANOVA followed by Bonferroni’s multiple comparisons test.
leptin levels were not changed in Furinsb–/– mice (Supplemental Figure 3G). Collectively, these observations suggest that Furinsb–/– mice do not develop insulin resistance as robustly as do Ocn–/– mice, in part because of a reduction in calorie intake, a condition shown previously to improve insulin sensitivity in mice (36).

To test this possibility directly, we pair fed 4-month-old control and Furinsb–/– mice for 2 to 4 weeks to ensure similar calorie intake in both groups (Supplemental Figure 3H) before assessing their glucose tolerance and insulin sensitivity. As shown in Figure 10A, Furinsb–/– mice were significantly heavier than the pair-fed control mice, and this was associated with an increase in the percentage of whole-body fat tissue (Figure 10B). When pair fed with their control littersmates, Furinsb–/– mice showed higher feeding blood glucose levels and a glucose intolerance phenotype (Figure 10, C and D). Finally, under these conditions, the Furinsb–/– mice were found to be insulin resistant in comparison with the control mice, as assessed by an ITT (Figure 10E). Importantly, most of these phenotypes were not observed at the same age when control and Furinsb–/– mice were fed ad libitum (Supplemental Figures 2 and 3).

Since furin deficiency leads to lethality between E10.5 and E11.5, this PC has been implicated in a limited number of other peptide hormones, is dependent on the activity of a specific PC.

Pro-OCN processing is dependent on furin in osteoblasts. With the use of a variety of approaches, we established that, in osteoblasts, pro-OCN maturation is exclusively performed by furin and not by the other members of the PC family. First, in vitro assays showed that pro-OCN is most efficiently processed by furin compared with the other members of the PC family. Second, inactivation of Furin only in osteoblasts, pro-OCN failed to be processed and accumulated in the bone ECM. PCs in another PC that is required for normal patterning of the axial skeleton through the processing of progrowth differentiation factor 11 (pro-GDF11) (35). PCs has been reported to be expressed in osteoblasts and osteocytes, where it may process FGF23, another bone-derived hormone regulating phosphate homeostasis (34). However, our data show that, although PC5A is expressed in primary osteoblasts and cleaves pro-OCN in vitro, the inactivation in this cell type of Pck5, encoding PC5A and PC5B isoforms, does not affect pro-OCN processing in cell culture or in vivo. PC5A and pro-OCN may not colocalize to the same cellular compartment, as PC5A is activated only after it reaches the cell surface (18).

Since furin deficiency leads to lethality between E10.5 and E11.5, this PC has been implicated in a limited number of
postnatal functions so far. Furin was shown to be essential for the maintenance of peripheral immune tolerance through its expression in T lymphocytes (24), to play a role in the granular acidification of pancreatic β cells (23), and to be required for cardiac development through its expression in endothelial cells (38). In contrast, studies of furin functions in liver showed that it might act redundantly with other PCs in this tissue (22, 30). In the present study, we show that furin is the cognate PC for pro-OCN activation in osteoblasts, providing genuine in vivo evidence for a nonredundant role of furin in this cell type. In classical endocrine cells, prohormones (e.g., proglucagon, proinsulin, and pro-opiomelanocortin) are generally converted to active hormones by either PC1 and/or PC2 (39, 40). Consistent with the notion that the osteoblast is a nonclassical endocrine cell, our data show that both PC1 and PC2 are not expressed in osteoblasts and that instead furin is responsible for pro-OCN conversion. Consequently, although furin was previously shown in cell culture to be responsible for proparathyroid hormone (pro-PTH) conversion (41) and in the stepwise generation of growth hormone–releasing hormone (GHRH) together with PC1 (42), our study is the first to our knowledge to reveal the sole requirement for furin in the activation of a specific peptide hormone and to imply that furin has a role in endocrine regulation in vivo.

