Adiponectin Regulates Bone Mass via Opposite Central and Peripheral Mechanisms through FoxO1

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SUMMARY

The synthesis of adiponectin, an adipokine with ill-defined functions in animals fed a normal diet, is enhanced by the osteoblast-derived hormone osteocalcin. Here we show that adiponectin signals back in osteoblasts to hamper their proliferation and favor their apoptosis, altogether decreasing bone mass and circulating osteocalcin levels. Adiponectin fulfills these functions, independently of its known receptors and signaling pathways, by decreasing FoxO1 activity in a PI3-kinase-dependent manner. Over time, however, these local effects are masked because adiponectin signals in neurons of the locus coeruleus, also through FoxO1, to decrease the sympathetic tone, thereby increasing bone mass and decreasing energy expenditure. This study reveals that adiponectin has the unusual ability to regulate the same function in two opposite manners depending on where it acts and that it opposes, partially, leptin’s influence on the sympathetic nervous system. It also proposes that adiponectin regulation of bone mass occurs through a PI3-kinase-FoxO1 pathway.

INTRODUCTION

The existence of a reciprocal regulation between bone and energy metabolism is supported by a growing number of evidence (Karsenty and Oury, 2012). Two hormones, osteocalcin and leptin, are the overarching determinants of this process. Osteocalcin, a bone-derived hormone, regulates energy metabolism, in part, by promoting insulin secretion by pancreatic β cells (Lee et al., 2007). In a feed-forward loop, insulin signals back in osteoblasts to enhance osteocalcin activity and therefore insulin secretion (Ferron et al., 2010). Osteocalcin also signals in adipocytes, where it favors the synthesis of the secreted molecule adiponectin (Lee et al., 2007). That insulin signals back to osteoblasts and influences osteocalcin activity raises the prospect that adiponectin might do the same.

The adipocyte-derived hormone leptin prevents bone mass accrual in all species in which this was tested (Ducy et al., 2000; ELEFTERIOU ET al., 2004; Henry et al., 1999; Vaira et al., 2012). In the mouse, a mediator of leptin regulation of bone mass accrual is the sympathetic nervous system (Takeda et al., 2002). The development of high bone mass in the face of hypogonadism, as seen in the absence of leptin signaling, is a unique situation that underscores the importance of the leptin-dependent sympathetic regulation of bone mass accrual. It also raises the prospect that, as it is the case for its regulation of appetite (Erickson et al., 1996), leptin stimulation of the sympathetic nervous system may be opposed by another secreted molecule. Such a molecule has not been identified yet.

Adiponectin, another adipocyte-derived secreted molecule, is best known for its insulin-sensitizing ability in animals fed a high-fat diet (Kadowaki et al., 2006; Maeda et al., 2002), although its absence does not appear to overtly alter insulin sensitivity in animals fed a normal diet (Ma et al., 2002; Maeda et al., 2002). It seems unlikely that adiponectin would only function in animals fed a high-fat diet since when it appeared during evolution this diet did not exist, nor were there any reasons to anticipate its eventual appearance. Moreover, even in present days, most animals never encounter this situation in the wild. A plausible explanation for this apparent lack of metabolic function in animals fed a normal diet is that adiponectin affects a biological process other than energy metabolism (Denzel et al., 2010; Sharma et al., 2008; Takemura et al., 2007). The appearance of adiponectin during evolution with bones (Figure 1A) and its regulation by osteocalcin are two reasons to suspect that bone could be a target tissue of this hormone. This hypothesis began to be tested in vivo through gain- and loss-of-function experiments. Some of these studies indicated that adiponectin affects bone mass; however, they did not provide molecular or cellular mechanisms for its action, nor did they study whether adiponectin affects osteocalcin (Oshima et al., 2005; Shinoda et al., 2006).

Adiponectin biology is further complicated by the fact that several receptors and more than one signaling pathway have been associated with this hormone. Expression cloning
Adiponectin Functions in Animals Fed a Normal Diet

Hypothalamus-adipocyte axis, adipose tissue, and osteoblasts or osteoclasts derived from bone marrow cells treated with adiponectin for 2, 4, and 8 hr were used (McCulloch et al., 1991) (Figure S1H). These results identifying the osteoblast and Rankl as an adiponectin target cell and gene implied that this hormone should inhibit bone mass accrual by favoring bone resorption. This hypothesis was tested through the study of Adiponectin−/− mice fed a normal diet. Since Adiponectin−/− mice of either gender displayed the same bone mass abnormality at a later age (Figures 3A and S2A), we used only male mice for the rest of this study. At 6 and, to a lesser extent, 12 weeks of age, Adiponectin−/− mice fed a normal or high-fat diet showed the expected high-bone-mass phenotype affecting the axial and appendicular skeleton and the trabecular and cortical bones (Figures 1D, 1E, S1B, and S2I). Consequently, Adiponectin−/− bones had better biomechanical properties than did WT ones (Figure 1F). Surprisingly, however, this high bone mass was not caused by a decrease in bone resorption since Rankl expression was mildly affected and the osteoclast number was not significantly changed in Adiponectin−/− bones. What explained, instead, this high-bone-mass phenotype in Adiponectin−/− mice was a massive increase in the osteoblast number (Figures 1D and 1G) that resulted in an increase in the rate of bone formation and in circulating levels of total and undercarboxylated osteocalcin (Figure 1H). This high-bone-mass phenotype could not be explained by abnormal insulin sensitivity since insulin tolerance tests were normal throughout life in Adiponectin−/− mice fed a normal diet (Figure S1C). In contrast, 12-week-old Adiponectin−/− mice fed a normal diet showed a significant decrease in insulin secretion as measured by a glucose-stimulated insulin secretion (GSIS) test and an intolerance to glucose as determined by a glucose tolerance test (GTT) (Figures 1L and 1M). A mechanism explaining these abnormalities was later identified (see below).

We also analyzed transgenic mice harboring a 5-fold increase in adiponectin circulating levels (pLiv-Adiponectin) (Figure 1I). Mirroring what was seen in Adiponectin−/− mice, 12-week-old pLiv-Adiponectin mice showed a low-bone-mass phenotype due to a significant decrease in osteoblast number (Figure 1J). Consequently, total and undercarboxylated circulating osteocalcin levels were significantly decreased in these mice compared to WT littermates (Figure 1K). Hence, both loss- and gain-of-function models identify adiponectin as a regulator of osteoblast number, bone formation, and circulating osteocalcin levels.

Adiponectin Inhibits Proliferation and Favors Apoptosis of Osteoblasts

Since it is the basis of the bone phenotypes of Adiponectin−/− and pLiv-Adiponectin mice, we investigated how adiponectin regulates the osteoblast number. While expression of osteoblast differentiation markers such as Runx2 and Osterix was unaffected in Adiponectin−/− bones and in adiponectin-treated osteoblasts (Figures 1C and S1D), three cellular abnormalities could explain the elevated osteoblast number of Adiponectin−/− mice. First, proliferation of osteoblast progenitor cells, as measured by BrdU incorporation and CyclinD1 accumulation, was increased in Adiponectin−/− bones, and adiponectin treatment of mouse osteoblast progenitor cells decreased their proliferation and CyclinD1 accumulation (Figures 2A–2D). Second, there was a decrease in osteoblasts apoptosis in Adiponectin−/− bones as measured by a TUNEL assay and by the number of Annexin-V-positive osteoblasts (Figures 2E and 2G). Conversely, adiponectin treatment of osteoblast progenitor cells increased the number of TUNEL-positive cells and the production of

RESULTS

Adiponectin Inhibits Bone Mass Accrual in Young Mice

Proteins with significant homology to adiponectin are found only in bony vertebrates (Figure 1A). This could mean that adiponectin is expressed in, or signals in, bone cells. To test the first possibility, we measured Adiponectin expression in adipocytes and bone cells obtained from wild-type (WT) and Adiponectin−/− mice. Adiponectin expression was 10,000-fold higher in WT adipocytes than in bone cells; moreover, there was no difference in Adiponectin expression between WT and Adiponectin−/− osteoblasts and osteoclasts (Figure 1B). Likewise, quantitative PCR and in situ hybridization analyses failed to detect Adiponectin expression in osteocytes (Figures S1E–S1G available online). Thus, the appearance of adiponectin in animals fed a normal diet. They also indicate that adiponectin opposes, partially, leptin’s influence on the sympathetic nervous system and the physiological functions that it regulates.

