

Orexin Regulates Bone Remodeling via a Dominant Positive Central Action and a Subordinate Negative Peripheral Action

Wei Wei,¹ Toshiyuki Motoike,^{2,3,6} Jing Y. Krzeszinski,¹ Zixue Jin,¹ Xian-Jin Xie,^{4,5} Paul C. Dechow,⁷ Masashi Yanagisawa,^{2,3,6} and Yihong Wan^{1,4,*}

¹Department of Pharmacology

²Howard Hughes Medical Institute

³Department of Molecular Genetics

⁴Simmons Cancer Center

⁵Department of Clinical Sciences

University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

⁶International Institute for Integrative Sleep Medicine (WPI-IIS), University of Tsukuba, Tsukuba 305-8575, Japan

⁷Department of Biomedical Sciences, Baylor College of Dentistry, Texas A&M University Health Sciences Center, Dallas, TX 75246, USA

*Correspondence: yihong.wan@utsouthwestern.edu

<http://dx.doi.org/10.1016/j.cmet.2014.03.016>

SUMMARY

Orexin neuropeptides promote arousal, appetite, reward, and energy expenditure. However, whether orexin affects bone mass accrual is unknown. Here, we show that orexin functions centrally through orexin receptor 2 (OX2R) in the brain to enhance bone formation. OX2R null mice exhibit low bone mass owing to elevated circulating leptin, whereas central administration of an OX2R-selective agonist augments bone mass. Conversely, orexin also functions peripherally through orexin receptor 1 (OX1R) in the bone to suppress bone formation. OX1R null mice exhibit high bone mass owing to a differentiation shift from marrow adipocyte to osteoblast that results from higher osseous ghrelin expression. The central action is dominant because bone mass is reduced in orexin null and OX1R2R double null mice but enhanced in orexin-overexpressing transgenic mice. These findings reveal orexin as a critical rheostat of skeletal homeostasis that exerts a yin-yang dual regulation and highlight orexin as a therapeutic target for osteoporosis.

INTRODUCTION

Orexin-A and -B (also known as hypocretin-1 and -2) are neuropeptides produced in the lateral hypothalamus that stimulate wakefulness, feeding, thermogenesis, and reward behaviors (Sakurai, 2007; Sakurai and Mieda, 2011). They function through two receptors: OX1R and OX2R. Orexin deficiency in human and mice leads to narcolepsy, hypophagia, and obesity (Chemelli et al., 1999; Hara et al., 2001; Lin et al., 1999; Peyron et al., 2000; Sellayah et al., 2011). Hence, there is tremendous pharmacological interest in developing orexin-targeting small molecules for the treatment of sleep and metabolic disorders such as

insomnia (Brisbare-Roch et al., 2007), obesity, and diabetes (Funato et al., 2009; Kotz et al., 2012; Sellayah et al., 2011), some of which has completed Phase III clinical trials.

In vertebrates, the adult skeleton is continuously regenerated through bone remodeling. This is a dynamic process that tightly couples osteoblast-mediated bone formation with osteoclast-mediated bone resorption. Osteoblasts are derived from bone marrow mesenchymal stem cells (MSCs) that can also differentiate into marrow adipocytes, the balance of which is controlled by an array of hormones and transcription factors (Bianco et al., 2013; Wan, 2013). In contrast, osteoclasts are differentiated from macrophage precursors in response to receptor activator of NF- κ B ligand (RANKL), depending on the ratio of RANKL to osteoprotegerin (OPG), a RANKL decoy receptor that inhibits osteoclast differentiation (Novack and Teitelbaum, 2008).

Emerging evidence reveal that neuropeptides, such as neuropeptide U (NMU) and neuropeptide Y (NPY), modulate skeletal homeostasis via both central and peripheral functions (Rosen, 2008). However, whether orexin regulates bone mass accrual is unknown. This is an important question in light of the therapeutic potential of orexin modulators in several diseases. Using both genetic and pharmacological strategies, we uncover orexin as a yin-yang dual regulator: on one hand, orexin enhances bone formation via a primary OX2R- and leptin-mediated neuroendocrine control; on the other hand, orexin also suppresses bone formation via a secondary OX1R- and ghrelin-mediated local regulation of bone cell differentiation.

RESULTS

Orexin Deletion Causes Low Bone Mass and Decreased Bone Formation

To determine the physiological roles of orexin in skeletal remodeling, we examined the orexin knockout mice (OX-KO) (Chemelli et al., 1999). MicroCT (μ CT) analysis of the trabecular bone in the proximal tibia (Figure 1A) reveals that the OX-KO mice displayed a low bone mass phenotype. The trabecular bone volume/tissue volume ratio (BV/TV) was decreased by 38% in OX-KO

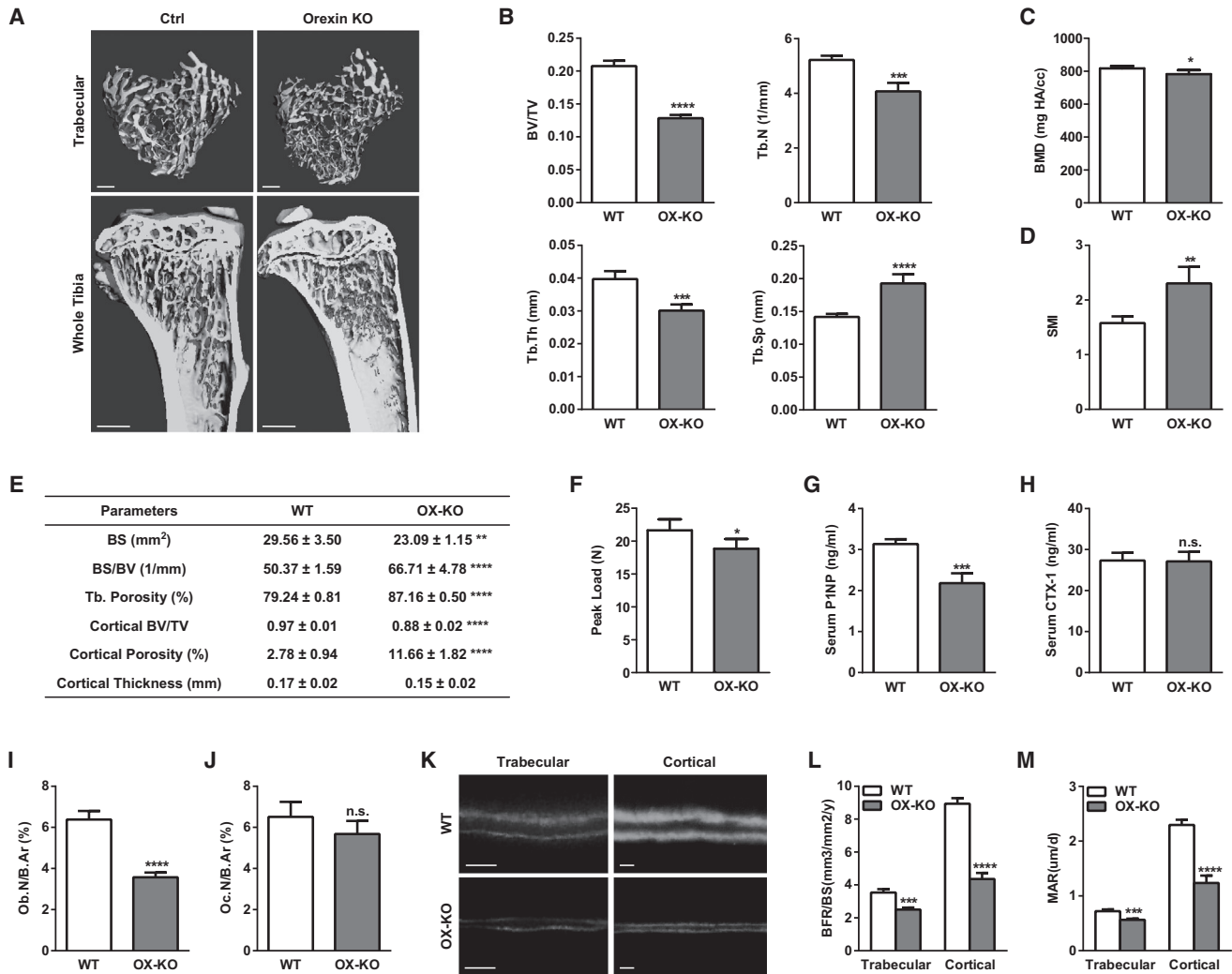


Figure 1. Orexin Deletion Causes Low Bone Mass and Decreased Bone Formation

(A–E) μ CT analysis of tibiae from OX-KO or WT controls (3 months old, male, $n = 9$). (A) Images of the trabecular bone of the tibial metaphysis (top) (scale bar represents 10 μ m) and the entire proximal tibia (bottom) (scale bar represents 1 mm). (B–D) Trabecular bone parameters. (B) BV/TV, bone volume/tissue volume ratio; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation. (C) BMD, bone mineral density. (D) SMI, Structure Model Index. (E) Additional trabecular and cortical bone parameters. BS, bone surface; BS/BV, bone surface/bone volume; Tb.Porosity, trabecular bone porosity.

(F) Three-point bending assay ($n = 9$).

(G) Serum P1NP ($n = 9$).

(H) Serum CTX-1 ($n = 9$).

(I and J) Static histomorphometry of osteoblast number (Ob.N/B.Ar) (I) and osteoclast number (Oc.N/B.Ar) (J) ($n = 9$); B.Ar, bone area.

(K–M) Dynamic histomorphometry (3 months old at endpoint, male, $n = 5$). (K) Images of the trabecular bone at metaphysis and the cortical bone at diaphysis. Scale bars represent 10 μ m. (L) Bone formation rate (BFR/BS). (M) Mineral apposition rate (MAR). Error bars represent SD.

