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The recent demonstration that osteoblasts have a role in controlling energy metabolism suggests that they express cell-specific regulatory genes involved in this process. Activating transcription factor 4 (ATF4) is a transcription factor that accumulates predominantly in osteoblasts, where it regulates virtually all functions linked to the maintenance of bone mass. Since Atf4−/− mice have smaller fat pads than littermate controls, we investigated whether ATF4 also influences energy metabolism. Here, we have shown, through analysis of Atf4−/− mice, that ATF4 inhibits insulin secretion and decreases insulin sensitivity in liver, fat, and muscle. Several lines of evidence indicated that this function of ATF4 occurred through its osteoblastic expression. First, insulin sensitivity is enhanced in the liver of Atf4−/− mice, but not in cultured hepatocytes from these mice. Second, mice overexpressing ATF4 in osteoblasts only [termed here α1(I)Collagen-Atf4 mice] displayed a decrease in insulin secretion and were insulin insensitive. Third, the α1(I)Collagen-Atf4 transgene corrected the energy metabolism phenotype of Atf4−/− mice. Fourth, and more definitely, mice lacking ATF4 only in osteoblasts presented the same metabolic abnormalities as Atf4−/− mice. Molecularly, ATF4 favored expression in osteoblasts of Esp, which encodes a product that decreases the bioactivity of osteocalcin, an osteoblast-specific secreted molecule that enhances secretion of and sensitivity to insulin. These results provide a transcriptional basis to the observation that osteoblasts fulfill endocrine functions and […]
The transcription factor ATF4 regulates glucose metabolism in mice through its expression in osteoblasts

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The recent demonstration that osteoblasts have a role in controlling energy metabolism suggests that they express cell-specific regulatory genes involved in this process. Activating transcription factor 4 (ATF4) is a transcription factor that accumulates predominantly in osteoblasts, where it regulates virtually all functions linked to the maintenance of bone mass. Since Atf4\(^{-/-}\) mice have smaller fat pads than littermate controls, we investigated whether ATF4 also influences energy metabolism. Here, we have shown, through analysis of Atf4\(^{-/-}\) mice, that ATF4 inhibits insulin secretion and decreases insulin sensitivity in liver, fat, and muscle. Several lines of evidence indicated that this function of ATF4 occurred through its osteoblastic expression. First, insulin sensitivity is enhanced in the liver of Atf4\(^{-/-}\) mice, but not in cultured hepatocytes from these mice. Second, mice overexpressing ATF4 in osteoblasts only [termed here α1(I)Collagen-Atf4 mice] displayed a decrease in insulin secretion and were insulin insensitive. Third, the α1(I)Collagen-Atf4 transgene corrected the energy metabolism phenotype of Atf4\(^{-/-}\) mice. Fourth, and more definitely, mice lacking ATF4 only in osteoblasts presented the same metabolic abnormalities as Atf4\(^{-/-}\) mice. Molecularly, ATF4 favored expression in osteoblasts of Esp, which encodes a protein that decreases the bioactivity of osteocalcin, an osteoblast-specific secreted molecule that enhances secretion of and sensitivity to insulin. These results provide a transcriptional basis to the observation that osteoblasts fulfill endocrine functions and identify ATF4 as a regulator of most functions of osteoblasts.

Introduction

The transcriptional control of osteoblast differentiation and function involves many players, some broadly expressed and others with a more restricted expression (1). At least 3 of these transcription factors accumulate only or mostly in osteoblasts: Runx2, Osterix, and activating transcription factor 4 (ATF4) (2–5). The first 2 are critical determinants of the transition from mesenchymal cells to the osteoblast lineage. The third one, ATF4, a member of the cAMP-responsive element-binding protein (CREB) family of basic zipper-containing proteins, regulates terminal differentiation and virtually all functions of the osteoblast related to the control of bone mass. Indeed, through its ability to regulate amino acid import, ATF4 is a critical determinant of the synthesis of proteins such as type I collagen in osteoblasts (4–6). Since type I collagen is the most abundant protein of the bone ECM, ATF4 is de facto a regulator of bone formation and of bone ECM mineralization (7). Additionally, ATF4 favors expression in osteoblasts of Rankl, a gene required for osteoclast differentiation; thus, through its osteoblastic expression, it also promotes bone resorption (8, 9). That ATF4 regulates these osteoblast functions raises the hypothesis that it may be an even more global regulator of osteoblast biology.