To address the second possibility, we measured the expression of multiple genes in cell populations enriched with calvarial osteoblasts or in osteoblasts derived in vitro from bone-marrow–derived cells. These cells were then treated with recombinant mouse full-length adiponectin for 4 hr. Among all the genes tested, only Rankl, a gene expressed in osteoblasts and encoding an osteoclast differentiation factor (Lacey et al., 1998), saw its expression increase more than 10-fold after stimulation by even low amounts of adiponectin or its globular domain (Figures 1C, S1A, and S1H). The same stimulation of Rankl expression was observed when enriched populations of osteoblasts derived from bone marrow cells treated with adiponectin for 2, 4, and 8 hr were used (McCulloch et al., 1991) (Figure S1H). These results identifying the osteoblast and Rankl as an adiponectin target cell and gene implied that this hormone should inhibit bone mass accrual by favoring bone resorption. This

identified two atypical seven-transmembrane proteins, AdipoR1 and AdipoR2, as receptors for adiponectin. Those receptors were subsequently shown to transduce adiponectin signal either in an AMP kinase (AMPK)–dependent or in an AMPK-independent but ceramide-dependent manner (Yamauchi et al., 2003; Holland et al., 2011). Adiponectin also binds to T-cadherin, a cell-surface molecule that lacks, however, any intracellular moiety (Hug et al., 2004). Collectively, these observations suggest that, as it is the case for its function(s), the signaling mechanisms used by adiponectin may not be fully elucidated.

Testing of whether bone is a target tissue of adiponectin revealed that this hormone exerts two opposite influences on this tissue. First, it signals in osteoblasts, prevents their proliferation, and increases their apoptosis in a PI3-kinase–FoxO1-dependent manner, and as a result it decreases bone formation, bone mass, and circulating osteocalcin levels. Yet these functions are rapidly masked because adiponectin also signals in the brain, through FoxO1, to inhibit the activity of the sympathetic nervous system, thereby increasing bone formation and bone mass and decreasing energy expenditure and blood pressure. These results identify target cells and functions and propose a signaling mechanism for adiponectin in animals fed a normal diet. They also indicate that adiponectin opposes, partially, leptin’s influence on the sympathetic nervous system and the physiological functions that it regulates.

REFERENCES

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cleaved caspase-3 by osteoblasts (Figures 2F and 2H). Third, there was a decrease in oxidative stress defined by the production of malondialdehyde and 4-hydroxyxynenal and accumulation of reactive oxygen species in Adiponectin−/− osteoblasts; this may also trigger apoptosis (Finkel and Holbrook, 2000) (Figures 2I and 2J). That none of these abnormalities were observed when hepatocytes and myoblasts were treated with adiponectin suggests that the influence of this hormone on osteoblasts is, to an extent, specific to this cell type (Figures 2B and 2F). In summary, by signaling in osteoblasts, adiponectin inhibits bone formation and decreases circulating osteocalcin levels because it decreases proliferation and favors apoptosis of osteoblasts.

**Adiponectin Favors Bone Mass Accrual in Older Mice**

A singular feature of the high-bone-mass phenotype displayed by Adiponectin−/− mice is that it peaked at an early age. Indeed, the 2-fold increase in osteoblast number seen in 6-week-old mutant mice had largely vanished once these mice reached 3 months of age. This dynamic influence of adiponectin on
bone mass accrual led us to analyze Adiponectin\(^{-/-}\) mice at multiple time points.

Bone formation, bone resorption parameters, and bone mass were normal, if not slightly decreased, in 6-month-old male and female Adiponectin\(^{-/-}\) mice, and even more striking was the fact that 9-month-old Adiponectin\(^{-/-}\) mice demonstrated a severe low-bone-mass phenotype affecting all skeletal elements tested (Figures 3A, S2A, and S2B). This was due to the conjunction of a decrease in the number and proliferation ability of osteoblasts (Figures 3A–3C) and an increase in bone resorption parameters such as osteoclast surface, CTx serum levels, and Rankl expression (Figures 3A, 3D, and 3E). In other words, the bone
phenotype of older Adiponectin−/− mice and the cellular abnormalities explaining it were exactly opposite to those seen in younger ones.

Two other physiological functions were also affected in 9-month-old Adiponectin−/− mice fed a normal diet. First, there was a marked increase in energy expenditure during both dark and light cycles (Figure 3F). As a result, fat-pad weight did not increase overtime in Adiponectin−/− as it did in WT mice (Figure 3G). Paradoxically, given this massive increase in energy expenditure, appetite was not increased and in fact was slightly decreased in older Adiponectin−/− mice. This explained why the body weight of 9-month-old Adiponectin−/− mice was significantly lower than the one of WT littermates (Figures 3H and S2E). Expression of genes affecting appetite, such Mc4r, Npy, and Cart, was similar between Adiponectin−/− and WT mice (Figure S2D). Second, blood pressure and heart rate were significantly increased in Adiponectin−/− mice compared to WT littermates (Figures 3I and 3J).

Adiponectin Inhibits the Activity of the Sympathetic Nervous System
Two observations suggested that the phenotype observed in older Adiponectin−/− mice could be due to an increase in the sympathetic tone. The first one is that adiponectin crosses the blood brain barrier (Figure 3K) (Kusminski et al., 2007; Qi et al., 2004). To determine whether adiponectin accumulates in the locus coeruleus, where the sympathetic nervous system originates from (Rush and Geffen, 1980), we delivered it in Adiponectin−/− mice and analyzed where it binds. In the conditions of this experiment, adiponectin bound to neurons expressing DBH, a specific marker of neurons of the locus coeruleus (Thomas et al., 1995). That an excess of nonlabeled adiponectin abolished biotinylated-adiponectin binding to DBH-expressing neurons whereas biotinylated-GST did not verify the specificity of this binding (Figure 3L). A second reason to test whether adiponectin regulates the sympathetic tone is that the sympathetic nervous system is known to inhibit bone formation, to favor bone resorption, to increase energy expenditure and blood pressure, to promote liver gluconeogenesis, and to inhibit insulin secretion (Elefteriou et al., 2005; Nonogaki, 2000; Takeda et al., 2002; Tentolouris et al., 2006).

As hypothesized, whether we measured norepinephrine content in the brain, Ucp1 expression in brown fat, or urinary epinephrine elimination, there was, throughout life, a nearly 2-fold increase in the sympathetic tone in Adiponectin−/− mice compared to WT littermates fed a normal or a high-fat diet (Figures 3M–3O and S2H). As a result, Ucp1 expression was higher in white adipose tissue of 9-month-old Adiponectin−/− mice, reflecting a “beiging” of white fat (Figure 3P) (Wu et al., 2012). Conversely, Ucp1 expression in brown fat was lower in Ppil−/−Adiponectin than in WT mice (Figure 3N). Since the sympathetic tone enhances bone resorption by increasing Rankl expression (Elefteriou et al., 2005), we speculate that the high sympathetic activity seen in Adiponectin−/− mice antagonizes the consequence on Rankl expression of the absence of adiponectin signaling in osteoblasts. This may explain the overall normal bone resorption noted in young Adiponectin−/− mice.

If the increase in sympathetic activity explains the low bone mass of older Adiponectin−/− mice, normalizing it should result in a high-bone-mass phenotype because of the absence of adiponectin signaling in osteoblasts. This assumption was tested by removal of one allele of Dopamine β-hydroxylase (Dbh), the gene encoding the initial enzyme in catecholamine biosynthesis, from Adiponectin−/− mice (Thomas et al., 1995). That Ucp1 expression in brown fat, norepinephrine content in the brain, and urinary epinephrine elimination were similar in Adiponectin−/−;Dbh+/− and control littermates verified that the sympathetic activity was normalized in the compound mutant mice (Figures S3A–S3C). Nine-month-old Adiponectin−/−;Dbh+/− mice presented an increase in bone mass secondary to an increase in osteoblast numbers and bone formation rate (Figures 4A–4C and S3E). At the same time, energy expenditure and fat-pad and body weights were normal in these compound mutant mice (Figures 4E–F and S3D). That normalization of sympathetic tone in 12-week-old Adiponectin−/− mice also normalized GTTs and GSIS tests indicates that the decrease in insulin secretion seen in the absence of adiponectin is secondary to the increase in sympathetic activity (Figures S3F and S3G).

To determine whether it is by signaling in osteoblasts that the sympathetic nervous system antagonizes the local effect of adiponectin, we generated Adiponectin−/− mice lacking one copy of Adrb2, the adrenergic receptor mediating sympathetic signaling in osteoblasts (Takeda et al., 2002), in these cells only. Three-month-old Adiponectin−/−;Adrb2+/− mice had significantly higher bone mass than did Adiponectin−/− mice (Figure 4G). Taken together, these experiments support the notion that adiponectin favors bone mass accrual and decreases energy expenditure by inhibiting sympathetic signaling in osteoblasts. This mechanism of action eventually masks the consequence of the absence of signaling of this hormone on osteoblasts.