See also [Figure S1](#).

compared to wild-type (WT) controls, accompanied by 22% less trabecular number (Tb.N), 24% less trabecular thickness (Tb.Th), 36% greater trabecular separation (Tb.Sp) (Figure 1B), and 4% lower bone mineral density (BMD) (Figure 1C). The Structure Model Index (SMI), which quantifies the 3D structure for the relative amount of plates (SMI = 0, strong bone) and rods (SMI = 3, fragile bone), was 46% higher (Figure 1D). Consistently, OX-KO mice had lower bone surface (BS), higher BS/BV ratio, and higher trabecular porosity (Tb.Porosity) (Figure 1E). Moreover, cortical BV/TV was also decreased, leading to higher cortical

porosity (Figure 1E). Consequently, three-point bending assay shows that the tibiae of OX-KO mice were weaker as the peak load at fracture was 13% lower (Figure 1F). Consistent with previous reports, body weight of OX-KO mice under chow diet was unaltered (Figure S1A available online).

Serum ELISA shows that the bone formation marker N-terminal propeptide of type I procollagen (P1NP) was 30% lower (Figure 1G), while the bone resorption marker C-terminal telopeptide fragments of the type I collagen (CTX-1) was unaltered (Figure 1H). Static histomorphometry shows that osteoblast number

was decreased (Figure 1I), whereas osteoclast number was unaltered (Figure 1J). Dynamic histomorphometry using double calcein labeling shows that OX-KO mice exhibited a lower bone formation rate (BFR/BS) and mineral apposition rate (MAR) in both trabecular bone at metaphysis and cortical bone (Figures 1K–1M). These results indicate that orexin augments bone mass mainly by promoting bone formation.

OX1R but Not OX2R Regulates Mesenchymal Stem Cell Differentiation

We next investigated whether the orexin regulation of bone mass is mediated by central and/or peripheral actions via OX1R and/or OX2R. Orexin, OX1R, and OX2R are all expressed in the brain (Sakurai, 2007), but it was unclear whether they are expressed in bone. We found that orexin and OX1R, but not OX2R, were expressed in mouse tibiae (Figures 2A, left, and S1B), indicating a specific local orexin regulation via OX1R. Tibial orexin expression, which was absent in OX-KO mice (Figure S1C), originated from MSCs, osteoblasts and marrow adipocytes but not macrophages or osteoclasts (Figures 2A, right, and S1D), suggesting an autocrine/paracrine regulation in the mesenchymal lineage.

OX1R expression was suppressed during osteoblast differentiation (Figures 2B and 2C) but elevated during adipocyte differentiation (Figures 2D and 2E). Again, OX2R was not expressed in either culture (Figures 2B and 2D). Marker gene expression confirmed complete differentiation (Figure S2). This indicates that OX1R may be proadipogenic and antiosteoblastogenic. Consistent with this notion, treatment with orexin-A (an agonist for OX1R and OX2R), but not orexin-B (an agonist for mainly OX2R), inhibited osteoblast differentiation (Figures 2F and S3A) and enhanced adipocyte differentiation (Figures 2G and S3B). Moreover, OX1R inhibitor (SB-408124) or OX1R2R dual inhibitor (ACT-078573) promoted osteoblast differentiation (Figures 2H and S3C) and attenuated adipocyte differentiation (Figures 2I and S3D), whereas OX2R inhibitor (compound 1) had no effect (Figures 2H, 2I, S3C, and S3D). In contrast, these inhibitors did not alter osteoclast differentiation (Figure 2J), in line with the absence of OX1R and OX2R expression in osteoclasts. An anti-OX-A antibody also increased osteoblast differentiation but decreased adipocyte differentiation (Figures S3E and S3F). These results indicate that activation of OX1R inhibits osteoblastogenesis from MSCs by favoring marrow adipogenesis.

OX1R Deletion Causes High Bone Mass and Increased Bone Formation

To investigate whether OX1R inhibits bone formation *in vivo*, we next analyzed OX1R-KO mice (Mieda et al., 2011). μ CT shows that OX1R-KO mice exhibited a high bone mass phenotype (Figure 2K). The trabecular bone in OX1R-KO mice had 40% higher BV/TV, 54% higher Tb.N, 29% higher Tb.Th, and 39% lower Tb.Sp (Figure 2L), as well as 5% higher BMD (Figure 2M) and 19% lower SMI (Figure 2N). Cortical bone BV/TV, porosity, and thickness were not significantly altered (Figure 2O). Moreover, OX1R-KO mice also had stronger bone as the peak load at fracture was 20% higher (Figure 2P). Serum P1NP was 37% increased (Figure 2Q, left), whereas serum CTX-1 was 35% decreased (Figure 2Q, right). Osteoblast number was higher; whereas marrow adipocyte number and osteoclast number were lower (Figure 2R). BFR/BS and MAR in the trabecular

bone at metaphysis was increased (Figure S2). Therefore, the high bone mass in OX1R-KO mice resulted from a combination of elevated bone formation and reduced bone resorption.

To determine the cellular mechanisms accounting for the higher bone formation in OX1R-KO mice, we next compared bone cell differentiation. Osteoblast differentiation from the marrow MSCs of OX1R-KO mice was enhanced compared to WT control mice, shown by the increased number of alkaline phosphatase⁺ (ALP⁺) cells, alizarin red⁺ cells and von Kossa⁺ cells (Figure 2T) and the higher expression of osteoblast markers including runx2, osterix, ALP, osteocalcin, and col1a1 (Figure 2U). In contrast, adipocyte differentiation was suppressed, shown by the decreased number of oil-red-o⁺ (ORO⁺) cells (Figure 2V) and the lower expression of adipocyte markers including PPAR γ 2, adiponectin and FABP4 (Figure 2W). These results are consistent with the findings in Figures 2B–2I, demonstrating that OX1R is antiosteoblastogenic but proadipogenic.

Because bone resorption was also decreased in OX1R-KO mice, we next compared osteoclastogenesis. When stimulated with the same concentration of RANKL, the bone marrow of OX1R-KO mice differentiated into osteoclasts to a similar extent as WT mice (Figure 2X), consistent with the results in Figure 2J. This indicates that the reduced bone resorption was caused by a non-cell-autonomous effect. We found that RANKL expression was lower (Figure 2Y), whereas OPG expression was higher (Figure 2Z) in OX1R-KO osteoblast differentiation cultures compared to WT cultures, indicating that the reduced bone resorption in OX1R-KO mice was mediated by a decreased RANKL/OPG ratio.

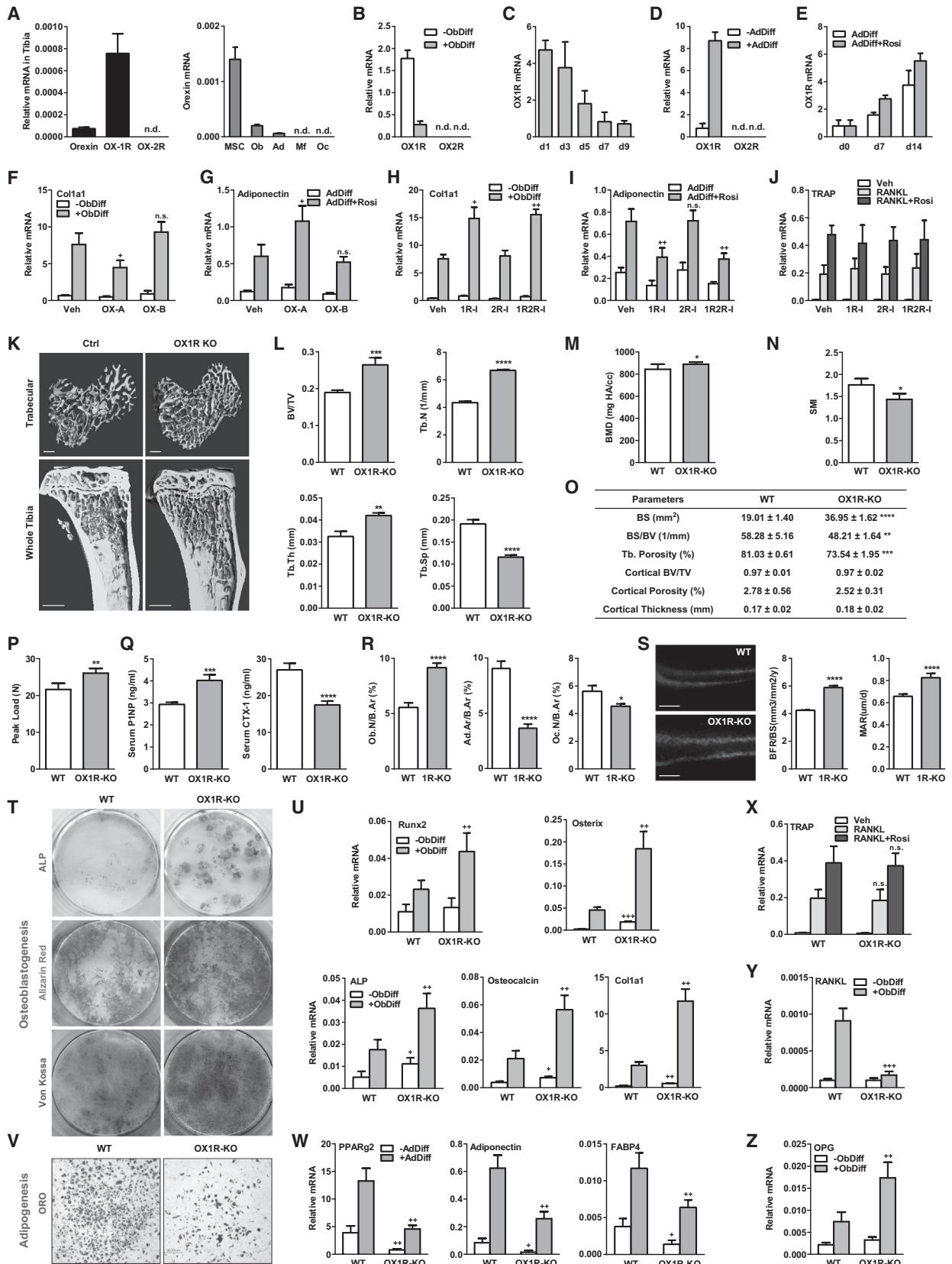
Together, these findings unexpectedly present a dichotomy where OX1R-KO mice exhibit a high bone mass that is the opposite of the low bone mass in the OX-KO mice, indicating that additional mechanisms may dominate over OX1R in orexin regulation of bone. Although OX2R is not expressed in bone, a possibility exists that OX2R may mediate a central regulation by orexin. Thus, we next investigated the consequences of OX2R deletion.