Recently, it was shown that the osteoblast is an endocrine cell type that, through the secreted molecule osteocalcin, favors insulin secretion by β cells and insulin sensitivity in liver, muscle, and adipocytes (10–12). The function of osteocalcin is regulated by at least one regulatory gene expressed in osteoblasts called embryonic stem cell phosphatase (Esp). The Esp gene product inhibits indirectly the biological activity of osteocalcin by favoring its carboxylation (10). The notion that the osteoblast may be an endocrine cell type raises the prospect that osteoblasts must express other regulatory genes such as those encoding transcription factors that may be implicated in this aspect of its biology.

While studying Atf4\(^{-/-}\) mice for another purpose, we were surprised to notice significantly lower fat mass and blood glucose levels. These observations echoed the glucose intolerance noted in humans lacking the eukaryotic translation initiation factor 2 kinase (EIF2AK3), which enhances ATF4 translation during amino acid starvation or ER stress (13, 14). In addition, it is known that phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) regulates glucose homeostasis (15–17). We show here that ATF4 is in fact a negative regulator of insulin secretion and of sensitivity to insulin in liver, muscle, and fat. Surprisingly, however, cell-based assays and analyses of classical and cell-specific loss-of-function and gain-of-function mouse models and of compound mutant mice reveal that ATF4 regulates glucose metabolism through its expression in osteoblasts. In these latter cells, ATF4 favors expression of Esp and as a result decreases the bioactivity of osteocalcin. This study reveals a further level of control of the endocrine function of osteoblasts and supports the notion that ATF4 regulates most functions of this cell type.

Authorship note: Tatsuya Yoshizawa and Eiichi Hinoi contributed equally to this work.

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**Results**

*Atf4* inactivation enhances secretion of and sensitivity to insulin. While studying whether ATF4 could mediate the effect of serotonin on osteoblasts (18), we noticed that *Atf4*–/– mice had smaller fat pads than their WT counterparts (Figure 1A). This feature prompted us to analyze their energy metabolism.

The first abnormality that this study revealed was that *Atf4*–/– mice displayed at 2 weeks, 1 month, and 2 months of age a significant decrease in blood glucose levels when compared with WT littermates (Figure 1B). This decrease in blood glucose levels was secondary to an increase in circulating insulin levels, which itself was secondary to an increase in insulin secretion as determined by a glucose-stimulated insulin secretion (GSIS) test (Figure 1, C and D). The decrease in blood glucose levels in the face of an increase in circulating levels of insulin suggested that *Atf4*–/– mice were more tolerant to glucose than WT littermates. To demonstrate that this was the case, we performed glucose tolerance tests (GTT) via i.p. injection of glucose (2 g/kg of body weight) after overnight fasting. These tests showed that *Atf4*–/– mice were indeed significantly more tolerant to a glucose load than WT mice (Figure 1F).

**Figure 1**

*Atf4* inactivation increases glucose tolerance. (A) Photograph of representative fat pad (16 weeks of age) and histogram showing fat pad weight over body weight in WT and *Atf4*–/– mice. (B and C) Blood glucose and serum insulin levels in WT and *Atf4*–/– mice at indicated ages. (D) Results of GSIS test in WT and *Atf4*–/– mice. (E) Insulin expression in pancreas of WT and *Atf4*–/– mice. (F) GTT in WT and *Atf4*–/– mice. (G and H) ITT and PTT in WT and *Atf4*–/– mice. (I) Insulin target gene and insulin sensitivity marker gene expression in *Atf4*–/– liver or cultured hepatocytes. (J) Phosphorylation of Akt in *Atf4*–/– liver (upper panels) or cultured hepatocytes (lower panels) at basal and insulin-stimulated conditions. (K) Insulin sensitivity marker gene expression in muscle and white adipose tissue (WAT) in *Atf4*–/– mice. (L) Phosphorylation of Akt in muscle in *Atf4*–/– mice at basal (upper panel) and insulin-stimulated (lower panel) conditions. Analysis of 8-week-old *Atf4*–/– mice is shown in D–L. Images in J and L were grouped from different parts of the same gel and film. Error bars show mean + SEM. **P < 0.01; *P < 0.05, WT versus *Atf4*–/– mice.
In principle, one would expect that an increase in insulin secretion would cause a decrease in insulin sensitivity in Atf4−/− mice. Remarkably, however, when we analyzed this aspect of glucose metabolism through an insulin tolerance test (ITT), we noticed that Atf4−/− mice were also more sensitive to insulin than WT littermates (Figure 1G). This increase in insulin sensitivity was next verified by molecular studies performed in various target organs of insulin (see below).