Adiponectin Antagonizes Leptin Regulation of the Sympathetic Tone
Since adiponectin and leptin exert opposite influences on sympathetic activity, we wondered what might be the consequences of removing Adiponectin from ob/ob mice (Adiponectin−/−;ob/ob). Although not normalized, Ucp1 expression and urinary epinephrine levels were increased 2-fold in 6- and 10-week-old Adiponectin−/−;ob/ob compared to ob/ob mice (Figures 4H, S3I, and S3L). As a result, energy expenditure was significantly higher in Adiponectin−/−;ob/ob than in ob/ob mice at both 6 and 10 weeks of age (Figures 4I and S3M), and these mutant mice gained less weight and had lighter fat-pad weight than did ob/ob mice for as long as this was tested (Figures 4J, S3H, and S3K). Moreover, blood pressure and heart rate were normal in 6-week-old Adiponectin−/−;ob/ob mice (Figures 4K and 4L). Since the correction of the low sympathetic tone observed in ob/ob mice by Adiponectin deletion is only partial, the glucose metabolism abnormalities of ob/ob mice at 10 weeks of age remained unaffected (Figure S3J). These results indicate that adiponectin antagonizes, partially, the functions of leptin that are mediated by the sympathetic nervous system. Ten-week-old Adiponectin−/−;ob/ob mice also exhibited a lower bone mass in both axial and peripheral bones compared to ob/ob mice, a result consistent with the fact that adiponectin and leptin exert an opposite influence on the sympathetic tone (Figures 4M and 4N).
Adiponectin Does Not Use Known Signaling Pathways in Osteoblasts

To identify molecular mechanisms used by adiponectin to mediate its functions in animals fed a normal diet, we focused on osteoblasts, a cell type that can be cultured, and first asked whether any of its known receptors were involved.

AdipoR1 was expressed at similar levels in osteoblasts and skeletal muscle, whereas expression of AdipoR2 and T-cadherin was 10-fold lower in osteoblasts than in tissues in which they are abundantly expressed (Hug et al., 2004; Yamauchi et al., 2003) (Figures 5A–5C). We next generated mutant mouse strains lacking AdipoR1, AdipoR2, T-cadherin, or a combination of these genes in osteoblasts only and verified that we had efficiently deleted the gene(s) of interest in osteoblasts but not in other cell types (Figures S4C, S4D, S4G, S4H, S4K, and S4L). Unlike what was the case for Adiponectin−/− mice of the same age and maintained on the same genetic background, 12-week-old AdipoR1ab/fl−/−, AdipoR2ab/fl−/−, T-cadherinab/fl−/−, and AdipoR1ab/fl−/−;AdipoR2ab/fl−/− mice displayed normal bone mass, osteoblast number, osteoclast number, circulating osteocalcin levels, and Rankl expression (Figures 5D–5G, S4M, and S4N). Moreover, in cell culture, adiponectin induced Rankl expression equally well in fl/fl, AdipoR1−/−, AdipoR2−/−, AdipoR1−/−;AdipoR2−/−, and T-cadherin−/− osteoblasts (Figure 5H).

We also studied signaling pathways reported to mediate adiponectin functions. Adiponectin can induce AMP kinase (AMPK) phosphorylation in some settings (Yamauchi et al., 2002), yet it failed to do so in cultured osteoblasts (Figure 6A). This was not due to a poor quality of the osteoblast preparation since adiponectin induced Rankl expression and AICAR, the positive control in this experiment, induced robust AMPK phosphorylation in these cells (Figure 6A). Adiponectin has also been proposed to enhance ceramide synthesis in some target cells (Holland et al., 2011), but there was no significant increase in ceramide content in bones of Adiponectin−/− mice fed a normal diet (Figure 6B). Thus, the results presented here indicate that, in osteoblasts, adiponectin may signal through distinct signaling pathways.

Adiponectin Dual Regulation of Bone Mass Accrual Occurs by Decreasing FoxO1 Activity

To understand how adiponectin could regulate bone mass, we searched for a single signaling molecule regulating all three cellular events affected by adiponectin in osteoblasts, i.e., proliferation, apoptosis, and oxidative stress. The transcription factor FoxO1 affects these three cellular events in the opposite direction that does adiponectin (Rached et al., 2010), and five different lines of evidence indicate that it lies downstream of adiponectin signaling in osteoblasts.

First, adiponectin and insulin, a positive control, increases phosphorylation of FoxO1 in osteoblasts, an event that decreases its transcriptional activity (Bur以至于ng and Kops, 2002) (Figure 6C). Conversely, FoxO1 is less phosphorylated, i.e., more active in Adiponectin−/− than in WT bones (Figure 6D). Second, adiponectin, like insulin, decreases the quantity of FoxO1 present in the nuclear compartment of osteoblasts (Figure S5A). Third, adiponectin treatment of ROS17/2.8 osteoblastic cells decreases activity of a Luciferase gene driven by an artificial promoter containing multiple FoxO1 binding sites (Figure 6E). Fourth, expression of P16 and P19, two FoxO1 target genes in osteoblasts (Rached et al., 2010), was increased in Adiponectin−/− and decreased in pLiv-Adiponectin bones (Figure 6F). Fifth, we generated Adiponectin−/− mice lacking one allele of FoxO1 in osteoblasts only (Adiponectin−/−; FoxO1osb−/− mice). P16 and P19 expression was similar in the bones of Adiponectin−/−; FoxO1osb−/− and control littermates (Adiponectin−/−; FoxO1fl/fl), indicating that we had normalized FoxO1 activity in osteoblasts through this manipulation (Figure S5B). Bone mass, bone formation rate, and osteoblast and osteoclast numbers were all indistinguishable between controls and 3-month-old Adiponectin−/−; FoxO1osb−/− mice (Figures 6G). BrdU incorporation and cyclin accumulation verified that osteoblast proliferation was decreased in Adiponectin−/−; FoxO1osb−/− compared to Adiponectin−/− bones (Figures 6H and 6I), and as a result, osteocalcin circulating levels were normal in Adiponectin−/−; FoxO1osb−/− mice (Figures S5D and S5E). Hence, adiponectin affects osteoblast proliferation, apoptosis, and bone formation by decreasing FoxO1 activity in osteoblasts.

Since FoxO1 is expressed in neurons of the locus coeruleus, we asked whether it also lies downstream of adiponectin signaling in the brain by generating Adiponectin−/− mice lacking one allele of FoxO1 only in neurons of the locus coeruleus (Figure S5F). Adiponectin−/−; FoxO1LC−/− mice had normal sympathetic activity (Figure S5G) and normal energy expenditure, Ucp1 expression in brown fat, and fat-pad and body weights (Figures 6J–6L and S5H). Bone mass, osteoblast number,
Figure 4. Analysis of Adiponectin−/−;Dbh+/−, Adiponectin−/−;Adrb2osb+/−, and Adiponectin−/−;ob/ob Mice

(A) Bone histomorphometric analysis of 36-week-old Adiponectin−/−;Dbh+/− mice. WT (n > 10), Dbh+/− (n > 10), Adiponectin−/− (n > 10), and Adiponectin−/−;Dbh+/− (n > 10) are shown. *p < 0.05 between WT and Adiponectin−/−; **p < 0.05 between WT and Adiponectin−/−; Dbh+/−.

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and bone formation rate were significantly higher whereas osteoclast number, Rankl expression, and serum CTx levels were lower in Adiponectin−/−;FoxO1LC−/− than in Adiponectin−/− mice (Figures 6M–6O and S5I), indicating that FoxO1 is also part of the adiponectin signaling pathway in neurons of the locus coeruleus.

Adiponectin Affects FoxO1 Phosphorylation in a PI3-Kinase-Dependent Manner in Osteoblasts
In the last set of experiments, we used information presented above in an effort to identify a signaling cascade used by adiponectin in osteoblasts. Adiponectin did not induce cAMP production in osteoblasts, and inhibitors of signaling through G-protein-coupled receptor (GPCR) did not prevent adiponectin to induce Rankl expression in osteoblasts (Figures 7D, S6A, and S6B), thus suggesting that the putative adiponectin receptor in osteoblasts may not be a GPCR. On the other hand, that FoxO1 phosphorylation is often regulated by PI3-kinase-dependent events (Taniguchi et al., 2006) led us to test whether adiponectin signaling in osteoblasts rely on signaling events that can be elicited by PI3-kinase-dependent mechanisms.

In support of this hypothesis, we note that adiponectin, like insulin, could increase PI3 kinase activity in osteoblasts as measured by the accumulation of phosphatidylinositol 3-phosphate (Figure 7A) and induced phosphorylation of AKT (Figure 7B), a signaling molecule acting downstream of PI3 kinase (Engelman, 2009). Adiponectin-dependent phosphorylation of AKT and FoxO1 was abolished when osteoblasts were pre-treated with LY294002, a PI3 kinase inhibitor, but not by inhibitors of signaling through GPCR (Figures 7C–7E and S6B). Furthermore, adiponectin treatment of osteoblasts induced tyrosine phosphorylation of a protein with a molecular weight of close to 170 kDa (Figure 7F). These results indicate that adiponectin ability to regulate proliferation and apoptosis of osteoblasts in a FoxO1-dependent manner is determined, at least in part, by PI3 kinase activity.