Processing of OCN is not dependent on its γ-carboxylation. The precursors of other γ-carboxylated proteins, such as thrombin and factor IX, also contain a propeptide that consists of a recognition signal for the γ-glutamyl carboxylase as well as a dibasic motif (RR) in their C-terminus (15). It is thought that processing of the propeptide in γ-carboxylated proteins could depend on their efficient γ-carboxylation (43). Although the OCN propeptide appears to contain a putative γ-glutamyl carboxylase recognition motif (44), mature OCN can be γ-carboxylated in vitro in the absence of its propeptide (16). Through several approaches, including mass spectrometry, we showed that preventing pro-OCN γ-carboxylation, either pharmacologically or genetically, did not affect its processing by furin, thereby establishing that these 2 posttranslational

Figure 9. Reduced energy expenditure and food intake in Furinosb–/– mice. (A–E) Metabolic parameters of 6-month-old Furinfl/fl (n = 11) and Furinosb–/– (n = 9) mice. O2 consumption (A), CO2 release (B), heat production (energy expenditure) (C), and activity on the x axis (D) and y axis (E). (F) Epididymal fat pad weight normalized to the body weights of 12-month-old Furinfl/fl (n = 12) and Furinosb–/– (n = 12) mice fed a normal chow diet. (G–J) Food intake by 6-month-old Furinfl/fl (n = 11), Furinosb–/– (n = 9), Ocn+/+ (n = 8), and Ocn–/– (n = 8) mice. (G and I) Food intake during the light and dark periods. (H and J) Cumulative food intake over a 3-day period. Results represent the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-way ANOVA for repeated measurements with Bonferroni’s multiple comparisons test (A–E and G–J) or by unpaired, 2-tailed Student’s t test (F).
Furinosb–/–. Beyond 9 months of age, part through OCN mature decarboxylated OCN (45, 46). the intracellular accumulation of pro-OCN, but also the release of using warfarin in the ROS 17/2.8 rat osteosarcoma cell line caused findings, earlier studies reported that inhibition of modifications occur independently of each other. In line with our Ocn–/– mice resulted in reduced glucose tolerance, hypoinsulinemia, metabolism. For instance, partial ablation of osteoblasts in adult appearances to be a major mediator of bone endocrine action on energy expenditure, and increased white adipose tissue mass compared with their control littermates. These phenotypes are consistent with those of Ocn–/– mice and support the conclusion that pro-OCN processing by furin is required for the activation of this hormone. Nonetheless, some metabolic anomalies in Furinosb–/– mice were delayed or not detected (e.g., insulin resistance) as compared with Ocn–/– mice (2).

Furin in osteoblasts regulates whole-body energy metabolism in part through OCN. Beyond 9 months of age, Furinosb–/– mice display reduced glucose tolerance, decreased serum insulin levels, lower energy expenditure, and increased white adipose tissue mass compared with their control littermates. These phenotypes are consistent with those of Ocn–/– mice and support the conclusion that pro-OCN processing by furin is required for the activation of this hormone. Nonetheless, some metabolic anomalies in Furinosb–/– mice were delayed or not detected (e.g., insulin resistance) as compared with Ocn–/– mice (2).

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Pro-OCN is less efficiently released during the process of bone resorption. Our cellular and in vivo experiments establish that, in the absence of furin, pro-OCN is not converted to mature OCN, but is still efficiently γ-carboxylated and secreted. These observations suggest that pro-OCN processing is not required for OCN to be secreted. Similarly, it was previously shown that profactor IX, which can be processed in vitro by furin, is normally secreted in its γ-carboxylated proform in heterologous cells. However, in contrast to mature factor IX, profactor IX was found to be unable to bind phospholipid vesicles in the presence of Ca2+ (48). We have previously demonstrated that γ-carboxylation of OCN is absolutely required for its binding to bone ECM, since in mice lacking Ggcx in osteoblasts, bones are depleted of OCN (12). In contrast, pro-OCN does accumulate normally in the bones of Furinosb–/– mice, suggesting that the propeptide does not prevent binding of the OCN Gla residues to the mineral component of bone ECM. Hence, retention of the propeptide has different effects on the calcium-binding properties of pro-OCN and profactor IX.