To determine whether gluconeogenesis, a process inhibited by insulin in the liver (19, 20), might be under the control of ATF4, we performed pyruvate tolerance tests (PTT) via i.p. injection of pyruvate (2 g/kg of body weight) in WT and Atf4−/− mice. Atf4−/− mice showed a marked reduction in glucose production after a pyruvate challenge compared with WT mice, indicating that gluconeogenesis was impaired by the absence of ATF4 (Figure 1H). To further show that gluconeogenesis is reduced in Atf4−/− mice, we analyzed the expression of phosphoenolpyruvate carboxykinase (Pck1) and glucose-6-phosphatase (G6pase), 2 well-known insulin target genes in the liver that are implicated in this process (21, 22). A significant reduction of the expression of both genes was detected in Atf4−/− mice (Figure 1I), further confirming the inhibition of hepatic gluconeogenesis in these animals. To determine whether glycolysis might be altered in Atf4−/− mice, we analyzed the expression of 2 key genes, glucokinase (Gck) and pyruvate dehydrogenase kinase 4 (Pdk4), involved in this process and whose expression is regulated by insulin in the liver (23, 24). In Atf4−/− mice, Gck expression was significantly increased, while Pdk4 expression was significantly decreased (Figure 1I), indicating that glycolysis was stimulated by the absence of ATF4. Furthermore, the expression of the transcription factor Foxa2, which regulates insulin sensitivity (25), was also increased in the liver of Atf4−/− mice (Figure 1I). To further confirm that ATF4 regulates insulin signaling in liver in vivo, we studied phosphorylation of Akt and GSK-3β in WT and Atf4−/− hepatocytes. As shown in Figure 1J, Akt phosphorylation in Atf4−/− hepatocytes was similar to that of WT hepatocytes in basal conditions, and insulin enhanced this phosphorylation to a similar extent in WT and Atf4−/− hepatocytes. The level of phosphorylation of GSK-3β in Atf4−/− hepatocytes was also similar to that of WT hepatocytes in both basal and insulin-stimulated conditions (Supplemental Figure 1B). Although we cannot rule out the possibility that the absence of effect of the Atf4 deletion in hepatocytes may be due to dedifferentiation of these cells in culture, these results suggest that insulin can signal normally in hepatocytes regardless of the presence or absence of ATF4 in this cell type. This raises the testable hypothesis that ATF4 affects insulin sensitivity, at least in liver, through its expression in another cell type.

Atf4 overexpression in osteoblasts hampers insulin secretion and insulin sensitivity. How could ATF4 inhibit insulin sensitivity in the liver in a non–cell-autonomous manner? That ATF4 accumulates mostly in osteoblasts (5) along with the metabolic functions recently ascribed to this cell type (10) suggested that it may be, at least in part, through its osteoblastic expression that Atf4 affects glucose metabolism.

As an initial means to addressing this question, we relied on the use of transgenic mice overexpressing Atf4. Specifically, we compared mice overexpressing Atf4 in osteoblasts but in no other tissues [α1(I)Collagen-Atf4 mice] to transgenic mice overexpressing Atf4 in all tissues but not in bone (CMV-Atf4 mice). We verified, prior to analyzing these mice, that the α1(I)Collagen-Atf4 transgene was not expressed in pancreas, white adipose tissue, liver, muscle, and brain, while the CMV-Atf4 transgene was expressed in all tissues examined but had a markedly weaker expression in bone compared with other tissues (Figure 2A and Supplemental Figure 2). The specificity of expression of Atf4 was further verified at the protein level (Supplemental Figure 2, A and B). We also determined that in the bones of α1(I)Collagen-Atf4 mice, Atf4 expression was 60% higher than in WT bones (Figure 2B).

α1(I)Collagen-Atf4 but not CMV-Atf4 mice displayed a significant increase in blood glucose levels and a significant decrease in circulating insulin levels compared with WT littermates. Hence, α1(I)Collagen-Atf4 mice had, in first approximation, metabolic abnormalities that were opposite to what was observed in Atf4−/− mice (Figure 2, C and D).

To assess whether this decrease in circulating insulin levels betrayed a decrease in insulin secretion in the α1(I)Collagen-Atf4 mice, we performed GSIS tests, which revealed that insulin

Table 1

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<th>Insulin contents, β cell area, and quantification of insulin/Ki67-positive cells in WT and Atf4−/− mice</th>
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<tr>
<td>Insulin content (ng/mg pancreas)</td>
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<tr>
<td>β cell area (%)</td>
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<td>Ki67-positive cells (%)</td>
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^aP < 0.05. Analysis is from 8-week-old Atf4−/− mice.
secretion following a glucose challenge was significantly lower in α1(I)Collagen-Atf4 mice than in WT littermates (Figure 2E). Consistent with the decrease in serum insulin levels, there was also a significant decrease in islet insulin content, in insulin immunoreactivity, in β cell area and mass, and in Ins1 expression (Figure 2, F and G, and Table 2). In addition, the number of Ki67-positive β cells and the expression of Cdk4, a regulator of cell cycle affecting β cell proliferation (29), were also significantly decreased in pancreas of α1(I)Collagen-Atf4 mice (Figure 2G and Table 2).