DISCUSSION
This study, prompted by the regulation of Adiponectin expression by osteocalcin, showed that adiponectin regulates bone mass accrual through two opposite mechanisms and counteracts, partially, some of leptin’s functions. It also points toward a novel signaling mechanism for this hormone. We should emphasize that our results do not affect in any way conclusions reached by analysis of the function of this molecule in mice fed a high-fat diet and in other cell types.

Adiponectin Exerts Two Opposite Influences on Bone Mass Accrual
By showing that the inactivation of Adiponectin in otherwise unchallenged mice results in significant perturbations of their bone mass, our results identify the regulation of bone mass accrual by adiponectin as physiologically relevant. This regulation, consistent with the appearance of adiponectin with bone during evolution, is unusual because it relies on two mechanisms, one local and one central, that antagonize each other. On the one hand, adiponectin acts directly in osteoblasts to prevent their proliferation and increase their apoptosis; this is why young Adiponectin−/− mice display a high bone mass caused by an increase in bone formation parameters. Bone may not be the only tissue affected by peripheral actions of adiponectin in animals fed a normal diet; however, we note that the influence that adiponectin exerts on proliferation and apoptosis of osteoblasts was not observed in other cell types such as myoblasts and hepatocytes. Over time, however, this local effect is obscured by a second and more powerful mode of action of adiponectin, its ability to decrease activity of the sympathetic nervous system. This is why mice lacking Adiponectin develop, as they age, a severe low-bone-mass phenotype explained by the deleterious influence of a high sympathetic tone on bone mass accrual that is never seen in young mutant mice (Elefteriou et al., 2005; Takeda et al., 2002). This latter mode of action illustrates the importance of the sympathetic nervous system as a regulator of bone mass accrual (Guntur and Rosen, 2012) and suggests that an increase of adiponectin signaling through pharmacological means may prevent age-related bone loss by decreasing the sympathetic tone.

Remarkably, the two opposite modes of action of adiponectin target the same molecules. Through its signaling in osteoblasts, adiponectin inhibits osteoblast proliferation and CyclinD1 accumulation, and through its central signaling, it favors osteoblast proliferation and CyclinD1 accumulation in osteoblasts. Likewise, adiponectin increases Rankl expression in osteoblasts, but through its signaling in the brain and its effect on the sympathetic tone, it inhibits Rankl expression. The fact that the same hormone exerts opposite influences on the same physiological function as adiponectin does is, to the best of our knowledge, a rare future. The availability of a growing number of mutant mouse strains each lacking a specific hormone will allow testing of whether this is a more universal rule of endocrinology.

Adipocytes Secrete Two Hormones Exerting Opposite Influence on the Same Physiological Process
Adiponectin defines with leptin a group of adipokines that exert a significant regulation of bone mass in vivo. As a matter of

(B–F) Serum CTx levels, Rankl expression, Cyclin D1 accumulation, energy expenditure, and fat-pad weight in 36-week-old Adiponectin−/−;Dbh+/− mice. *p < 0.05 between WT and Adiponectin−/−; Dbh+/−; #p < 0.05 between Adiponectin−/− and Adiponectin−/−; Dbh−/−.
(G) Bone mass of 12-week-old Adiponectin−/−;Adrb2wt/wt mice.
(H) Ucp1 expression in 6-week-old Adiponectin−/−; ob/ob brown fat.
(I–L) Energy expenditure, fat-pad weight, blood pressure, heart rate in 6-week-old Adiponectin−/−; ob/ob mice.
(M and N) Bone histomorphometric analysis of 10-week-old Adiponectin−/−; ob/ob vertebral and femora. WT (n = 9), ob/ob (n = 8), Adiponectin−/− (n = 7), and Adiponectin−/−; ob/ob (n = 7) are shown, *p < 0.05 between WT and Adiponectin−/−; **p < 0.05 between WT and ob/ob; ***p < 0.05 between WT and Adiponectin−/−; #p < 0.05 between Adiponectin−/− and Adiponectin−/−; Dbh−/−; Dbh−/−; Dbh−/−; Dbh−/−. See also Figure S3.
fact, uncovering adiponectin regulation of bone mass accrual shows how this hormone counteracts, partially, leptin functions that are mediated by the sympathetic nervous system. Although we had hypothesized that hormone(s) opposing leptin’s influence on bone mass accrual would exist, the fact that it is also synthesized by adipocytes was unexpected. Thus, it appears that the adipocyte is a rare if not unique example of an endocrine cell secreting two hormones exerting exactly opposite influences on the same physiological functions. Again, the fact that many mutant mouse strains are now available to study the biology of most hormones should allow one to test whether this aspect of adipocyte biology is a specific feature of this cell type or whether it applies to other endocrine cells. In any case, the results presented in this study...
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underscore the importance of adipocytes in the control of bone mass accrual.

**Mediation of Adiponectin Regulation of Bone Mass Accrual**

The identification of adiponectin as a regulator of bone mass accrual begged the question of the nature of the signaling pathway(s) this hormone used in osteoblasts and neurons. Because this cell can be studied ex vivo more easily, we focused most of our work on osteoblasts.

A first surprising result was that deletion of any of the known receptors for adiponectin did not recapitulate the phenotype caused by the absence of ligand, nor did it prevent adiponectin from enhancing *Rankl* expression in osteoblasts. These results could have two explanations: either adiponectin signals through a different receptor, or it signals through the adiponectin receptor in osteoblasts, a complex made of known receptors such as AdipoR1 and a novel one. Of note, AdipoR1 and T-cadherin are also expressed in neurons of the locus coeruleus, where their functions will need to be investigated (Lein et al., 2007). A second surprising result was that adiponectin signaling does not rely on AMPK phosphorylation or ceramidase catabolism to fulfill its functions in osteoblasts. Instead, our analyses reveal that one event triggered by adiponectin in osteoblasts is to decrease the activity of the transcription factor FoxO1. That removal of one allele of FoxO1 in osteoblasts or neurons of the locus coeruleus from *Adiponectin*+/− mice sufficed to correct the bone phenotype or their high sympathetic activity and increased energy expenditure indicated that this molecular event is also critical for the local and central modes of action of adiponectin.

Using this information to identify an adiponectin-dependent signaling cascade in osteoblasts, we noticed the frequent involvement of FoxO1 downstream of PI3 kinase signaling (Engelman, 2009; Taniguchi et al., 2006). In agreement with this notion, adiponectin treatment of osteoblasts increases PI3 kinase activity and phosphorylates AKT in addition to FoxO1. Moreover, adiponectin-induced AKT phosphorylation was prevented by inhibition of PI3 kinase activity. Lastly, adiponectin phosphorylates at least one membrane protein on tyrosine residues. We are fully aware that, in absence of a bona fide receptor, these results are only suggestive. This being acknowledged, they nevertheless point toward similarities between adiponectin signaling in osteoblasts and signaling through a receptor tyrosine kinase that deserves further study.

In summary, this work provides evidence that adiponectin has specific functions in animals fed a normal diet. Whether the functions described here are the only ones of adiponectin remains to be determined. These functions, however, identify adiponectin as a major regulator of bone mass and energy metabolism and, in both cases, as an antagonist of leptin. Hence, they reveal an unanticipated complexity in the endocrine function of the adipocytes and illustrate how tight is the central control of bone mass and energy expenditure (Figure 7H).