OX2R Deletion Causes Low Bone Mass and Decreased Bone Formation

μ CT shows that OX2R-KO mice (Willie et al., 2003) exhibited a low bone mass (Figures 3A–3E) as in the OX-KO mice, leading to a reduced peak load at fracture (Figure 3F). P1NP and osteoblast number were decreased, whereas CTX-1 and osteoclast number were unaltered (Figures 3G–3I). Consistently, BFR/BS and MAR were reduced in both males (Figure 3J) and females (Figure S4). In agreement with the lack of OX2R expression in bone, osteoblast and adipocyte (Figures 2A, 2B, and 2D), marrow MSCs from OX2R-KO mice displayed normal capacity to differentiate into osteoblasts (Figure 3K) and adipocytes (Figure 3L). These results show that OX2R deletion causes low bone mass that recapitulates the bone defects in OX-KO mice, due to a reduction in bone formation via potentially a central mechanism.

Central Administration of an OX2R-Selective Agonist Augments Bone Mass

To further test the hypothesis that OX2R acts centrally to regulate skeletal homeostasis, we performed intracerebroventricular



(legend on next page)

(i.c.v.) injection. An OX2R selective agonist (OX2R-AG) (Ala11, D-Leu15) orexin-B (Asahi et al., 2003) was continuously infused in the lateral ventricles of WT mice at 0.5 nmol/day for 35 days as described (Funato et al., 2009). μ CT shows that OX2R-AG remarkably enhanced bone mass (Figure 3M), leading to 66% higher BV/TV, 32% higher Tb.N, 30% higher Tb.Th and 33% lower Tb.Sp (Figure 3N); as well as 8% higher BMD (Figure 3O) and 25% lower SMI (Figure 3P). The cortical bone had 1% higher BV/TV and 30% less porosity (Figure 3Q). Moreover, OX2R-AG also increased bone strength as the peak load at fracture was 11% higher (Figure 3R). P1NP was 100% increased (Figure 3S), whereas CTX-1 was unaltered (Figure 3T). These results suggest that central activation of OX2R enhances bone formation and augments bone mass.

We next investigated whether OX2R-AG i.c.v. injection can serve as a therapeutic strategy to treat postmenopausal osteoporosis in an ovariectomy (OVX) mouse model. Three days after surgery, we began the i.c.v. infusion at 0.5 nmol/day for 35 days. Uterine weight was reduced by ~87% in all ovariectomized mice compared to sham controls, indicating effective estrogen depletion (Figure 3U). OVX-mediated reduction in P1NP was significantly abolished by OX2R-AG (Figure 3V); whereas OVX-mediated increase in CTX-1 was not significantly altered (Figure 3W). Moreover, OVX-mediated reduction in osteoblast number and bone formation rate was also rescued by OX2R-AG (Figure 3X). Consequently, OVX-induced bone loss was attenuated in OX2R-AG-treated mice (Figures 3X–3Z). This bone anabolic effect of OX2R-AG was abolished in OX2R-KO mice (Figure S5), confirming that it is OX2R dependent. These results suggest that central activation of OX2R may be a strategy to ameliorate bone degenerative diseases such as osteoporosis.

Central Action Is Dominant over Peripheral Action in Orexin Regulation of Bone

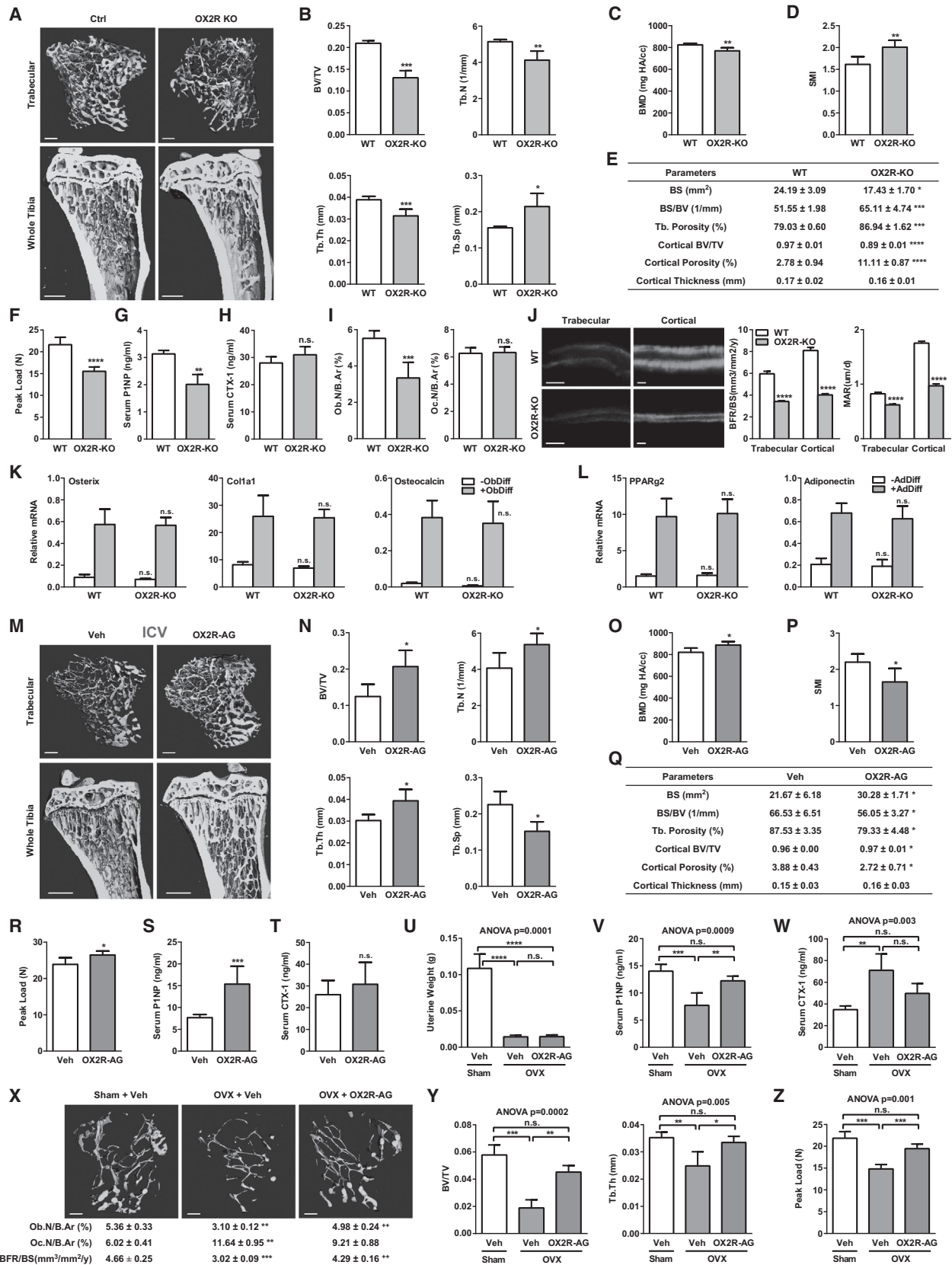
The observation that OX-KO mice exhibited a similar bone phenotype as OX2R-KO mice rather than OX1R-KO mice sug-

gests that the OX2R-mediated central control is dominant over the OX1R-mediated peripheral regulation. To further test this hypothesis, we analyzed the OX1R2R double knockout mice (1R2R-DKO). μ CT shows that 1R2R-DKO mice also exhibited a low bone mass (Figures 4A–4F). P1NP was decreased whereas CTX-1 was unaltered (Figures 4G and 4H). Osteoblast number, BFR/BS, and MAR were reduced whereas osteoclast number was unaltered (Figures 4I and 4J). OX1R2R double heterozygous mice did not exhibit any significant bone phenotype (Figure S6). These results indicate that simultaneous deletion of both OX1R and OX2R presents an OX2R null phenotype.

As a complimentary gain-of-function approach, we examined the effects of global orexin overexpression by analyzing the CAG/orexin-transgenic mice (OX-Tg) (Mieda et al., 2004). The pattern of orexin overexpression in the OX-Tg mice has been described (Funato et al., 2009; Mieda et al., 2004): ectopic orexin immunoreactivity was detected in several CNS regions as well as in a limited set of peripheral tissues, including thyroid gland, adrenal cortex, and some pancreatic islets, but not in other metabolic tissues such as brown and white adipose, liver, or skeletal muscle. In agreement, the percentage of brown adipose tissue weight in body weight was unaltered in OX-Tg mice ($0.306\% \pm 0.017\%$) compared with WT littermate controls ($0.302\% \pm 0.019\%$). Tibial orexin expression was elevated by 115% in the OX-Tg mice compared with controls (Figure S1B). μ CT shows that OX-Tg mice exhibited high bone mass (Figures 4K–4P). Bone formation was elevated as P1NP was 83% higher (Figure 4Q). Interestingly, chronic global orexin overexpression also suppressed bone resorption as CTX-1 was 31% lower (Figure 4R). Osteoblast number, BFR/BS and MAR were higher, adipocyte number was lower, and osteoclast number was not significantly altered (Figures 4S and 4T). Together, the results that bone mass is reduced in OX-KO and 1R2R-DKO mice but enhanced in OX-Tg mice strongly supports the notion that the OX2R-mediated central regulation is dominant over the OX1R-mediated peripheral modulation of bone cell differentiation.