To determine how this decrease in insulin secretion could affect the ability of α1(I)Collagen-Atf4 mice to dispose of a glucose load, we performed a GTT. This test showed that α1(I)Collagen-Atf4 mice had a significantly lower tolerance to glucose than WT mice (Figure 2H). We next asked whether the glucose intolerance of the α1(I)Collagen-Atf4 mice was caused in part by a decrease in insulin sensitivity. An ITT showed that insulin sensitivity was significantly decreased in α1(I)Collagen-Atf4 mice compared with WT mice (Figure 2I), although the circulating levels of adipokines affecting this process, such as leptin, resistin, and adiponectin, were not affected in α1(I)Collagen-Atf4 mice (Supplemental Figure 3). In addition, α1(I)Collagen-Atf4 mice showed increased expression of Pck1 and G6pase and decreased Gck expression in the liver and decreased expression of Pparg in fat cells and Mcad in muscle (Figure 2J). Consistent with the virtual absence of the CMV-Atf4 transgene in bone, none of these metabolic and histological abnormalities were observed in CMV-Atf4 mice (Figure 2, B–D, H, and I, and Table 2). Thus, Atf4 overexpression in osteoblasts only results in a metabolic phenotype that is the mirror image of the one observed in Atf4−/− mice, while its overexpression in other tissues does not.

We next introduced the α1(I)Collagen-Atf4 transgene in Atf4+/− mice [α1(I)Collagen-Atf4 Atf4+/− mice], reasoning that the extent of

**Figure 2**

Overexpression of Atf4 in osteoblasts only decreases glucose tolerance. (A) CMV-Atf4 and α1(I)Collagen-Atf4 (α1(I)-Atf4) transgene expression in several tissues. (B) Endogenous Atf4 expression in bone of CMV-Atf4 and α1(I)Collagen-Atf4 mice. (C and D) Blood glucose and serum insulin levels in CMV-Atf4 and α1(I)Collagen-Atf4 mice at indicated ages. (E) GSIS test in α1(I)Collagen-Atf4 mice. (F) Insulin immunostaining in pancreas of α1(I)Collagen-Atf4 mice. Arrows indicate islets. Scale bars: 500 μm. (G) Insulin and Cdk4 expression in pancreas of α1(I)Collagen-Atf4 mice. (H and I) GTT and ITT in CMV-Atf4 and α1(I)Collagen-Atf4 mice. (J) Insulin target genes and insulin sensitivity marker genes expression in liver, muscle and white adipose tissue in α1(I)Collagen-Atf4 mice. Analysis of 24 week-old CMV-Atf4 and α1(I)Collagen-Atf4 mice is shown in E–J. Error bars show mean ± SEM. **P < 0.01; *P < 0.05, WT versus α1(I)Collagen-Atf4 mice.
the rescue of the metabolic phenotype of the Atf4<sup>–/–</sup> mice induced by this transgene would be a reliable, albeit suggestive, indicator of the role that Atf4 expression in osteoblasts plays in regulating glucose metabolism. Remarkably, Atf4 expression was restored to a WT level but not above it in the bones of osb<sup>–/–</sup>–/– mice (Figure 3A). As shown in Figure 3, B–L, whether we looked at blood glucose, circulating insulin levels, metabolic tests (GTT, ITT, GSIS test, and PTT), pancreas histology, or gene expression in islets, liver, and muscle, the α1(I)Collagen-Atf4 transgene completely rescued the metabolic abnormalities of the Atf4<sup>–/–</sup> mice.

### Table 2

<table>
<thead>
<tr>
<th>Insulin content</th>
<th>WT (n ≥ 6)</th>
<th>α1(I)Collagen-Atf4 (n ≥ 6)</th>
<th>CMV-Atf4 (n ≥ 4)</th>
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<tr>
<td>(ng/mg pancreas)</td>
<td>134.1 ± 10.9</td>
<td>97.1 ± 12.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>β cell area (%)</td>
<td>1.07 ± 0.30</td>
<td>0.58 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95 ± 0.20</td>
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<tr>
<td>β cell mass (mg)</td>
<td>3.87 ± 0.18</td>
<td>1.95 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.45 ± 0.37</td>
</tr>
<tr>
<td>Ki67-positive cells (%)</td>
<td>1.42 ± 0.21</td>
<td>0.81 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
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<sup>a</sup>P < 0.05, WT versus α1(I)Collagen-Atf4 mice. Analysis is of 24-week-old mice. ND, not determined.