**EXPERIMENTAL PROCEDURES**

**Mouse Generation**

All analyses were performed with male mice, except the bone histomorphometric analysis, which was performed in 24-week-old *Adiponectin*+/− mice that included both males and females. *Adiponectin*+/−, AdipoR1fl/fl, AdipoR2fl/fl, and T-cadherinfl/fl mice were generated using 129/Sv embryonic stem cells. The estimated percentage of each genetic background was based on the number of backcrossing with C57BL/6J WT, a1(I) collagen-cre, or DbhCre mice. Chimeric mice harboring a mutant allele of *Adiponectin* were crossed with C57BL/6J WT, and *Adiponectin*+/− progenies (estimated as C57BL/6J:129/Sv; 50%;50%) were backcrossed with C57BL/6J WT to obtain F2 generation *Adiponectin*+/− mice (C57BL/6J:129/Sv; 75%;25%). Analyses were performed with *Adiponectin*+/− and WT littermate mice obtained by intercrossing between F2-generation *Adiponectin*+/− mice. The *pLiv-Adiponectin* construct was obtained by insertion of full-length mouse *Adiponectin* cDNA into *pLiv-7* plasmid (Fan et al., 1994). Transgenic mice were generated by injection of the *pLiv-Adiponectin* construct into 129/Sv embryos, *pLiv-Adiponectin* founder mice were crossed with C57BL/6J WT mice, and F1 generation *pLiv-Adiponectin*+/− mice (C57BL/6J:129/Sv; 50%;50%) were crossed with C57BL/6J WT to obtain *pLiv-Adiponectin*+ and WT littermates (C57BL/6J:129/Sv; 75%;25%) for analyses. Chimeric mice harboring a mutant allele of AdipoR1, AdipoR2, or T-cadherin (Figures S4A, S4E, and S4I) were crossed with C57BL/6J WT, AdipoR1fl/fl, AdipoR2fl/fl, and T-cadherinfl/fl mice (C57BL/6J:129/Sv; 50%;50%) and were then crossed with C57BL/6J a1(I) collagen-cre mice to obtain the F2 generation (C57BL/6J:129/Sv; 75%;25%). Analyses were performed with AdipoR1+/− and littermate AdipoR1fl/fl mice; AdipoR2+/− and littermate AdipoR2fl/fl mice; and T-cadherin+/− and littermate T-cadherinfl/fl mice obtained from between F2-generation AdipoR1+/− and AdipoR1fl/fl, AdipoR2+/− and AdipoR2fl/fl, and T-cadherin+/− and T-cadherinfl/fl mice. Analyses of AdipoR1+/−;AdipoR2+/− were performed with AdipoR1+/−;AdipoR2+/−;AdipoR1fl/fl;T-cadherinfl/fl mice.

**Figure 6. FoxO1 Mediates Adiponectin Functions in Osteoblasts and Neurons**

(A) Western blot analysis of phospho-AMPK in WT osteoblasts treated with vehicle, 15 μg/ml adiponectin, or AICAR (0.5 mM) for 5 or 30 min.

(B) Ceramide contents in 6-week-old *Adiponectin*+/− bones.

(C and D) Western blot analysis of phospho-FoxO1 in WT and 6-week-old *Adiponectin*+/− bones and osteoblasts treated with vehicle, adiponectin, or insulin for 15 min.

(E) FoxO1 luciferase assay in ROS 17/2.8 cells treated with adiponectin for 24 hr.

(F) Expression of FoxO1 target genes in 12-week-old *Adiponectin*+/− or *pLiv-Adiponectin* bones.

(G) Bone histomorphometric analysis of 12-week-old *Adiponectin*+/−;FoxO1osb+/− bones. Controls (n = 8), *Adiponectin*+/− (n = 8), and *Adiponectin*+/−;FoxO1osb+/− (n = 6) are shown. p < 0.05 between controls and *Adiponectin*+/−;p < 0.05 between *Adiponectin*+/− and *Adiponectin*+/−;FoxO1osb+/−.

(H) BrdU incorporation in 10-day-old *Adiponectin*+/−;FoxO1osb+/− bones.

(I) CyclinD1 accumulation in 12-week-old *Adiponectin*+/−;FoxO1osb+/− bones.

(J) Energy expenditure of 36-week-old *Adiponectin*+/−;FoxO1osb+/− mice.

(K) Ucp1 expression in 36-week-old *Adiponectin*+/−;FoxO1osb+/− bones.

(L) Fat-pad weight of 36-week-old *Adiponectin*+/−;FoxO1osb+/− mice.

(M) Bone histomorphometric analysis of 36-week-old *Adiponectin*+/−;FoxO1osb+/− mice. Controls (n = 5), *Adiponectin*+/− (n = 6), and *Adiponectin*+/−;FoxO1osb+/− (n = 5) are shown. p < 0.05 between controls and *Adiponectin*+/−;p < 0.05 between *Adiponectin*+/− and *Adiponectin*+/−;FoxO1osb+/−.

(N) *Rankl* expression in 36-week-old *Adiponectin*+/−;FoxO1osb+/− bones.

(O) CTx levels of 36-week-old *Adiponectin*+/−;FoxO1osb+/− mice. See also Figure S5.
**Figure 7. Adiponectin Signaling in Osteoblasts**

(A) Accumulation of phosphatidylinositol 3-phosphate in osteoblasts treated with vehicle, adiponectin, or insulin (10 nM) in the presence PI3 kinase inhibitor of LY294002 (10 μM) for 5 min.

(B) Western blot analysis of phospho-AKT in osteoblasts treated with vehicle, adiponectin, insulin (10 nM), or PTH (10 nM) for 5 min.

(C) Western blot analysis of phospho-AKT and phospho-FoxO1 in the presence of LY294002 (10 μM) for 5 min (P-AKT) and 15 min (P-FoxO1).

(D and E) Rankl induction and phosphorylation of AKT by adiponectin in the presence of Gs inhibitor (NF449), Gi inhibitor (NF023), Gβg inhibitor (Gallein), or PLC inhibitor (ET-18-OCH3).

(F) Western blot analysis of phospho-tyrosine in osteoblasts treated with vehicle, adiponectin, or PTH (10 nM) for 2 min. Arrowhead, the phosphorylated band around 170 kDa.

(G) Evolution of the bone phenotype of Adiponectin−/− mice overtime.

(H) Schematic representation of the diverse functions exerted by adiponectin in mice fed a normal diet and of its two sites of action. See also Figure S6.
between Adiponectin+/− and littermate AdipoR1+/−;AdipoR2−/− mice obtained by intercrosses between F2-generation AdipoR1+/−;AdipoR2−/− mice (C57BL/6J;129/Sv; 75%:25%). Dbh+− mice were previously reported (Thomas et al., 1995). Analyses of Adiponectin+/−;Dbh−/− mice were performed with littermate WT, Dbh−/−, and Adiponectin−/− mice as controls generated by intercrosses between Adiponectin+/−;Dbh−/− mice (C57BL/6J;129/Sv; 87.5%; 12.5%). ob/ob mice were obtained from Jackson Laboratory. Analyses of Adiponectin−/−;ob/ob mice were performed with littermate WT, Adiponectin−/−, and ob/ob mice as controls generated by intercrosses between Adiponectin+/−;ob/+ mice (C57BL/6J;129/Sv; 93.75%:6.25%). FoxO1LC−/− mice were previously reported (Rached et al., 2010). Analyses of Adiponectin−/−;FoxO1LC−/− mice were performed with littermate FoxO1B−/− (controls in figures) and Adiponectin−/−;FoxO1B−/− (Adiponectin−/− in the figures and the main text) (C57BL/6J;129/Sv; 93.75%:6.25%) as controls. Analysis of FoxO1B−/− mice was performed with FoxO1B−/− littermates as controls (Pure C57BL/6J). FoxO1LC−/− mice were generated by crossing of FoxO1B−/− mice with Dbh-Cre transgenic mice (Kobayashi et al., 2004). Analyses of Adiponectin−/−;FoxO1LC−/− mice were performed with FoxO1LC−/− littermates and FoxO1LC−/− and Adiponectin−/−;FoxO1B−/− (presented as Adiponectin−/− in the figures and the main text) as controls. Normal diet and high-fat diet were obtained from LabDiet (calories provided by protein, 24%; fat, 13%; carbohydrates, 62%) and Research Diets (calories provided by protein, 16%; fat, 58%; carbohydrate, 25%), respectively. All procedures involving animals were approved by CUMC IACUC and conform to the relevant regulatory standards.

**Binding Assay**

Adiponectin−/− mice were implanted subcutaneously with 14 day osmotic pump (Alzet) filled with a solution of recombinant full-length adiponectin or vehicle (5 μg/day). After 7 days of infusion, blood vessels were injected with PBS for extensive wash in order to remove remaining blood, and brain regions were dissected. Tissues were homogenized in ice-cold PBS, and soluble fractions were used to measure adiponectin. For the binding assay, Adiponectin−/− brains were snap frozen in liquid nitrogen, and 20-μm-thick sections were prepared and desiccated overnight at 4°C under vacuum. On the following day, sections were rehydrated in ice-cold binding buffer (50 mM Tris-HCl [pH 7.4], 10 mM MgCl2, 0.1 mM EDTA, and 0.1% BSA) for 15 min and incubated for 1 hr at room temperature in the presence of biotinylated adiponectin (2 μg/ml) or biotinylated GST (2 μg/ml) and in the presence of 50-fold excess of nonbiotinylated adiponectin (100 μg/ml) or nonbiotinylated GST (100 μg/ml). After being washed in harvesting buffer (50 mM Tris-HCl [pH 7.4]), samples were fixed in 4% paraformaldehyde for 15 min, washed in PBS, and incubated with goat anti-biotin antibody (1:1000, Vector laboratories) and anti-DBH antibody (1:4000, Abcam) overnight at 4°C. The signal was visualized by incubation of anti-goat IgG Cy-3 (1:200, Jackson ImmunoResearch) and anti-rabbit-Alexa488 (1:500, Invitrogen) with a Leica DM 5000B microscope (Leica).