Figure 2. OX1R Decreases Bone Mass via a Local Regulation of Bone Cell Differentiation

- (A) Left: expression of orexin, OX1R, and OX2R in mouse tibiae (bone + marrow) (n = 3); n.d., not detected. Right: orexin expression in various cell types (n = 10). MSC, mesenchymal stem cell; Ob, osteoblast; Ad, marrow adipocyte; mf, macrophage; Oc, osteoclast.
- (B) Expression of OX1R and OX2R in marrow osteoblast differentiation cultures (n = 3). ObDiff, osteoblast differentiation cocktail.
- (C) Expression of OX1R during a time course of osteoblast differentiation (n = 3); d, day.
- (D) Expression of OX1R and OX2R in marrow adipocyte differentiation cultures (n = 3). AdDiff, adipocyte differentiation medium.
- (E) Expression of OX1R during a time course of adipocyte differentiation (n = 3).
- (F and G) Effects of OX-A or OX-B (10 nM) on osteoblast (F) and adipocyte (G) differentiation from WT marrow (n = 3).
- (H–J) Effects of OX1R inhibitor (OX1R-I), OX2R inhibitor (OX2R-I), or OX1R2R dual inhibitor (1R2R-I) (10 nM) on osteoblast (H), adipocyte (I), and osteoclast (J) differentiation from WT marrow (n = 3).
- (K–O) μ CT analysis of tibiae from OX1R-KO or WT controls (3 months old, male, n = 9). (K) Representative images. (L–O) Trabecular and cortical bone parameters.
- (P) Three-point bending assay (n = 9).
- (Q) Serum P1NP (left) and CTX-1 (right) (n = 9).
- (R) Static histomorphometry (n = 9).
- (S) Dynamic histomorphometry of the trabecular bone at metaphysis (3 months old, male, n = 5).
- (T and U) Osteoblast differentiation from the bone marrow of OX1R-KO or WT control mice (n = 3). (T) Alkaline phosphatase (ALP, top), alizarin red (middle), or von Kossa (bottom) stained osteoblast differentiation cultures. (U) Expression of osteoblast markers (n = 3).
- (V and W) Adipocyte differentiation from the bone marrow of OX1R-KO or WT control mice (n = 3). (V) Oil red O (ORO) stained adipocyte differentiation culture. Scale bar represents 100 μ m. (W) Expression of adipocyte markers (n = 3).
- (X) Osteoclast differentiation quantified by TRAP expression (n = 3).
- (Y and Z) Expression of RANKL (Y) and OPG (Z) in the osteoblast differentiation culture (n = 3). “+” and “n.s.” in (F–I) compares treatment with vehicle control; “+” and “n.s.” in (U) and (W–Z) compares OX1R-KO with WT control under the same treatment conditions. Error bars represent SD. See also Figures S1–S3.



(legend on next page)

OX1R Inhibits Osteoblastogenesis by Suppressing Local Ghrelin Expression

We next set out to elucidate the molecular mechanisms underlying the local and central bone regulation by orexin. We found that the expression of ghrelin protein was markedly upregulated in the tibiae of OX1R-KO and 1R2R-DKO mice (Figure 5A). In contrast, the level of serum ghrelin protein, which largely derives from the stomach, was unaltered (Figure 5B). This suggests that OX1R regulation of ghrelin expression occurs locally in the bone. Indeed, ghrelin mRNA was also higher in the tibiae of OX1R-KO and 1R2R-DKO mice (Figure 5C).

Ghrelin expression was induced during osteoblast differentiation, which was enhanced in the culture from OX1R-KO mice compared to WT controls (Figure 5D). In contrast, ghrelin expression was reduced during adipocyte differentiation, which was also elevated in the culture from OX1R-KO mice compared to WT controls (Figure 5E). In line with these findings, the ghrelin induction during osteoblast differentiation was abolished by OX-A treatment but potentiated by OX1R inhibitor or OX1R2R dual inhibitor (Figure 5F). Ghrelin expression in adipocyte differentiation cultures was also inhibited by OX-A, but enhanced by OX1R inhibitor or OX1R2R dual inhibitor (Figure 5G). Furthermore, ghrelin secretion was elevated by OX1R inhibitor or OX1R2R dual inhibitor in both osteoblast (Figure 5H) and adipocyte (not shown) cultures. Previous studies have shown that ghrelin promotes osteoblastogenesis (Delhanty et al., 2006; Fukushima et al., 2005; Kim et al., 2005; Maccarinelli et al., 2005). Thus, these results indicate that OX1R inhibition of osteoblast differentiation may be mediated by the suppression of local ghrelin expression in bone.

To further investigate whether ghrelin upregulation was required for the pro-osteoblastogenic and antiadipogenic effects of OX1R deletion, we performed ghrelin small interfering RNA (siRNA) knockdown experiments. Marrow MSCs from WT or OX1R-KO mice were transfected with ghrelin siRNA (si-Ghrl) or control siRNA (si-Ctrl) before differentiation. Ghrelin knockdown in WT cells decreased osteoblast differentiation (Figures S7A–S7C), supporting the pro-osteoblastogenic role of ghrelin. Importantly, ghrelin knockdown in OX1R-KO cells to a level similar to WT cells (Figures 5I and S7D) abolished their ability to increase osteoblastogenesis, decrease adipogenesis, or alter RANKL and OPG expression (Figures 5J–5M). These findings indicate that osseous ghrelin is an essential mediator of the local bone regulation by OX1R.

OX2R Augments Bone Formation by Suppressing Serum Leptin Level

Leptin suppresses bone formation, and serum leptin level is a critical determinant of bone mass. We found that leptin protein levels in both bone and serum were elevated in OX-KO, OX2R-KO, and OX1R2R-DKO mice but reduced in OX-Tg mice (Figures 6A–6C). Leptin mRNA showed a similar pattern in white adipose tissue (WAT) (Figure 6D) but was undetectable in bone (not shown), indicating that the changes in leptin protein in bone mainly originated from peripheral fat via circulation. Consistent with the genetic evidence, i.c.v. injection of an OX2R agonist also decreased serum leptin in WT mice (Figure 6E). These results indicate that orexin may enhance bone formation by suppressing leptin levels.

Leptin has been shown to decrease trabecular bone mass at least in part by activating the sympathetic nerves (Ducy et al., 2000; Elefteriou et al., 2004). Interestingly, recent studies suggest that leptin paradoxically increases cortical bone mass at least in part by downregulating hypothalamic expression of NPY, a neuropeptide that causes bone loss (Baldock et al., 2002; Lee and Herzog, 2009; Wong et al., 2013). Because both trabecular and cortical bone mass was decreased in OX-KO and OX2R-KO mice but increased in OX-Tg mice, we next examined the sympathetic outflow and hypothalamic NPY expression in these mice. Expression analyses of UCP1, PGC1 α and Dio2 in brown adipose tissue (BAT) show that the sympathetic tone was increased in OX2R-KO mice but decreased in OX-Tg mice (Figure 6F). In contrast, expression of NPY in hypothalamus was unaltered (Figure 6G). These results suggest that orexin augments bone mass mainly by suppressing leptin activation of the sympathetic nerves.

To further elucidate whether leptin is required for OX2R regulation of bone formation, we performed i.c.v. injection of OX2R-AG in ob/ob mice that lack functional leptin protein (Zhang et al., 1994) (Figure 6E). In contrast to WT mice (Figures 3M–3T), the bone enhancing effects of OX2R-AG was completely abolished in ob/ob mice, as OX2R-AG was no longer able to increase BV/TV, Tb.N, Tb.Sp, or P1NP (Figures 6H–6L). These results indicate that leptin is a critical mediator of the central bone regulation by OX2R.

DISCUSSION

This study has identified orexin as a critical yet previously unrecognized regulator of bone mass accrual that functions via a

Figure 3. OX2R Increases Bone Mass via a Central Regulation

(A–E) μ CT of tibiae from OX2R-KO or WT controls (3 months old, male, n = 9). (A) Representative images. (B–E) Trabecular and cortical bone parameters.

(F) Three-point bending assay (n = 9).

(G) Serum P1NP (n = 9).

(H) Serum CTX-1 (n = 9).

(I) Static histomorphometry (n = 9).

(J) Dynamic histomorphometry of the trabecular bone at metaphysis and the cortical bone at diaphysis (3 months old, male, n = 5).

(K and L) Osteoblast differentiation (K) and adipocyte differentiation (L) from the marrow of OX2R-KO mice and WT controls (n = 3).

(M–T) Effects of i.c.v. injection of an OX2R agonist (OX2R-AG) on bone in 12-week-old WT male mice (n = 5). (M–Q) μ CT analysis of tibiae. (M) Representative images. (N–Q) Trabecular and cortical bone parameters. (R) Three-point bending assay. (S) Serum P1NP. (T) Serum CTX-1.

(U–Z) Effects of i.c.v. injection of an OX2R-AG on OVX-induced bone loss in 6-month-old WT female mice (n = 4). (U) Uterine weight. (V) Serum P1NP. (W) Serum CTX-1. (X and Y) μ CT and histomorphometry. (X) Top: μ CT images; bottom: histomorphometry analyses. p < 0.05 by ANOVA; “***” compares “OVX+Veh” with “Sham+Veh”; “+” compares “OVX+OX2R-AG” with “OVX+Veh.” (Y) Trabecular bone parameters. (Z) Three-point bending assay. “n.s.” in (K) and (L) compares OX2R-KO with WT control under the same treatment conditions. (U–Z) Statistical analysis was performed by ANOVA and the post hoc Tukey pairwise comparisons. Error bars represent SD.

See also Figures S4 and S5.