was reduced in these mutant mice (Figure 4G). Moreover, there was also, as we had observed in Atf4<sup>–/–</sup> mice, a significant increase in the expression of Gck<sup>–/–</sup> mice (Figure 4G). None of these abnormalities were observed in Creb<sup>–/–</sup> mice (Figure 4, A, B, D, and E, and Supplemental Figure 3). Last, to further assess insulin sensitivity, we performed a 2-hour hyperinsulinemic-euglycemic clamp in conscious mice (Supplemental Table 1). Atf4<sup>–/–</sup> mice had lower basal glucose levels than WT littermates (95 ± 9 vs. 120 ± 13 mg/dl in WT mice). During the clamp, plasma glucose levels were maintained at euglycemia (∼110 mg/dl) in both groups of mice. Steady-state rates of glucose infusion to maintain euglycemia during the clamp were significantly elevated in Atf4<sup>–/–</sup> mice compared with the WT mice (69 ± 3 vs. 47 ± 4 mg/kg/min in WT mice; P = 0.01). This was mostly due to a 40% increase in insulin-stimulated whole-body glucose turnover in Atf4<sup>–/–</sup> mice (70 ± 3 vs. 50 ± 4 mg/kg/min in WT mice; P = 0.02). Insulin-stimulated whole-body glycogen synthesis was markedly elevated in Atf4<sup>–/–</sup> mice (42 ± 4 vs. 17 ± 7 mg/ kg/min in WT mice; P = 0.04). Taken together, these data are consistent with the notion that ATF4 decreases insulin secretion and hampers insulin sensitivity through its expression in osteoblasts.

**ATF4 directly regulates Esp expression in osteoblasts.** Since ATF4 is a known regulator of the expression of osteocalcin, a gene favoring insulin secretion and insulin sensitivity (4, 10), the observation that Atf4<sup>–/–</sup> mice had a glucose metabolism phenotype mirroring the one seen in osteocalcin<sup>–/–</sup> mice was counterintuitive and raised the hypothesis that ATF4 could regulate expression in osteoblasts of a gene or genes that oppose the metabolic function of osteocalcin. One such gene is Esp, which acts upstream of osteocalcin (10). Remarkably, the metabolic phenotypes of Esp<sup>–/–</sup>, Atf4<sup>–/–</sup>, and Atf4<sup>–/–</sup> mice are strikingly similar.

Analysis of the Esp promoter revealed the existence of a CAMP-responsive element (CRE) at −340. To assess the importance of this cis-acting element in regulating Esp expression in osteoblasts, we performed DNA transfection assays in ROS17/2.8 cells, an osteoblastic cell line expressing Esp<sup>–/–</sup> promoter, including the CRE, were necessary for its activity (Figure 4). These α1(I)Collagen-Cre;Atf4<sup>–/–</sup> mice were subjected to the same tests as Atf4<sup>–/–</sup> and α1(I)Collagen-Atf4 mice. As a negative control, we used mice lacking, in osteoblasts only, another member of the same family of transcription factors, CREB [α1(I)Collagen-Cre;Creβ<sup>–/–</sup> (Cre<sup>β<sup>–/–</sup>β<sup>–/–</sup>) mice].

Starting at 2 weeks of age, we noticed the same significant decrease in blood glucose levels in Atf4<sup>–/–</sup> mice that we had observed in mice lacking Atf4 in all cells. These markedly lowered blood glucose levels were also observed at 1 month of age in Atf4<sub>osb</sub>–/– mice (Figure 4A). In addition, Atf4<sup>–/–</sup> but not Creβ<sup>–/–</sup> mice showed a significant increase in circulating insulin levels and in insulin secretion following a glucose challenge (GSIS test) compared with control littermates (Figure 4, B and C). To determine whether Atf4<sub>osb</sub>–/– mice were more tolerant to a glucose load than control littermates, we performed GTT after overnight fasting. The GTT showed that Atf4<sub>osb</sub>–/– mice, like Atf4<sup>–/–</sup> mice, were significantly more tolerant to a glucose challenge than control littermates (Figure 4D; compare with Figure 1F).