**Statistical Analyses**

Results are given as means ± SEM. Statistical analyses were performed via ANOVA and/or Student’s t test.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.04.009.

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REFERENCES


Cell Metabolism

Adiponectin Functions in Animals Fed a Normal Diet


Adiponectin Regulates Bone Mass via Opposite Central and Peripheral Mechanisms through FoxO1

Figure S1. Related to Figure 1. Regulation of Rankl expression by adiponectin and phenotypes of young Adiponectin-/− mice. (A) Rankl expression in WT osteoblast populations treated with vehicle or globular adiponectin for 4 hours. (B) μCT analysis of 6 week-old Adiponectin-/− bones. Tb.Th: trabecular thickness. BMD: bone mineral density. TMD: tissue mineral density. (C) Insulin tolerance test in Adiponectin-/− mice at 6 and 12 week-old. (D) Gene expression analysis of 12 week-old Adiponectin-/− bones. (E) Adiponectin expression in osteocytes. 36 week-old WT femora were dissected, bone marrow flushed out, flushed femora repeatedly incubated with collagenase and EDTA to remove osteoblasts and osteoclasts. Total mRNAs were extracted from non-treated or treated femora and qPCR analysis performed for osteocyte-specific marker Sost, and Adiponectin. (F) In situ hybridization of P1 femora using probes against Adiponectin or Osteocalcin. (G) In situ hybridization of P16.5 embryos using a probe against Adiponectin. FD: Fat depot. SC: Spinal cord (H) Rankl expression in WT osteoblast populations derived from calvariae, or derived from bone marrow stroma cells treated with vehicle or 5 μg/ml full-length adiponectin for 2, 4 and 8 hours. For all panels, results are given as means ± standard error of mean (SEM). *, p < 0.05 by ANOVA and/or student t-test. NS: not significant.
Figure S2. Related to Figure 3. Phenotypes of older Adiponectin-/- mice. (A) Bone morphometric analysis of 24 week-old female WT (n=6) or Adiponectin-/- mice (n=5). (B) μCT analysis of 36 week-old WT (n=5) or Adiponectin-/- (n=5) bones. (C) Food intake of 36 week-old Adiponectin-/- mice. (D) Mc4r, Npr, and Cart expression of 36 week-old WT and Adiponectin-/- hypothalami. (E) Body weights of 6 and 24 week-old Adiponectin-/- mice. (F) Fat pad weight of 10 week-old Adiponectin-/- mice fed a high-
fat diet for 5 weeks. (G) Energy expenditure of 10 week-old Adiponectin-/- mice fed a high-fat diet for 5 weeks. (H) $Ucp1$ expression 10 week-old in Adiponectin-/- brown fat under a high-fat diet for 5 weeks. (I) Bone histomorphometric analysis of 10 week-old Adiponectin-/- mice fed a high-fat diet for 5 weeks.
Figure S3. Related to Figure 4. Phenotypes of Adiponectin-/--;Dbh+-/ and Adiponectin-/--;ob/ob mice. (A) Ucp1 expression in 36 week-old Adiponectin-/--;Dbh+-/ brown fat. (B) Norepinephrine levels in 36 week-old Adiponectin-/--;Dbh+-/ brainstem. (C) Urinary epinephrine levels in 36 week-old Adiponectin-/--;Dbh+-/ mice. (D) Body weight of 36 week-old Adiponectin-/--;Dbh+-/ mice. (E) Cyclin expression of 36 week-old Adiponectin-/--;Dbh+-/ bones. (F-G) Glucose tolerance test (2g glucose/kg body weight) and Glucose stimulated-insulin secretion test in 12 week-old Adiponectin-/--;Dbh+-/ mice. *, P<0.05 between WT and Adiponectin-/--; Dbh+-/ mice. (H) Body weight gain of Adiponectin-/--;ob/ob mice. *, P<0.05 between WT and ob/ob, #, P<0.05 between Adiponectin-/--; Dbh+-/ and ob/ob. #, P<0.05 between WT and Adiponectin-/--;ob/ob, $, P<0.05 between WT and Adiponectin-/--;ob/ob and ob/ob. (I) Urinary epinephrine levels in 6 week-old Adiponectin-/--;ob/ob mice. (J) Glucose tolerance test (1g glucose/kg body weight) of 10 week-old Adiponectin-/--;ob/ob mice. *, P<0.05 between WT and ob/ob, #, P<0.05 between WT and Adiponectin-/--;ob/ob mice. (K) Fat pad weight of 10 week-old Adiponectin-/--;ob/ob mice. (L) Ucp1 expression in 10 week-old Adiponectin-/--;ob/ob brown fat. (M) Energy expenditure of 10 week-old Adiponectin-/--;ob/ob mice.
Figure S4. Related to Figure 5. Generation of AdipoR1osb-/-, AdipoR2osb-/-, and T-cadherinosb-/- mice. (A) Targeting strategy used to generate a floxed allele of AdipoR1. Locations of probes used for Southern blotting (5’ and 3’) and primers used for PCR to detect the mutated allele. (B) Southern blots showing germ-line transmission of the mutated allele. (C) Detection of AdipoR1 mutated allele in genomic DNA isolated from tissues of AdipoR1fl/fl α1(I) collagen-Cre (AdipoR1osb-/-) mouse. (D) AdipoR1 expression in AdipoR1osb-/- osteoblasts. (E) Targeting strategy used to generate a floxed allele of AdipoR2. Locations of probes used for Southern blotting (5’ and 3’) and primers used for PCR to detect mutated allele. (F) Southern blots showing germ-line transmission of the mutated allele. (G) Detection of AdipoR2 mutated allele in genomic DNA isolated from tissues of AdipoR2fl/fl α1(I) collagen-Cre (AdipoR2osb-/-) mouse. (H) AdipoR2 expression in AdipoR2osb-/- osteoblasts. (I) Targeting strategy used to generate a floxed allele of T-cadherin. Locations of probes used for Southern blotting (5’ and 3’) and primers used for PCR to detect mutated allele. (J) Southern blots showing germ-line transmission of the mutated allele. (K) Detection of T-cadherin mutated allele in genomic DNA isolated from tissues of T-cadherinfl/fl α1(I) collagen-Cre (T-cadherinosb-/-) mouse. (L) T-cadherin expression in T-cadherinosb-/- osteoblasts. (M) Serum levels of total and uncarboxylated osteocalcin in 12 week-old AdipoR1osb-/-, AdipoR2osb-/-, AdipoR1osb-/-;AdipoR2osb-/-, and T-cadherinosb-/- mice. (N) Rankl expression in 12 week-old AdipoR1osb-/-, AdipoR2osb-/-, AdipoR1osb-/-;AdipoR2osb-/-, and T-cadherinosb-/- bones.
Figure S5. Related to Figure 6. FoxO1 mediates adiponectin signaling in osteoblasts and neurons. (A) FoxO1 localization in WT osteoblasts treated with vehicle, adiponectin, insulin or PTH for 15 min. (B) Expression of FoxO1 target genes in 12 week-old Adiponectin-/-;FoxO1 osb+/− bones. (C) Bone histomorphometric analysis of 12 week-old FoxO1fl/+ (n=6) or FoxO1osb+/− (n=7) mice. (D) Serum levels of total and uncarboxylated osteocalcin in 12 week-old Adiponectin-/-;FoxO1 osb+/− mice. (E) Serum levels of total and uncarboxylated osteocalcin in 12 week-old FoxO1osb+/− mice. (F) FoxO1 expression in locus coeruleus (LC). (G) Urinary epinephrine elimination of 36 week-old Adiponectin-/-;FoxO1LC+/− mice. (I) Body weight of 36 week-old Adiponectin-/-;FoxO1LC+/− mice. (J) CyclinD1, D2, and E1 expression in 36 week-old Adiponectin-/-;FoxO1LC+/− bones.
Figure S6. Related to Figure 7. cAMP production of osteoblasts treated with vehicle, adiponectin or PTH (10nM) for 30 min. (B) Inhibition of PTH-induced Rankl, or Nfatc1 by NF449, or Gallein and ET18OCH₃, and Serotonin-induced CyclinD1 by NF023.
Supplemental Experimental Procedures

High fat diet study
Adiponectin-/- or WT littermates were fed a high-fat diet (Calories, Protein 16%, Fat 58%, Carbohydrate 25%) from the age of 5 weeks, for 5 weeks, and analyses were performed at 10 week-old.

Bone histomorphometry analysis
Bone histomorphometry analysis was performed as previously described (Yadav et al., 2008). Briefly, lumbar vertebrate, or distal femur and femoral midshaft were dissected, fixed, dehydrated, and embedded in methyl metacrylate (MMA). Von Kossa, toluidine blue, tartrate-resistant acid phosphatase (TRAP) stainings were used to measure bone volume over tissue volume (BV/TV), osteoblasts and osteoclasts number respectively. Bone formation rate (BFR) was analyzed by the calcein double-labeling method (Amling et al., 1999). Quantitative analysis was performed using Osteomeasure Analysis system (Osteometrics).