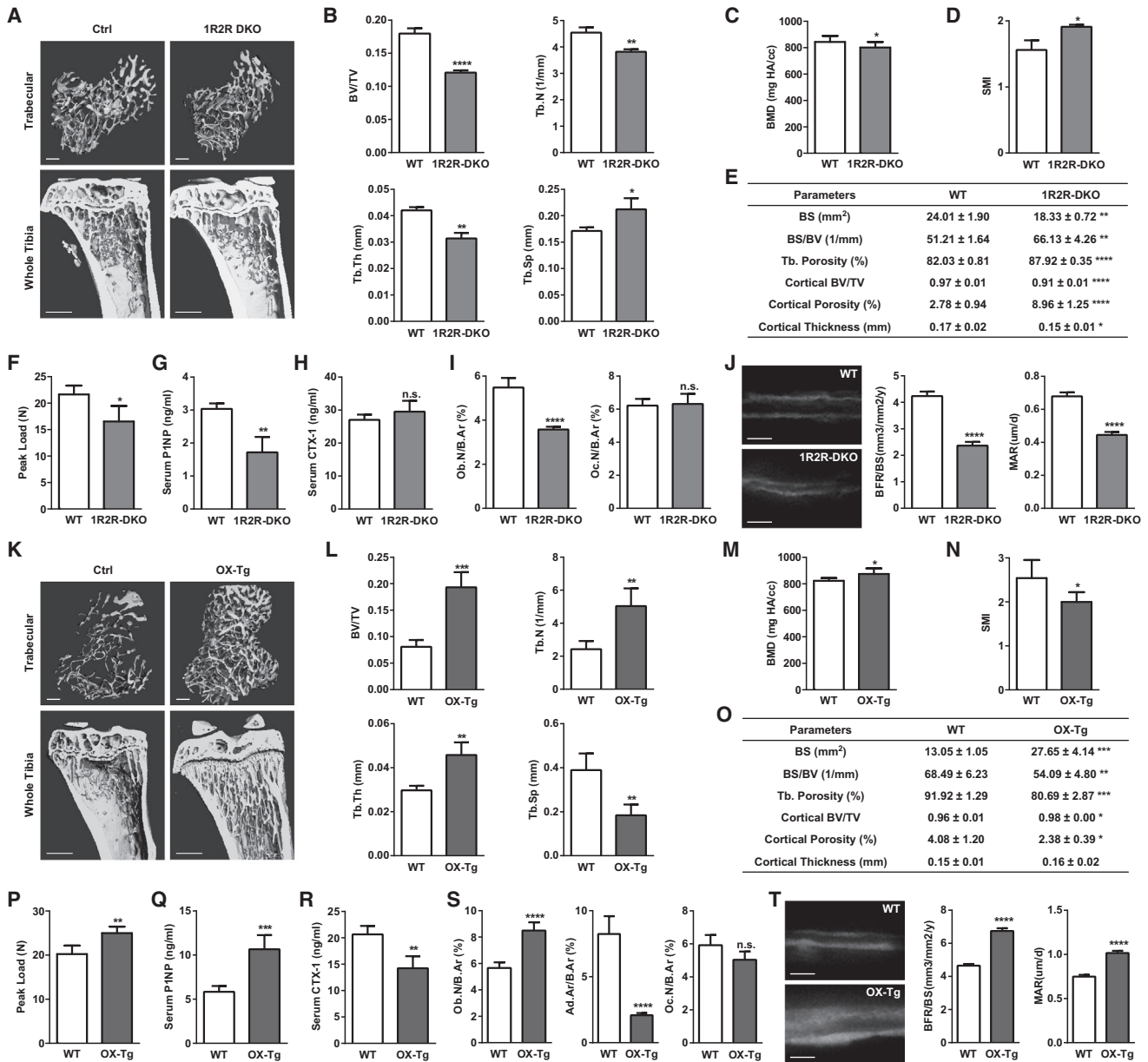


Figure 4. Central Action Is Dominant over Peripheral Action in Orexin Regulation of Bone

(A–J) Analysis of 1R2R-DKO mice and WT controls (3 months old, male, n = 9). (A–E) μ CT of tibiae. (A) Representative images. (B–E) Trabecular and cortical bone parameters. (F) Three-point bending assay. (G) Serum P1NP. (H) Serum CTX-1. (I) Static histomorphometry. (J) Dynamic histomorphometry (3 months old, male, n = 5).

(K–T) Analysis of OX-Tg mice (n = 10) and WT littermate controls (n = 8) (3–4 months old, male). (K–O) μ CT of tibiae. (K) Representative images. (L–O) Trabecular and cortical bone parameters. (P) Three-point bending assay. (Q) Serum P1NP. (R) Serum CTX-1. (S) Static histomorphometry. (T) Dynamic histomorphometry (3 months old, male, n = 5). Error bars represent SD.

See also Figure S6.

yin-yang dual mechanism (Figure 6M). On one hand, orexin activation of OX2R in the brain centrally enhances bone formation by lowering circulating leptin level. On the other hand, orexin activation of OX1R in the bone locally suppresses bone formation and enhances bone resorption by lowering osseous ghrelin expression. Importantly, the central action is dominant over local action so that systemic orexin overexpression increases bone

mass whereas complete deletion of orexin or orexin receptors decreases bone mass. It is remarkable how orexin achieves a physiological balance in the regulation of skeletal homeostasis by differentially utilizing two different receptors at distinct anatomic sites.

Orexin deficiency in humans causes behavior abnormalities including sleep and mood disorders. Both OX-KO and OX2R-KO

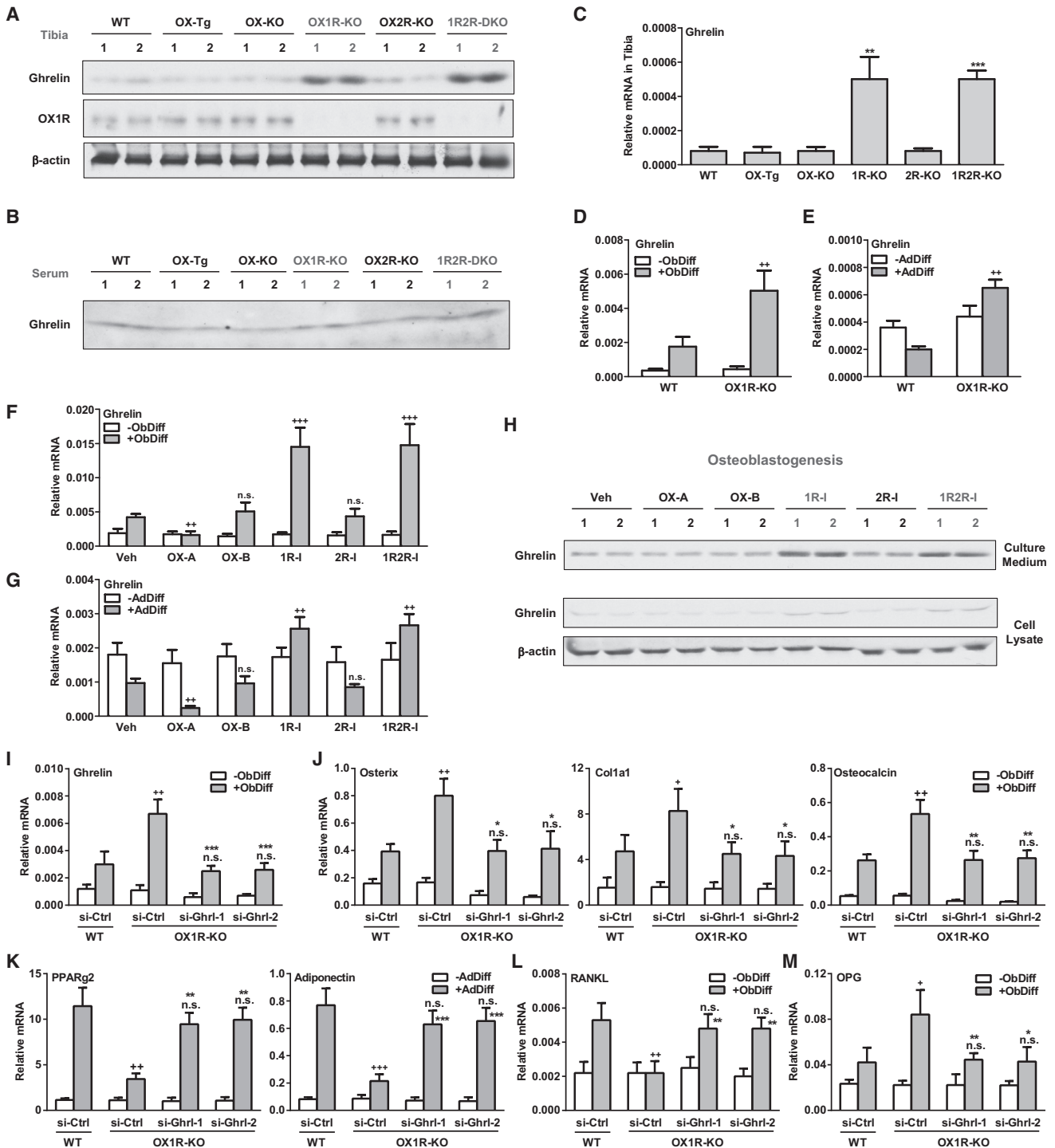


Figure 5. OX1R Inhibits Osteoblastogenesis by Suppressing Local Ghrelin Expression

(A and B) Ghrelin protein levels in tibiae (A) and serum (B) of WT or mutant mice (n = 2).

(C) Ghrelin mRNA levels in tibiae of WT or mutant mice (n = 3).

(D and E) Ghrelin mRNA in osteoblast (D) and adipocyte (E) differentiation cultures from the marrow of OX1R-KO or WT mice (n = 3).

(F–H) Effect of OX-A, OX-B, OX1R-I, OX2R-I, or 1R2R-I (10 nM) on ghrelin expression in marrow differentiation cultures. (F and G) Ghrelin mRNA in osteoblast (F) and adipocyte (G) cultures (n = 3). (H) Ghrelin protein expression and secretion in osteoblast culture (n = 2).