To assess insulin sensitivity in the Atf4<sub>osb</sub>–/– mice, we first performed an ITT, which showed that insulin sensitivity was increased in Atf4<sub>osb</sub>–/– mice to the same extent as in Atf4<sup>–/–</sup> mice when compared with control littermates (Figure 4E; compare with Figure 1G). Again, this increase in insulin sensitivity occurred in the face of normal levels of circulating adipokines (Supplemental Figure 3). We next performed PTT in Atf4<sub>osb</sub>–/– mice. There was also, in Atf4<sub>osb</sub>–/– mice, a reduction in glucose production after pyruvate challenge similar to the one seen in Atf4<sup>–/–</sup> mice (Figure 4F; compare with Figure 1H). That the expression of Pck1 and G6pase was reduced in the liver of Atf4<sub>osb</sub>–/– mice further confirmed that gluconeogenesis
ChIP assays confirmed that ATF4 but not CREB binds to the CRE element in the Esp promoter (Figure 5D). The specificity of the binding of ATF4 to the CRE element located at −340 in the Esp promoter was verified by electric mobility shift assay. In that assay, we used as a source of proteins nuclear extracts of 293 cells transfected with either an empty vector or a vector expressing a FLAG-tagged version of ATF4. As shown in Figure 5E, a protein-DNA complex formed upon incubation of a labeled double-stranded oligonucleotide encompassing the sequence of the −340 CRE elements with nuclear extracts of 293 cells transfected with the ATF4 expression vector (Figure 5E) but not when nuclear extracts of cells transfected with the empty vector were used (data not shown). As a control of specificity we also performed “supershift” experiments using various antibodies. As shown in Figure 5E, an antibody against the FLAG sequence could alter the mobility of the protein-DNA complex, while an antibody against an unrelated sequence could not (Figure 5E).

Taken together, these data indicate that the CRE element present at −340 bp in the Esp promoter is required, at least in cell culture, for the osteoblast-specific activity of the Esp promoter. These molecular observations are consistent with the existence of a metabolic phenotype in Atf4osb–/– mice but not in Crebosb–/– mice and with the fact that Esp expression was significantly increased in α1(I)Collagen-Atf4 mice and decreased in Atf4osb–/– mice but not affected in Crebosb–/– mice (Figure 5F).

ATF4 modulates glucose metabolism via osteocalcin by favoring Esp expression in osteoblasts. To further ascertain that it is through its regulation of Esp expression that ATF4 achieves its function on metabolism, we performed 3 additional experiments.
First, we generated compound heterozygous mice lacking 1 allele of Atf4 and 1 allele of Esp. As shown in Figure 6, A–C, while Atf4<sup>–/–</sup> mice and Esp<sup>–/–</sup> mice were indistinguishable from WT mice, Atf4<sup>–/–</sup> Esp<sup>–/–</sup> mice displayed a metabolic phenotype similar to that of the Esp<sup>–/–</sup> mice whether we looked at blood glucose levels, serum insulin levels, or the number of Ki67/Insulin-positive β cells, and Esp expression was significantly decreased in the compound heterozygous mice (data not shown). Furthermore, the percentage of uncarboxylated osteocalcin was significantly increased in Atf4<sup>–/–</sup> Esp<sup>–/–</sup> compared with control mice (Supplemental Figure 6). These data indicate that Atf4 and Esp are in the same genetic pathway. Second, since Esp favors osteocalcin carboxylation, a posttranslational modification that hampers osteocalcin metabolic function (10), we measured osteocalcin carboxylation in WT, α1(I)Collagen-Atf4, and Atf4<sub>osb</sub><sup>–/–</sup> mice. As shown in Figure 6D, the percentage of uncarboxylated osteocalcin, i.e., bioactive osteocalcin, present in serum was decreased in α1(I)Collagen-Atf4 mice compared with WT. In contrast, this percentage was increased in Atf4<sub>osb</sub><sup>–/–</sup> mice to the same extent as in Esp<sup>–/–</sup> mice, although the level of serum total osteocalcin was decreased in Atf4<sub>osb</sub><sup>–/–</sup> mice (Supplemental Figure 5).

If uncarboxylated osteocalcin is a mediator of the metabolic functions of ATF4 present in osteoblasts, then one would expect that adding exogenous uncarboxylated osteocalcin to α1(I)Collagen-Atf4 mice would rescue their metabolic phenotypes. Indeed, and as shown in Figure 6, E–G, the metabolic abnormalities noticed in the α1(I)Collagen-Atf4 mice were rescued by long-term perfusion of uncarboxylated osteocalcin. Taken together, these data indicate that ATF4 regulates glucose metabolism, at least in part, by favoring expression of Esp in osteoblasts and, as a result, by decreasing osteocalcin bioactivity. That a single injection of warfarin (77 μg/kg), a compound that inhibits the carboxylation of osteocalcin (31), decreased blood glucose levels in WT mice and did so even more severely in α1(I)Collagen-Atf4 mice, but not in Osteocalcin<sup>–/–</sup> mice (Figure 6H), added further credence to this contention.

**Discussion**

In this report, we provide in vivo evidence indicating that the transcription factor ATF4 negatively regulated insulin secretion and decreases sensitivity to insulin in liver, muscle, and fat cells; we show that it achieves this function, in large part, through its expression in osteoblasts. Last, we provide a molecular explanation for this set of observations (see Figure 7).