Although bone OCN content was normal in Furinosb–/– mice, the circulating levels of total and ucOCN were reduced. γ-Carboxylated OCN was shown to be partially decarboxylated and activated during the process of bone resorption in a nonenzymatic reaction requiring the acidic pH of the resorption lacuna generated by the osteoclasts (13, 14). Other data presented here show that pro-OCN is less efficiently decarboxylated than is mature OCN during bone resorption and following incubation at pH 4.5, suggesting that the retention of the propeptide protects OCN Gla residues from the effect of the low pH generated by osteoclasts.
What is the role of OCN propeptide if it is not required for OCN γ-carboxylation? The propeptide domain might facilitate the entry of the 96-aa-long pre–pro-OCN in the ER, its cleavage by the signal peptidase, the exit of the 72-aa-long pro–OCN from the ER, and its transport through the secretory pathway until it reaches the TGN, where furin is active (26). In support of this notion, expression of OCN without its propeptide sequence in osteoblasts greatly reduces the amount of mature OCN secreted in the media (El-Rifai and Ferron, unpublished observations). Finally, it was reported that a polypeptide must have a minimum size of at least 50 aa in order to be recognized by the signal recognition particle and threaded through the membrane of the ER (49), making the 46-aa-long OCN too small to be efficiently secreted on its own.

The lumen of the TGN is characterized by an acidic pH, i.e., as low as pH 5.9 in HeLa cells (50). Interestingly, consistent with its preferential action in the TGN, furin is more active in vitro at pH 6 than at pH 7.5 (51). Hence, we can also speculate that the normal function of OCN propeptide could be to protect OCN from premature decarboxylation while it is transiting through the TGN and the constitutive secretory pathway. Alternatively, the presence of the propeptide may prevent OCN from premature folding in the TGN, since it was reported that OCN can fold in the absence of calcium ions at pH 6 and below (52). Further investigation will be needed to specifically address these possibilities.

In summary, this work provides in vitro and in vivo evidences that the processing of the OCN prohormone by furin regulates the activation and endocrine function of OCN. Moreover, we established that furin in osteoblasts plays an important role in the regulation of energy metabolism by bone through OCN-dependent and -independent mechanisms.

Methods

Animal models. The Furin<sup>−/−</sup> mice were generated by introducing loxP sites upstream and downstream of exon 2 of the Furin gene (30). Ggcx<sup>−/−</sup> mice were generated by introducing loxP site upstream of exon 1 and a loxP site downstream of exon 2 of the Ggcx gene (12). Psk<sup>5fl/fl</sup> mice were generated by introducing loxP sites on each side of Psk<sup>5</sup> exon 1 (35). Each of these strains was crossed with OCN-Cre–transgenic mice, which express Cre recombinase under the control of a fragment of the human OCN promoter (31) and which were previously shown not to display any metabolic anomalies, in order to generate, respectively, Furin<sup>−/−</sup> OCN-Cre (Furin<sup>−/−</sup>OCN-Cre (Furin<sup>−/−</sup>OCN-Cre (Ggcx<sup>−/−</sup>)), Ggcx<sup>−/−</sup>OCN-Cre (Ggcx<sup>−/−</sup>)), and Psk<sup>5fl/fl</sup>OCN-Cre (Psk<sup>5fl/fl</sup>OCN-Cre). Ocn<sup>−/−</sup> mice were generated by replacing Ocn1 (Bglap1) and Ocn2 (Bglap2) genes in the mouse Ocn cluster with a neomycin resistance cassette through homologous recombination (53). All strains used were backcrossed on a C57BL/6j genetic background more than 10 times and maintained in an IRBM specific pathogen–free animal facility (SPF) under 12-hour dark/12-hour light cycles. Male mice fed a normal chow diet were used in all experiments, unless otherwise noted. Tissue DNA was extracted by standard procedure, and PCR was performed with the genotyping PCR primers listed in Supplemental Table 1.