(I–M) Ghrelin knockdown abolished the enhanced osteoblastogenesis, reduced adipogenesis, and altered RANKL/OPG expression in OX1R-KO differentiation cultures (n = 3). Marrow MSCs were transfected with ghrelin-siRNA (si-Ghr1) or control siRNA (si-Ctrl) (n = 3). The results are shown as mRNA expression of ghrelin (I), osteoblast markers (J), adipocyte markers (K), RANKL (L), and OPG (M). “+” and “n.s.” in (D) and (E) compares OX1R-KO with WT control under the

(legend continued on next page)

mice exhibit a narcolepsy phenotype, which is characterized by daytime sleepiness that is accompanied by a sudden loss of muscle tone known as cataplexy, often after laughter or excitement (Chemelli et al., 1999; Lin et al., 1999). Orexin is undetectable in most human narcolepsy patients (Nishino et al., 2000). Interestingly, older women with sleep disorders are reported to suffer from greater risk of osteoporotic fractures (Stone et al., 2006). Moreover, orexin deficiency and sleep disorders are also frequently associated with major mood disorders (MMD), especially depression (Allard et al., 2004; Brundin et al., 2007). In humans, orexin-A levels in amygdala are maximal during positive emotion but minimal during depression, suggesting that boosting orexin function could elevate mood (Blouin et al., 2013). In mice, orexin neurons are also maximally active during performance of rewarded behaviors; OX-KO mice are deficient in conducting rewarded behaviors; and OX2R-KO mice display increased behavioral despair, indicating a similar involvement of orexin in positive reinforcement (Borgland et al., 2009; McGregor et al., 2011; Scott et al., 2011). In humans, depression is associated with low bone mass and increased incidence of osteoporotic fractures (Bab and Yirmiya, 2010). A study using a mouse stress model shows that depression induces bone loss by inhibiting bone formation via the stimulation of the sympathetic nervous system (Yirmiya et al., 2006). However, the neural circuitry underlying the connection of narcolepsy, depression, and bone loss is not well understood. Our findings that OX-KO and OX2R-KO mice exhibit lower bone mass and higher leptin levels provide a potential mechanism for the increased fracture risk in narcolepsy and depression.

Orexin deficiency is also associated with metabolic abnormalities including obesity and hypophagia (Sakurai, 2007; Sakurai and Mieda, 2011). The obese phenotype in young mice occurs only under high-fat diet feeding, but not under chow diet feeding (Funato et al., 2009; Sellayah et al., 2011), and at least in part due to decreased energy expenditure and impaired development of BAT (Sellayah et al., 2011), which have recently been reported to promote bone formation (Rahman et al., 2013). Interestingly, the decreased energy expenditure in OX-KO mice was caused by an OX1R-dependent direct BAT differentiation defect rather than defects in sympathetic nervous system (Sellayah et al., 2011). Thus, even if leptin and sympathetic tone are increased in OX-KO mice due to a central effect, leading to decreased bone mass, energy expenditure is still lower due to the lack of BAT. Consistent with this notion, our results show that leptin levels are only elevated in OX2R-KO, but not OX1R-KO mice, and reduced by i.c.v. delivery of OX2R-agonist (Figures 6A–6E), indicating that orexin suppresses leptin via a central regulation. Of note, we found that orexin and orexin receptors can regulate bone remodeling in the absence of body weight change under chow diet (Figure S1A). In addition, orexin expression in the brain (Mieda et al., 2011; Willie et al., 2003) and tibiae (Figure S1B) are unaltered in the receptor knockout mice. Furthermore, our previous study shows that OX-Tg mice have no ectopic orexin protein expression in BAT and WAT (Funato

et al., 2009), suggesting that orexin regulation of bone can be independent from its regulation of BAT. Nonetheless, it is possible that these other metabolic and behavior changes may indirectly contribute to the skeletal phenotype observed in orexin and orexin receptor knockout mice.

Global knockout mice have the advantage of revealing the net effects of loss-of-function, including both cell-autonomous and systemic/non-cell-autonomous effects. Indeed, our findings uncover OX1R in the osteoblasts and OX2R in the brain as two potential mechanisms contributing to the peripheral and central bone regulation by orexin, respectively. We have identified a cell-autonomous role of orexin and OX1R in osteoblast differentiation and thus provided a key mechanism for how OX1R regulates bone. It is possible that other non-cell-autonomous effects may also contribute to the bone phenotype of OX1R-KO mice. Although i.c.v. injection delivers OX2R agonist to several CNS regions, it is known that endogenous orexin peptides are also widely projected in the brain (Sakurai, 2007; Sakurai and Mieda, 2011). The finding that the bone anabolic effects of OX2R agonist is abolished in the ob/ob mice indicates that leptin may be an essential mediator of the central bone regulation by orexin and OX2R; although other indirect mechanisms in ob/ob mice, such as the altered metabolism, may also contribute to their resistance to OX2R agonist. Future studies to generate and characterize conditional knockout mice for orexin and orexin receptors using flox mice and cre drivers specific for MSCs, pre-osteoblasts, mature osteoblasts, and specific CNS regions will further delineate the functional requirement of each tissue and cell type.

Our *in vivo* genetic studies using OX1R knockout mice, in combination with our *in vitro* bone marrow osteoblast differentiation assays using orexin peptides and orexin receptor inhibitors, demonstrate that OX1R suppresses osteoblast differentiation and bone formation. Orexin regulation of osteoblastogenesis has also been implicated in a previous study using rat calvarial osteoblast-like cells (Ziolkowska et al., 2008). We found that OX1R inhibits osteoblast differentiation by specifically reducing osseous ghrelin expression without altering circulating ghrelin levels (Figure 5), suggesting that distinct mechanisms may account for the regulation of ghrelin expression in bone and stomach. Our ghrelin siRNA knockdown experiments (Figure 5) and previous pharmacological experiments (Delhanty et al., 2006; Fukushima et al., 2005; Kim et al., 2005; Maccarinelli et al., 2005) show that ghrelin promotes osteoblastogenesis. Moreover, pharmacological studies also show that ghrelin stimulates growth and appetite. Interestingly however, it is reported that ghrelin knockout mice are normal with unaltered body weight, food intake and bone density (Sun et al., 2003). A possible explanation is that developmental compensation in the knockout mice may mask the physiological role of ghrelin.

The elucidation of the intricate mechanisms underlying orexin regulation of bone also presents exciting opportunities for the treatment of bone degenerative diseases such as osteoporosis.

same culture conditions; “+” and “n.s.” in (F) and (G) compares treatment with vehicle control under the same culture conditions. (I–M) “***” compares si-Ghrl with si-Ctrl in OX1R-KO cells, “+” and “n.s.” compares OX1R-KO cells transfected with si-Ghrl or si-Ctrl with WT cells transfected with si-Ctrl. Error bars represent SD.

See also Figure S7.

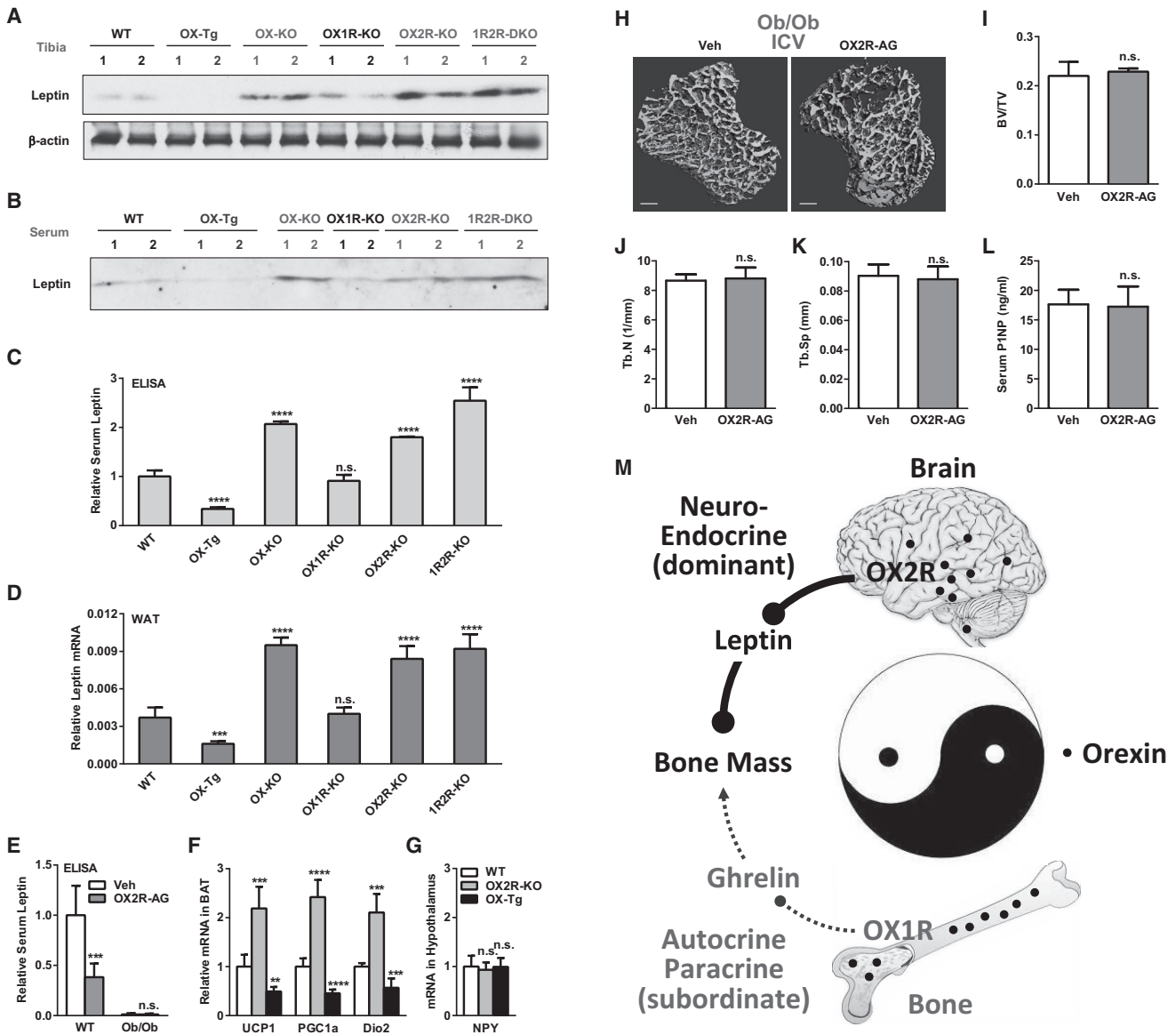


Figure 6. OX2R Augments Bone Formation by Suppressing Serum Leptin Level

(A–C) Leptin protein levels in tibiae (A) and serum (B and C), quantified by western blot (A and B) (n = 2) and ELISA (C) (n = 5).