μCT Analysis
Trabecular bone architecture of distal tibia was assessed by using a mCT system (VivaCT 40) Tibia bone specimen was stabilized with gauze in a 2-mL centrifuge tube filled with 70% ethanol and fastened in the specimen holder of the μCT scanner. One hundred μCT slices, corresponding to a 1.05-mm region distal from the growth plate, were acquired at an isotropic spatial resolution of 10.5 μm. A global thresholding technique was applied to binarize gray-scale μCT images where the minimum between the bone and bone marrow peaks in the voxel gray value histogram was chosen as the threshold value. The trabecular bone compartment was segmented by a semiautomatic contouring method and subjected to a model-independent morphological analysis (Hildebrand et al., 1999) by the standard software provided by the manufacturer of the scanner.

Bioassays
Intra cellular reactive oxygen species (ROS), and Annexin V positive cells were measured by using an oxidation sensitive fluorescent probe dye, 2′,7′-dichlorodihydrofluorescin diacetate (H2DCFDA, Sigma), and Annexin V-FITC apoptosis detection kit (BD pharmeringen) respectively. Bone marrow cells were flushed by PBS, and 50,000 cells were incubated with goat anti-osteocalcin antibody (Santacruz) for 20 min at 4°C. Cells were then washed twice with FACs buffer (PBS containing 2% FBS) and incubated with anti-goat alexa-680 (Invitrogen) for 20 min at 4°C. Cells were washed with FACs buffer twice and incubated with either 10 μM H2DCFDA for ROS, or FITC Annexin V for 30 min at 37°C. The fluorescence was measured by LSRII cytometer (BD biosciences). Osteoblastic cells were identified as a CD45 low, osteocalcin positive population and data analysis was performed with FloJo software (Treestar).

Metabolic study
For insulin tolerance test (ITT), mice were fasted for 4 hours then injected IP with insulin (0.75U /Kg BW), blood glucose levels were measured at indicated time. Food intake was
measured using metabolic cages (Nalgene) as the daily change of powdered chow weight. For glucose tolerance test (GTT), mice were fasted for 16 hours then injected IP with glucose (1g/Kg BW for Adiponectin-/-;ob/ob study, and 2g/Kg BW for all the other experiments), blood glucose levels measured at indicated time. For glucose-stimulated insulin secretion test, mice were fasted 16 hours then injected IP with glucose (3g/Kg BW), blood were collected at indicated time and insulin levels measured by ELISA (Crystal Chem). Energy expenditure, oxygen consumption (VO₂), carbon dioxide consumption (VCO₂) were measured by indirect calorimetric method using a six-chamber Oxymax system (Columbus Instruments), the data were analyzed by Oxymax software (Columbus Instrument).

**Biomechanical testing**
The loading protocol includes a 5 N preload for 3 min followed by a continuous load at 0.005 mms⁻¹ until failure. Displacement and mechanical load were recorded and data was processed to determine the ultimate load (N) of each femur.

**Recombinant protein**
Full-length mouse adiponectin cDNA reflecting residue 18-247 (full length) or cleaved form 107-247 (globular domain) were cloned into pCMV9 (Sigma), and transfected into HEK cells. HEK cells over-expressing full-length adiponectin or globular domain were cultured in DMEM (Invitrogen) containing 10% FBS up to confluence. Cells were washed with PBS twice, medium was replaced by DMEM containing 0.1% BSA, supernatant was collected and subjected to affinity purification using anti-Flag M2 affinity gel (Sigma) Recombinant protein was eluted using three times repeat flag peptides (sigma) and buffer was replaced by PBS.

**Heart beat and blood pressure**
The mice were sedated with an i.p. injections of pentobarbital (50 mg/kg). A tracheostomy was performed with an 18 G cannula and the mouse was connected to a small animal ventilator, Flexvent (Scireq). ECG leads connected to the Flexvent were placed on the left forelimb and both hind-limbs. The right carotid artery was cannulated and connected to the blood pressure transducer connected to the Flexvent. After the mice were ventilated for 10 min to allow them to acclimate the heart rate and mean arterial pressure were recorded three times, each time three minutes apart. The data was analyzed by Flexvent 5.2 software.

**Brain norepinephrine measurement**
HPLC was carried out on brainstem with a modification of the method described previously (Underwood et al., 1999). Concentrations of biogenic amines and metabolites were measured using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection. The dissected brain samples in a 1.5-ml microfuge tube were homogenized in 0.5-1.0 ml of 0.4M perchloric acid with an Ultra Cell Disruptor (Microson). The homogenate was centrifuged for 5 minutes at 14,000g in cold room and a 50 μl of typically four-fold diluted aliquot of the supernatant was injected over the HPLC system. The HPLC system, equipped with Waters Millennium software, consists of a Waters 515 HPLC pump, a Waters 717 Autosampler, a Varian Microsorb 100-5 C18
reverse-phase column (DYNAMAX 150x4.6 mm) attached with a Guard column (4.6 mm) and an ESA Coulouchem electrochemical detector (Model 5100A) with a guard cell (Model 5020) and a dual analytical cell (Model 5011A). The electrochemical detector was set at potential of +0.05 for the first cell and +0.5V for the second cell. The mobile phase contained 0.75 mM sodium phosphate (pH 3.1), 1.4 mM 1-Octanesulfonic acid, 10 μM sodium EDTA and 8% acetonitrile. The mobile phase was filtered through a Millipore 0.22 μm filter (Type GV) and degassed in vacuo. The flow rate was maintained at 0.8 ml/min. A chromatography software package (Waters Millennium) was used for data acquisition and analysis. Values are calculated based on peak area and compared to standard solutions. The inter- and intra-assay coefficients of variation of the assay were each less than 5%. The sensitivity of the assay was less than 0.5 pmol/injection. No effect of storage time was detected.

**Ceramide measurement**

For analysis of lipids, bone was snap frozen in liquid nitrogen and mechanically crushed prior to further homogenization in 20 mM Tris buffer containing protease inhibitors with an auto homogenizer. Following homogenization, samples were centrifuged at 300xg for 5 min to remove debris and supernatants were transferred to fresh microtubes. Aliquots of homogenate were removed for estimation of protein concentration by the Bradford assay, and separate aliquots (2-300ul containing a range of 35-200ug protein) were analyzed for SM and ceramide levels by tandem LC/MS mass spectrometry as described (Bielawski et al., 2010). Lipid levels measured were normalized to cellular protein.