Metabolic analysis. For GTTs, adult male mice fed a normal chow diet were fasted overnight for 16 hours. Blood glucose levels were measured after fasting and at 15, 30, 60, and 120 minutes following i.p. injection with 2 g/kg glucose. In some experiments, mice were fed a 60% high-fat diet (D12331-5I; Research Diets) for 10 weeks starting from 3-day-old mouse calvariae through serial collagenase (Worthington Biochemical Corp.) digestion as previously described (12). To generate Furin<sup>−/−</sup> or Psk<sup>5fl/fl</sup> osteoblasts, Furin<sup>−/−</sup> or Psk<sup>5fl/fl</sup> osteoblasts were infected with Cre-GFP–expressing adeno virus (University of Iowa) at 200 MOI per well. Control osteoblasts were obtained by infecting Furin<sup>−/−</sup> or Psk<sup>5fl/fl</sup> osteoblasts with GFP-expressing adeno virus. Osteoblast differentiation was induced by supplementing osteoblast culture media (αMEM plus 10% FBS and 2mM L-glutamine) with 5 mM β-glycerophosphate and 100 μg/ml L-ascorbic acid for 21 days. Bone marrow–derived osteoblasts were prepared as previously described (12). Briefly, long bones of 3-week-old mice were dissected, and bone marrow cells were flushed with αMEM. Cells were then plated in 6-well plates at a density of 5 × 10<sup>4</sup> cells per well. After 7 days, media were changed to differentiation media (osteoblast media supplemented with 100 μg/ml L-ascorbic acid, 5 mM β-glycerophosphate, and 10 μg/ml dexamethasone). After 14 days of differentiation, cells were used for mRNA or protein analysis.

Biochemical studies. For OCN immunoprecipitation, Furin<sup>−/−</sup> or Furin<sup>−/−</sup>OCN–serum was diluted 1:50 with 1× PBS containing 1 mM PMSF,
and 1× protease inhibitor cocktail and incubated overnight at 4°C in the presence of the OCN antibodies indicated in Figure 5D, Figure 6E, and in Supplemental Figure 1F. Protein G agarose beads prewashed with 1× PBS were added and incubated for 4 hours at 4°C. Beads were spun down and washed 4 times with 1× PBS before Western blot analysis. Proteins were resolved on 15% Tris-tricine SDS-PAGE gel for OCN detection, whereas 7.5% Tris-glycine gel was used for the detection of furin and the insulin receptor. The antibodies used in this study were: anti-OCN (total) polyclonal goat antibody, which recognize aa 26–46 corresponding to mature mouse OCN (55); anti-Gla OCN polyclonal goat antibody, which recognize Gla13 OCN aa 11–26 corresponding to carboxylated mature OCN (55); anti-β-actin (mouse, clone AC-15, A5441; Sigma-Aldrich); anti-V5 (mouse, clone V5-10, V8012; Sigma-Aldrich); anti-furin (rabbit, PAI-062; Thermo Fisher Scientific); anti-insulin receptor β (mouse, clone L55B10, 3020; New England BioLabs); and anti-LCN2 (goat, AF1857; R&D Systems). Serum and cell supernatant levels of carboxylated, undercarboxylated, and total OCN were measured using ELISAs as described previously (55). Briefly, the Gla ELISA detects OCN only when it is carboxylated on the Glu13 residue. The Glu ELISA quantifies the uncarboxylated form of OCN. The total ELISA detects OCN, whether it is carboxylated or not. OCN measurements in bone homogenate were normalized to total protein content, as assessed by Bradford assay.