(D) Leptin mRNA in WAT (n = 3). Asterisk in (C) and (D) compares mutant mice with WT controls.

(E) Serum leptin levels were decreased by i.c.v. injection of OX2R-AG in WT mice (n = 5) but not in ob/ob mice (n = 4).

(F) Expression of UCP1, PGC1 α , and Dio2 in BAT (n = 5).

(G) NPY expression in hypothalamus (n = 5). Asterisk and n.s. in (F) and (G) compares mutant mice with WT control mice.

(H–L) The bone enhancing effects of OX2R-AG via i.c.v. injection was abolished in ob/ob mice (12 weeks old, male, n = 4). (H–K) μ CT of tibiae. (H) Images of the trabecular bone of the tibial metaphysis (scale bar represents 10 μ m). (I–K) Trabecular bone parameters. (L) Serum P1NP. Error bars represent SD.

(M) A simplified working model for how orexin regulates skeletal homeostasis via a yin-yang dual mechanism. On one hand, orexin activation of OX2R in the brain centrally enhances bone mass via a dominant neuroendocrine mechanism by decreasing circulating leptin levels, thus ameliorating the bone-suppressive effects of leptin. On the other hand, orexin activation of OX1R in the bone peripherally reduces bone mass via a subordinate autocrine/paracrine mechanism by decreasing local ghrelin expression, thus compromising the bone-augmenting effects of ghrelin. Consequently, OX1R deletion causes bone gain whereas OX2R deletion causes bone loss; global orexin deletion leads to bone loss whereas global orexin overexpression leads to bone gain.

First, OX2R-specific agonists hold tremendous potential as bone anabolic therapeutics. Second, OX1R-specific antagonists may present anabolic and anticatabolic dual benefits to enhance bone formation and suppress bone resorption. Interestingly, OX2R activation also confers resistance to obesity and diabetes

(Funato et al., 2009; Kotz et al., 2012), hence OX2R agonists may promote metabolic and skeletal fitness simultaneously. This prospect is even more exciting in light of the bone loss side effects observed in the current drugs or drug candidates for obesity/diabetes such as rosiglitazone (Bilezikian et al.,

2013; Grey, 2009; Home et al., 2009; Kahn et al., 2006, 2008; Zinman et al., 2010) and fibroblast growth factor 21 (FGF21) (Wan, 2013; Wei et al., 2012).

Several pharmaceutical companies including Merck and GlaxoSmithKline have developed small molecule orexin antagonists for the treatment of insomnia. These include OX1R antagonists, OX2R antagonists, and OX1R2R dual antagonists such as almoxant (ACT-078573) (Brisbare-Roch et al., 2007) and suvorexant (MK-4305) (Cox et al., 2010; Willyard, 2012). Suvorexant is under FDA review after completion of Phase III clinical trials (Mieda and Sakurai, 2013; Willyard, 2012). Our findings suggest that OX1R-specific antagonist may be bone protective whereas OX2R antagonists and OX1R2R dual antagonists may risk bone loss.

More fundamentally, the finding of orexin as a key regulator of skeletal homeostasis provides insight to our understanding of how bone remodeling is controlled by neuronal and endocrine mechanisms. It also raises a provocative question of how skeletal physiology may crosstalk with sleep/wake, fast/feeding, energy store/expenditure cycles, as well as reward, addiction, anxiety, and motivation behaviors, via the common orexin pathway in vertebrates.

EXPERIMENTAL PROCEDURES

Mice

OX-KO mice (Chemelli et al., 1999), OX1R-KO mice (Mieda et al., 2011), OX2R-KO mice (Willie et al., 2003), OX-Tg mice (Mieda et al., 2004), and littermate WT controls that were backcrossed to the C57BL/6J background for more than ten generations have been previously described. WT and ob/ob mice on a C57BL/6J background in the i.c.v. experiments were purchased from Jackson Laboratory. Mice were fed standard chow containing 4% fat ad libitum. All protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center.

Reagent

OX1R inhibitor (SB-408124) (Langmead et al., 2004) was from Sigma. OX2R inhibitor (compound 1) (Aissaoui et al., 2008) and OX1R2R dual inhibitor (ACT-078573, almoxant) (Brisbare-Roch et al., 2007) were synthesized in the Yanagisawa laboratory. Human orexin-A, orexin-B, and (Ala11, D-Leu15) Orexin-B (OX2R-specific agonist) peptides were from American Peptide. Anti-OXR1 (C-19) and anti-leptin (A-20) antibodies were from Santa Cruz Biotechnology. Anti-Ghrelin (clone 1ML-1D7) and anti-orexin-A were from Millipore. Ghrelin siRNA and negative control siRNA were from Sigma.

Bone Analyses

μ CT and histomorphometry were performed as described (Wei et al., 2010, 2012). Calcein (20 mg/kg) were injected 2 and 10 days before bone collection. For strength measurement, tibiae were tested by three-point bending, and a strength parameter (peak load at failure) was assessed with a Test Resources DDL200 axial loading machine outfitted with an Interface SMT1-22 force transducer. Cross-head displacement rate was 0.1 mm/s. Tests were conducted on the mid-diaphyses with the bones resting on two supports 5 mm apart and the tibial anterior margins facing upward toward the actuator. Serum P1NP and CTX-1 were measured with the Rat/Mouse P1NP EIA kit and the RatLaps EIA kit, respectively (Immunodiagnostic Systems) (Wei et al., 2012).

Ex Vivo Bone Marrow Differentiation

Bone marrow cells were cultured for 4 days in MSC media (Mouse MesenCult Proliferation Kit, StemCell Technologies), then differentiated into osteoblast with α -MEM containing 10% FBS, 5 mM β -glycerophosphate, and 100 μ g/ml ascorbic acid for 9 days, or differentiated into adipocytes with adipogenesis medium (MesenCult Basal Medium + Mouse MesenCult Adipogenic Stimulatory Supplement, StemCell Technologies) for 7 days (Wei et al., 2011a,

2011b, 2012). For ghrelin knockdown, siRNA was transfected twice with FuGENE HD (Roche) the day before and 3 days after differentiation. Osteoclast differentiation was performed as described (Wan et al., 2007; Wei et al., 2010).

Chronic i.c.v. Injection

Mice were single-housed 1 week before surgery. After anesthetizing with 2%–3% isoflurane, a cannula (3300PM/SPC; PlasticsOne) was implanted into the right lateral ventricle (0.3 mm posterior from the bregma, 0.9 mm lateral from the midline, and 2.4 mm from the surface of skull) using standard sterile stereotaxic techniques as described (Funato et al., 2009). An osmotic minipump (model 1004; Alzet) was attached to the cannula and implanted in the subcutaneous space. The OX2R selective agonist ([Ala11, D-Leu15] Orexin-B; American Peptide) (Asahi et al., 2003) or PBS control was continuously injected in the lateral ventricle for 35 days (0.5 nmol/day) before analyses. Ovariectomy or sham operation was performed 3 days before i.c.v. infusion as described (Wei et al., 2011b).

Statistical Analyses

All statistical analyses were performed with Student's t test and represented as mean \pm SD unless stated otherwise. The p values were designated as: *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001; n.s. nonsignificant (p > 0.05).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cmet.2014.03.016>.

ACKNOWLEDGMENTS

We thank Shelley Dixon for mouse colony maintenance and Hidetoshi Kumagai for reagents. Y.W. is a Virginia Murchison Linthicum Scholar in Medical Research. M.Y. is an investigator of the Howard Hughes Medical Institute. This work was in part supported by the UT Southwestern Endowed Scholar Startup Fund (to Y.W.), NIH (RO1DK089113, to Y.W.), The Welch Foundation (I-1751, to Y.W.), the Howard Hughes Medical Institute (to M.Y.), and the Japan Society for the Promotion of Science through the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST) (to M.Y.).

Received: November 4, 2013

Revised: February 3, 2014

Accepted: February 26, 2014

Published: May 1, 2014

REFERENCES

- Aissaoui, H., Koberstein, R., Zumburn, C., Gattfield, J., Brisbare-Roch, C., Jenck, F., Treiber, A., and Boss, C. (2008). N-Glycine-sulfonamides as potent dual orexin 1/orexin 2 receptor antagonists. *Bioorg. Med. Chem. Lett.* *18*, 5729–5733.
- Allard, J.S., Tizabi, Y., Shaffery, J.P., Truth, C.O., and Manaye, K. (2004). Stereological analysis of the hypothalamic hypocretin/orexin neurons in an animal model of depression. *Neuropeptides* *38*, 311–315.
- Asahi, S., Egashira, S., Matsuda, M., Iwaasa, H., Kanatani, A., Ohkubo, M., Ihara, M., and Morishima, H. (2003). Development of an orexin-2 receptor selective agonist, [Ala(11), D-Leu(15)]orexin-B. *Bioorg. Med. Chem. Lett.* *13*, 111–113.
- Bab, I.A., and Yirmiya, R. (2010). Depression and bone mass. *Ann. N Y Acad. Sci.* *1192*, 170–175.
- Baldock, P.A., Sainsbury, A., Couzens, M., Enriquez, R.F., Thomas, G.P., Gardiner, E.M., and Herzog, H. (2002). Hypothalamic Y2 receptors regulate bone formation. *J. Clin. Invest.* *109*, 915–921.
- Bianco, P., Cao, X., Frenette, P.S., Mao, J.J., Robey, P.G., Simmons, P.J., and Wang, C.Y. (2013). The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat. Med.* *19*, 35–42.
- Bilezikian, J.P., Josse, R.G., Eastell, R., Lewiecki, E.M., Miller, C.G., Wooddell, M., Northcutt, A.R., Kravitz, B.G., Paul, G., Cobitz, A.R., et al. (2013).