ATF4 has already been implicated in many critically important cellular processes. For instance, its translation increases in the case of amino acid starvation and ER stress following phosphorylation of eIF2α. ATF4 then initiates a gene expression program promoting amino acid import into cells (6). Although Atf4 is a broadly expressed gene (4), the main phenotypic abnormalities noted in Atf4<sup>–/–</sup> mice after birth are in the skeleton. This is due, to a large extent, to the fact that the Atf4 protein is far more abundant in osteoblasts than in any other cell type analyzed (5). This restricted accumulation in osteoblasts explains why Atf4 is such an impor...
research article

Figure 5
ATF4 directly regulates Esp expression in osteoblasts. (A) DNA transfection assay in ROS17/2.8 osteoblasts with different length of Esp promoters. (B) DNA cotransfection assay in COS cells using Esp promoter and indicated expression vectors. (C) DNA cotransfection assay in COS cells using Esp promoter containing 6 copies of the CRE site or mutated CRE site and indicated expression vectors. (D) ChIP assay in primary calvarial osteoblasts using ATF4 and CREB antibodies. (E) Electric mobility shift assay using labeled CRE site located at –340 in the Esp promoter and FLAG-Atf4 protein. (F) Esp expression in bone of 8-week-old α1(I)Collagen-Atf4, Atf4osb–/–, Crebosb–/–, and Atf4–/– mice. **P < 0.01; *P < 0.05, WT versus α1(I)Collagen-Atf4 or Atf4osb–/– mice (E).

Tant determinant of collagen synthesis, bone formation, and through its regulation of Rankl, osteoclast differentiation (4, 9). The paramount importance of ATF4 in bone biology before and even more after birth is also illustrated by the fact that its activity is affected in several human diseases and mouse models of these conditions. For instance, ATF4 transcriptional activity is decreased and bone formation is hampered in a mouse model of Coffin-Lowry syndrome (4), a rare skeletal dysplasia, while it is increased, as is bone formation, in a mouse model recapitulating some of the skeletal manifestation of neurofibromatosis type 1 (7).

That ATF4 regulates such a large number of functions fulfilled by osteoblasts suggested that this transcription factor may also affect other aspects of osteoblast biology such as, for instance, the recently described ability of this cell type to improve glucose metabolism (10). In indirect support of this hypothesis, one other disease, Wolcott-Rallison syndrome (WRS), affecting among other organs the skeleton, is caused by inactivating mutations in the gene encoding EIF2AK3 (13). This mutation should prevent any increased translation of Atf4 following ER stress or amino acid starvation, although it may change many other functions. WRS patients are also diabetic, suggesting that ATF4 could be needed for insulin secretion (13).

Results of the present analysis demonstrate that, unlike what could have been anticipated, ATF4 inhibits insulin secretion as well as insulin sensitivity. Furthermore, cell-specific gain-of-function and loss-of-function experiments in mice establish that it is, to a large extent, through its osteoblastic expression that ATF4 regulates these 2 functions. In this cell type, ATF4 regulates the expression of Esp, a gene inhibiting the metabolic functions of osteocalcin, a bone-derived secreted molecule promoting insulin secretion and insulin sensitivity (10). Thus, although ATF4 regulates the expression of both Osteocalcin and Esp, it is through the regulation of the latter gene that ATF4 inhibits insulin secretion and insulin sensitivity. Indeed, by increasing Esp expression, ATF4 decreases the ability of osteocalcin to increase insulin secretion (12). This function of the osteoblast-expressed Atf4 is consistent with the fact that ATF4 function is regulated by the sympathetic tone, which also regulates Esp expression, and by protein intake (7, 9, 12).

We remain aware, however, that our results do not exclude the formal possibility that ATF4 may also exert an influence on glucose metabolism in addition to the mechanism of action presented here through its expression in other cell types such as the adipocyte. This note of caution is an important one since, for instance, the levels of adiponectin are not affected in Atf4–/– or in α1(I)Collagen-Atf4–overexpressing mice. This may be an indication that ATF4, through its expression in osteoblasts, modulates glucose metabolism via an osteocalcin-independent mechanism. Alternatively, we cannot exclude the possibility that osteocalcin regulates insulin sensitivity in adipocytes, hepatocytes, and myoblasts directly, without any intervention of adiponectin. That Atf4 and Esp lie in the same genetic cascade provides some support to the latter hypothesis. These are the 2 important issues that will need to be addressed once a specific receptor for osteocalcin has been molecularly identified.
As mentioned above, ATF4 was already known to regulate bone formation and mineralization as well as osteoclast differentiation through its osteoblast expression (7–9). By showing that it is also involved in the endocrine function of this cell, this study underscores the critical importance of ATF4 as a regulator of many, but not all, the functions of this cell type. To date, the only function of the osteoblast that is not influenced by ATF4 is the regulation of phosphate metabolism (data not shown). By identifying another regulatory gene involved in this pathway, the observations presented in the paper add further credence to the notion that the osteoblast is a bona fide endocrine cell.