**GST pro-OCN preparation and in vitro digestion assay.** Mouse pro-OCN cDNA (WT or R48A/R49A, i.e., RR/AA mutant) was cloned into pGEX4T3 in BamHI and EcoRI restriction sites (Supplemental Table 1). Plasmids were transformed in Rosetta (DE3) pLysS competent bacteria (EMD Millipore), and recombinant GST proteins were purified using glutathione resin (Genscript). Recombinant full-length LCN2 protein was provided by S. Kousteni (Columbia University, New York, NY, USA). The cDNA of the soluble form of various PCs were cloned into the pIRES2-EGFP plasmid and transfected into HEK293 cells (Columbia University, New York, NY, USA). The cDNA of the soluble form of various PCs were cloned into the pIRES2-EGFP plasmid and transfected into HEK293 cells (Columbia University, New York, NY, USA). The cDNA of the soluble form of various PCs were cloned into the pIRES2-EGFP plasmid and transfected into HEK293 cells (Columbia University, New York, NY, USA). The cDNA of the soluble form of various PCs were cloned into the pIRES2-EGFP plasmid and transfected into HEK293 cells (Columbia University, New York, NY, USA).

**Gene expression.** Osteoblasts and calvaria total RNA were isolated using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific). RNAs were reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific), and real-time quantitative PCR (qPCR) was performed using SYBR Green qPCR Master Mix (Biorad) and gene-specific primers (see Supplemental Table 1). Expression levels were normalized to Actb expression levels.

Additional details on the methods used in this study are provided in the supplemental material.

**Statistics.** Statistical analyses were performed using GraphPad Prism software version 7.03. Results are shown as the mean ± SEM. For single measurements, an unpaired, 2-tailed Student’s t test was used, while 1-way ANOVA followed by Bonferroni’s post test was used for comparison of more than 2 groups. For repeated measurements (e.g., GTTs and ITTs), a repeated-measures 2-way ANOVA followed by Bonferroni’s post test was used. A P value of less than 0.05 was considered statistically significant. All experiments were repeated at least 3 times or performed on at least 3 independent animals.

**Study approval.** All animal use complied with the guidelines of the Canadian Committee for Animal Protection and was approved by IRCM Animal Care Committee.

**Author contributions**

MF and JL designed experiments. OER performed most experiments, with crucial help from JC (genetic and pharmacological studies with primary osteoblasts), JL (metabolic cages and metabolic tests), CJ (in vitro PC assay), DF (LC-MS/MS analysis), and DSR (PC activity). RE and NGS provided Pck5o/b mice and JWC provided Furino/b mice. MF and OER wrote the manuscript with suggestions from JL, JWC, and NGS. All authors discussed the results and commented on the manuscript.

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**Table 1**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Description</th>
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<tbody>
<tr>
<td>OCN</td>
<td>Mouse</td>
<td>Secreted and membrane protein</td>
</tr>
<tr>
<td>Furin</td>
<td>Mouse</td>
<td>Prohormone convertase</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>Mouse</td>
<td>Transmembrane receptor</td>
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**Figure 1**

- **A**: Cartoon of the osteocalcin gene (OCN) expression and metabolism in bone. OCN is synthesized as a prohormone and undergoes proteolytic cleavage by furin to generate the active form, pro-OCN. Cleavage by furin releases the carboxylated form of OCN, which is subsequently cleaved by furin to generate the uncarboxylated form. OCN is then secreted into the circulation and binds to its receptor, the insulin receptor. The insulin receptor is a transmembrane protein that mediates signaling pathways involved in glucose metabolism.

- **B**: Western blot analysis of furin expression in the pancreas. The blot shows the presence of furin protein in the pancreas, indicating its role in the processing of pro-OCN in this tissue.

- **C**: ELISA analysis of OCN levels in serum. The results demonstrate that OCN levels are significantly higher in diabetic mice compared to control mice, indicating that OCN plays a role in glucose metabolism.

**References**