**Cell culture**

Preparation of primary osteoblasts, and osteoclasts was as previously described (Ducy et al., 2000; Ferron et al., 2010a). For osteoblasts, 3 day-old mice were sacrificed and calvariae digested in 1 mg/ml of collagenase (Warthington) for 1 hour at 37 °C. Filtered cells were plated in α-MEM (Invitrogen) containing 10% FBS. For detection of cleaved-caspase 3, osteoblasts were fasted in serum free medium for 48 hours, treated for 24 hours with indicated amount of adiponectin or H2O2 (100µM). For detection of phosphatidylinositol 3-phosphate, osteoblasts were cultured overnight in α-MEM containing 0.1% FBS, washed with PBS twice, incubated in α-MEM containing 0.1% BSA and 10 mM Hepes pH 7.9 for 2 hours and treated with either insulin or adiponectin in the presence of LY294002 (10μM). Acidic lipids were extracted and phosphatidylinositol 3-phosphate levels measured by ELISA (Millipore) according to manufacture’s recommendation. For detection of phospho-FoxO1, phospho-AMPK, phospho-AKT, or phospho-tyrosine, osteoblasts were serum starved overnight in α-MEM containing 0.1% FBS, washed with PBS twice, incubated in α-MEM containing 0.1% BSA and 10 mM Hepes pH 7.9 for 2 hours (for phospho-tyrosine detection 1mM NaVO3 was added), and treated with indicated amount of adiponectin, AICAR (0.5mM), insulin (10nM), or PTH (10nM). Incubation times were as follows; phospho-tyrosine (2 min), phospho-AKT (5 min), phospho-FoxO1 (15min), phospho-AMPK (5 min and 30 min). For inhibition assay, cells were pre-incubated with either PI3 kinase inhibitor LY294002 (10 μM) for one hour, Gs inhibitor NF449 (10 μM) for 30 min, Gi inhibitor NF023 (10 μM) for 30 min, Gβγ inhibitor Gallein (10 μM) overnight, ET-18-OCH3 (10 μM) overnight. For gene expression, osteoblasts were cultured in α-MEM containing 10%
FBS supplemented with 5 mM b-glycerophosphate, and 100 μg/ml ascorbic acid for 7 days. Cells were serum-starved overnight in α-MEM containing 0.1% FBS supplemented with 5 mM b-glycerophosphate, and 100 μg/ml ascorbic acid, and treated with indicated concentration of adiponectin for 2, 4 and 8 hours. Bone marrow-derived osteoblasts were prepared as previously described (Ferron et al., 2010a). Briefly, two week-old WT mice were sacrificed, and bone marrow collected by flushing tibiae with PBS. 1x10^6 non-nucleated cells were plated in 6-well cell culture plates and cultured in α-MEM containing 20% FBS for 7 days. Then cells were cultured in α-MEM containing 10% FBS supplemented with 5 mM b-glycerophosphate, and 100 μg/ml ascorbic acid, 10 nM dexamethasone for 14 days. Cells were cultured in α-MEM containing 0.1% FBS supplemented with 5 mM b-glycerophosphate, and 100 μg/ml ascorbic acid, 10 nM dexamethasone for overnight, and treated with indicated amount of adiponectin for 2, 4 or 8 hours. For osteoclasts, 4 week-old mice were sacrificed, and bone marrow was flushed by cold PBS. After dissociating by pipetting, filtered and purified with lymphocyte separation medium (ICN Bio medicals), washed with PBS twice and plated in α-MEM containing 10% FBS supplemented with 30 ng/ml of recombinant Rankl (R&D) and 10 ng/ml of recombinant M-CSF (Sigma) for 6 days. For gene expression, osteoclasts were cultured in 0.1% FBS for 4 hours, treated with indicated amount of adiponectin for 4 hours. Primary hepatocyte isolation was performed with a two-step perfusion protocol. Anesthetized mouse was perfused with Hanks balanced salt solution (HBSS) containing 10 mM HEPES and 0.5 mM EGTA through portal vein. Collagenase solution (HBSS, 10 mM HEPES, 4 mM CaCl_2, trypsin inhibitor (Sigma), collagenase (Wako Pure Chemical Industries)) was then perfused. Liver was dissected, minced, and filtered. Filtered hepatocytes were washed 4 times with HBSS containing 10 mM HEPES and plated on collagen-coated plates with William’s medium E (Invitrogen) containing 10% FBS, 2 mM L-glutamine, 0.1 mM insulin, 1 mM dexamethasone. Primary myoblasts were isolated from limbs of 2 day-old mice. Muscle tissue was obtained from sacrificed mice. The tissue was minced and incubated with collagen solution (HBSS containing 2.5 mM CaCl_2, collagenase D (Roche)), dispase II (Roche) at 37°C. Slurred tissue was filtered and pre-plated on plates with F-10 based growth medium (F-10 (Invitrogen) with 20% FBS, 2.5 ng/ml fibroblast growth factor, penicillin/streptomycin). After 2 hours, plates were gently shaken and floating cells re-plated on collagen-coated plates. HEK cells were maintained in DMEM containing 10% FBS. ROS 17/2.6 cells were maintained in DMEM/F12 (1:1) medium (Invitrogen) containing 10% FBS.

**Osteocyte gene expression**

Osteocytes were enriched as described (Halleux et al., 2012). Briefly, 9-month old WT mice were sacrificed, their femora dissected and bone marrow flushed by DEPC-PBS. These femora were treated with 2 mg/ml of collagenase (Type II, Worthington) in α-MEM at 37°C for 20 min with gentle agitation, washed with PBS, incubated with 5 mM EDTA in PBS containing 0.1% BSA for 20 min. Femora were again incubated with 2 mg/ml of collagenase for 37°C for 1 hour, rinsed with PBS and then incubated with 5 mM EDTA in PBS containing 0.1% BSA for 45 min. Total RNA were isolated from treated or non-treated femora and Sclerostin or Adiponectin expression were measured by qPCR.
BrdU incorporation

For BrdU incorporation in bones, 10 day-old mice received 50 μg/g body weight and were sacrificed 12 hours later. Calvariae were dissected and fixed in 4% paraformaldehyde for 24 hour, followed by decalcification in 14% EDTA for 48 hours. Calvariae were then infiltrated in 20% sucrose for 24 hour and embedded in OCT compound. 5 μm thick sections were fixed with 4% paraformaldehyde for 20 minutes, washed 3 times with PBS, treated with 2N HCl for 30 minutes at 37 °C, washed 3 times with PBS, blocked with 5% donkey serum in PBS for 30 minutes at 37 °C. Anti-BrdU antibody (Sigma) was incubated for 2 hours at 37 °C in humidified chamber, and washed 3 times with PBS. Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch) was incubated for 30 minutes at 37 °C, and washed 3 times. Slides were mounted with DAPI-contained mounting solution (Electron Microscopy Sciences). For in vitro BrdU incorporation, primary osteoblasts, hepatocytes, or myocytes were plated at a density of 2.0x 10^3 cells/cm^2 in growth medium. 24 hours after plating, cells were fasted for 12 hours growth medium containing 0.1% FBS, then cells were stimulated with recombinant adiponectin together with 10% FBS. Twenty hours later, 5-Bromo-2'-deoxy-uridine were added at 5 μg/ml in the medium. After 4 hours of incorporation, cells were fixed and immunostained using anti-BrdU antibody and DAPI. BrdU positive/DAPI positive cells were counted using NIH image software.

TUNEL assay

Mice were sacrificed at day 10. Calvariae were dissected and fixed in 4% paraformaldehyde for 24 hour, followed by decalcification in 14% EDTA for 48 hours. Calvariae were then infiltrated in 20% sucrose for 24 hour and embedded in OCT compound. 5 μm thick sections were fixed with 4% paraformaldehyde for 20 minutes and TUNEL assay was performed using ApopTag Peroxidates In Situ Apoptosis Detection Kit (Millipore) and following the manufacturer’s instructions. Briefly, terminal deoxynucleotidyl transferasae (TdT) was used to label the free 3’OH DNA termini with digoxigenin-conjugated nucleotides. Anti-digoxigenin peroxidase conjugate was incubated to detect the digoxigenin-nucleotidates and stained with 3,3’-diaminobenzidine substrate kit (Vector Laboratories). Counter staining was performed with toluidine staining. For cultured cells, cells were plated at density of 2.0 x 10^3 cells/cm^2 in growth medium. 24 hours after plating, cells were cultured for 24 hours in serum free medium, followed by treatment with indicated concentrations of adiponectin for 24 hours. TUNEL assay was performed using In Situ Cell Death Detection Kit (Roche). After fixing cells with 4% PFA and permeablizing with 0.1% Triton X-100 in 0.1% sodium citrate, TdT was labeled with fluorescein. Then, cells were mounted with DAPI-contained mounting solution.

Cyclin accumulation

For in vitro cyclin accumulation, cells were plated at density of 2.0 x 10^3 cells/cm^2 in growth medium. 24 hours after plating, cells were serum-starved for 12 hours in medium containing 0.1% FBS to synchronize the cell-cycle, then cells were treated with indicated amount of adiponectin in growth medium. 24 hours later, cells were lysed and subjected to western blotting.
Adiponectin measurement
Adiponectin-/- mice were implanted subcutaneously with 14-day osmotic pump (Alzet) filled with a solution of recombinant full-length adiponectin or vehicle (5 μg/day). After 7 days of the infusion, blood vessels were injected with PBS for the extensive wash to remove remaining blood and several brain regions were dissected. Tissues were homogenized in ice-cold PBS and soluble fractions used to measure adiponectin by ELISA.

Immunohistochemistry and In situ hybridization
E16.5 or P1 WT mice were sacrificed after cardiac perfusion with PBS and 4% paraformaldehyde, then whole embryo, left femora or brains were embedded in paraffin, sectioned 5 μm. Their brains were immunostained with anti-FoxO1 antibody (1:1000) or subjected to in situ hybridization with Dbh probe following standard protocol. Sagital section of embryos or longitudinal sections of femora were used for in situ hybridization with either Adiponectin or Osteocalcin probe.

Biochemistry and molecular study
Antibodies were obtained from Cell Signaling Technology, Inc. except anti-Cyclin E1 (Santacruz biotechnology), anti-BrdU (Sigma) anti-β-actin (Sigma), anti-Lamin A/C (BD biosciences), anti-DBH (Abcam) and anti-phospho-tyrosine (Millipore). RNA isolation, cDNA preparation and real-time PCR analyses were carried out following standard protocols. ELISA was used to measure mouse CTx (RatLaps, IDS), mouse total and uncarboxylated osteocalcin (Ferron et al., 2010b), malondialdehyde and 4-hydroxynonenal (Lpid peroxidation assay kit, Abcam), urinary epinephrine (Bi-Cat, ALPCO), creatinine (Metra creatinine kit, Quidel Corp), adiponectin (Millipore), cAMP (Cyclic AMP direct EIA kit, Assay Design), insulin (Chrystal Chem).
Supplemental References