**Methods**

*Animals.* α1(I)Collagen-Atf4, Esp+/-, Creb-flx/flx, Atf4-/-, and α1(I)Collagen-Cre mice were previously described (4, 7, 30, 32, 33). Creb-flx/flx mice were a gift from G. Schütz (German Cancer Research Center, Heidelberg, Germany). Osteoblast-specific Atf4-deficient mice (Atf4osb-/-) mice were generated through homologous recombination in ES cells and the use

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**Figure 6**

ATF4 modulates glucose metabolism through regulation of osteocalcin bioactivity by favoring Esp expression in osteoblasts. (A–C) Blood glucose, serum insulin levels, and quantification of insulin/Ki67-immunoreactive cells in islets of 2-week-old Esp+/+Atf4-/- mice. (D) Serum uncarboxylated osteocalcin levels in α1(I)Collagen-Atf4 and Atf4osb-/- mice. (E and F) Blood glucose and serum insulin levels in uncarboxylated osteocalcin-treated α1(I)Collagen-Atf4 mice. (G) GTT in uncarboxylated osteocalcin-treated α1(I)Collagen-Atf4 mice. (H) Blood glucose (percentage of DMSO-treated control) at 120 minutes after injection in warfarin-treated α1(I)Collagen-Atf4 mice and Ocn-/- mice. Analysis shows 12-week-old α1(I)Collagen-Atf4 mice in E–G. Analysis shows 24-week-old α1(I)Collagen-Atf4 mice and Ocn-/- mice in H. Error bars show mean ± SEM. **P < 0.01; *P < 0.05, WT versus Esp+/+Atf4-/- or Esp-/- mice (A–C), WT versus α1(I)Collagen-Atf4 or Atf4osb-/- mice (D) WT versus α1(I)Collagen-Atf4 mice (E–G), DMSO-treated mice as control versus warfarin-treated mice (H), *P < 0.05, α1(I)Collagen-Atf4 versus osteocalcin-treated α1(I)Collagen-Atf4 mice (E–G), WT mice plus warfarin versus α1(I)Collagen-Atf4 mice plus warfarin (H).

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**Figure 7**

Model showing how ATF4 controls glucose handling through its expression in osteoblasts. ATF4 controls the expression of Osteocalcin (Ocn) and to a larger extent of Esp, a gene encoding an inhibitor of osteocalcin bioactivity. This causes a chronic decrease in osteocalcin-mediated insulin production and induces a long-term negative effect on pancreatic β cell proliferation. At the same time, the sensitivity to insulin of its target tissues (liver, fat, and muscle) is decreased.
of Atf4(Cre)Collagen-Cre transgenic mice (30). Genotyping was performed by PCR analysis of genomic DNA. All mutant mouse strains were on the same genetic background (C57BL/6J) except for the e1(I)Collagen-Atf4 transgenic mice, which were generated on an Fvb background. All procedures involving animals were approved by the Columbia University IACUC. The Columbia University IACUC meets national standards.

**Metabolic studies.** For GTT, glucose (2 g/kg) was injected i.p. after overnight fast, and blood glucose was monitored using blood glucose strips and the Accu-Check glucometer (Roche) at indicated times. For PTT, pyruvate (2 g/kg) was injected i.p. after overnight fast, and blood glucose levels were measured at indicated times. For ITT, insulin (0.5 or 0.75 U/kg) was injected i.p. after 4 hours of fasting, and blood glucose levels were measured at indicated times. ITT data are presented as percentage of insulin signaling study in vivo, insulin (0.05 U/kg) was injected into the mice for 5 minutes, followed by homogenization with lysis buffer containing 4% PMSF, 50 mM NaCl, 1 mM EDTA, and 1% Triton X-100. For insulin signaling study in vitro, hepatocytes were cultured in William’s E medium without FBS for 24 hours and subsequently treated with insulin at 2 nM and 

**Molecular studies.** Primary osteoblasts were prepared from calvaria of 5-day-old pups as previously described (4) and were cultured in αMEM/10% FBS in the presence of 100 μM ascorbic acid and 5 mM β-glycerophosphate for 5 days. Real-time PCR was performed on DNAse-treated total RNA converted to cDNA using Taq SYBR Green Supermix with ROX (Bio-Rad) on an MX3000 instrument (Stratagene); 

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