- Rosiglitazone decreases bone mineral density and increases bone turnover in postmenopausal women with type 2 diabetes mellitus. *J. Clin. Endocrinol. Metab.* **98**, 1519–1528.
- Blouin, A.M., Fried, I., Wilson, C.L., Staba, R.J., Behnke, E.J., Lam, H.A., Maidment, N.T., Karlsson, K.A.E., Lapiere, J.L., and Siegel, J.M. (2013). Human hypocretin and melanin-concentrating hormone levels are linked to emotion and social interaction. *Nat. Commun.* **4**, 1547.
- Borgland, S.L., Chang, S.J., Bowers, M.S., Thompson, J.L., Vittoz, N., Floresco, S.B., Chou, J., Chen, B.T., and Bonci, A. (2009). Orexin A/hypocretin-1 selectively promotes motivation for positive reinforcers. *J. Neurosci.* **29**, 11215–11225.
- Brisbare-Roch, C., Dingemans, J., Koberstein, R., Hoever, P., Aissaoui, H., Flores, S., Mueller, C., Nayler, O., van Gerven, J., de Haas, S.L., et al. (2007). Promotion of sleep by targeting the orexin system in rats, dogs and humans. *Nat. Med.* **13**, 150–155.
- Brundin, L., Björkqvist, M., Petersén, A., and Träskman-Bendz, L. (2007). Reduced orexin levels in the cerebrospinal fluid of suicidal patients with major depressive disorder. *Eur. Neuropsychopharmacol.* **17**, 573–579.
- Chemelli, R.M., Willie, J.T., Sinton, C.M., Elmquist, J.K., Scammell, T., Lee, C., Richardson, J.A., Williams, S.C., Xiong, Y., Kisanuki, Y., et al. (1999). Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* **98**, 437–451.
- Cox, C.D., Breslin, M.J., Whitman, D.B., Schreier, J.D., McGaughey, G.B., Bogusky, M.J., Roecker, A.J., Mercer, S.P., Bednar, R.A., Lemaire, W., et al. (2010). Discovery of the dual orexin receptor antagonist [(7R)-4-(5-chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3-triazol-2-yl)phenyl]methanone (MK-4305) for the treatment of insomnia. *J. Med. Chem.* **53**, 5320–5332.
- Delhanty, P.J., van der Eerden, B.C., van der Velde, M., Gauna, C., Pols, H.A., Jahr, H., Chiba, H., van der Lely, A.J., and van Leeuwen, J.P. (2006). Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a. *J. Endocrinol.* **188**, 37–47.
- Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A.F., Beil, F.T., Shen, J., Vinson, C., Rueger, J.M., and Karsenty, G. (2000). Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* **100**, 197–207.
- Eleftheriou, F., Takeda, S., Ebihara, K., Magre, J., Patano, N., Kim, C.A., Ogawa, Y., Liu, X., Ware, S.M., Craigen, W.J., et al. (2004). Serum leptin level is a regulator of bone mass. *Proc. Natl. Acad. Sci. USA* **101**, 3258–3263.
- Fukushima, N., Hanada, R., Teranishi, H., Fukue, Y., Tachibana, T., Ishikawa, H., Takeda, S., Takeuchi, Y., Fukumoto, S., Kangawa, K., et al. (2005). Ghrelin directly regulates bone formation. *J. Bone Miner. Res.* **20**, 790–798.
- Funato, H., Tsai, A.L., Willie, J.T., Kisanuki, Y., Williams, S.C., Sakurai, T., and Yanagisawa, M. (2009). Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity. *Cell Metab.* **9**, 64–76.
- Grey, A. (2009). Thiazolidinedione-induced skeletal fragility—mechanisms and implications. *Diabetes Obes. Metab.* **11**, 275–284.
- Hara, J., Beuckmann, C.T., Nambu, T., Willie, J.T., Chemelli, R.M., Sinton, C.M., Sugiyama, F., Yagami, K., Goto, K., Yanagisawa, M., and Sakurai, T. (2001). Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* **30**, 345–354.
- Home, P.D., Pocock, S.J., Beck-Nielsen, H., Curtis, P.S., Gomis, R., Hanefeld, M., Jones, N.P., Komajda, M., and McMurray, J.J.; RECORD Study Team (2009). Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial. *Lancet* **373**, 2125–2135.
- Kahn, S.E., Haffner, S.M., Heise, M.A., Herman, W.H., Holman, R.R., Jones, N.P., Kravitz, B.G., Lachin, J.M., O'Neill, M.C., Zinman, B., and Viberti, G.; ADOPT Study Group (2006). Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *N. Engl. J. Med.* **355**, 2427–2443.
- Kahn, S.E., Zinman, B., Lachin, J.M., Haffner, S.M., Herman, W.H., Holman, R.R., Kravitz, B.G., Yu, D., Heise, M.A., Aftring, R.P., and Viberti, G.; Diabetes Outcome Progression Trial (ADOPT) Study Group (2008). Rosiglitazone-associated fractures in type 2 diabetes: an Analysis from A Diabetes Outcome Progression Trial (ADOPT). *Diabetes Care* **31**, 845–851.
- Kim, S.W., Her, S.J., Park, S.J., Kim, D., Park, K.S., Lee, H.K., Han, B.H., Kim, M.S., Shin, C.S., and Kim, S.Y. (2005). Ghrelin stimulates proliferation and differentiation and inhibits apoptosis in osteoblastic MC3T3-E1 cells. *Bone* **37**, 359–369.
- Kotz, C., Nixon, J., Butterick, T., Perez-Leighton, C., Teske, J., and Billington, C. (2012). Brain orexin promotes obesity resistance. *Ann. N Y Acad. Sci.* **1264**, 72–86.
- Langmead, C.J., Jerman, J.C., Brough, S.J., Scott, C., Porter, R.A., and Herdon, H.J. (2004). Characterisation of the binding of [3H]-SB-674042, a novel nonpeptide antagonist, to the human orexin-1 receptor. *Br. J. Pharmacol.* **141**, 340–346.
- Lee, N.J., and Herzog, H. (2009). NPY regulation of bone remodelling. *Neuropeptides* **43**, 457–463.
- Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., de Jong, P.J., Nishino, S., and Mignot, E. (1999). The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* **98**, 365–376.
- Maccarinelli, G., Sibilia, V., Torsello, A., Raimondo, F., Pitto, M., Giustina, A., Netti, C., and Cocchi, D. (2005). Ghrelin regulates proliferation and differentiation of osteoblastic cells. *J. Endocrinol.* **184**, 249–256.
- McGregor, R., Wu, M.F., Barber, G., Ramanathan, L., and Siegel, J.M. (2011). Highly specific role of hypocretin (orexin) neurons: differential activation as a function of diurnal phase, operant reinforcement versus operant avoidance and light level. *J. Neurosci.* **31**, 15455–15467.
- Mieda, M., and Sakurai, T. (2013). Orexin (hypocretin) receptor agonists and antagonists for treatment of sleep disorders. Rationale for development and current status. *CNS Drugs* **27**, 83–90.
- Mieda, M., Willie, J.T., Hara, J., Sinton, C.M., Sakurai, T., and Yanagisawa, M. (2004). Orexin peptides prevent cataplexy and improve wakefulness in an orexin neuron-ablated model of narcolepsy in mice. *Proc. Natl. Acad. Sci. USA* **101**, 4649–4654.
- Mieda, M., Hasegawa, E., Kisanuki, Y.Y., Sinton, C.M., Yanagisawa, M., and Sakurai, T. (2011). Differential roles of orexin receptor-1 and 2 in the regulation of non-REM and REM sleep. *J. Neurosci.* **31**, 6518–6526.
- Nishino, S., Ripley, B., Overeem, S., Lammers, G.J., and Mignot, E. (2000). Hypocretin (orexin) deficiency in human narcolepsy. *Lancet* **355**, 39–40.
- Novack, D.V., and Teitelbaum, S.L. (2008). The osteoclast: friend or foe? *Annu. Rev. Pathol.* **3**, 457–484.
- Peyron, C., Faraco, J., Rogers, W., Ripley, B., Overeem, S., Charnay, Y., Nivamsalova, S., Aldrich, M., Reynolds, D., Albin, R., et al. (2000). A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat. Med.* **6**, 991–997.
- Rahman, S., Lu, Y., Czernik, P.J., Rosen, C.J., Enerback, S., and Lecka-Czernik, B. (2013). Inducible brown adipose tissue, or beige fat, is anabolic for the skeleton. *Endocrinology* **154**, 2687–2701.
- Rosen, C.J. (2008). Bone remodeling, energy metabolism, and the molecular clock. *Cell Metab.* **7**, 7–10.
- Sakurai, T. (2007). The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. *Nat. Rev. Neurosci.* **8**, 171–181.
- Sakurai, T., and Mieda, M. (2011). Connectomics of orexin-producing neurons: interface of systems of emotion, energy homeostasis and arousal. *Trends Pharmacol. Sci.* **32**, 451–462.
- Scott, M.M., Marcus, J.N., Pettersen, A., Birnbaum, S.G., Mochizuki, T., Scammell, T.E., Nestler, E.J., Elmquist, J.K., and Lutter, M. (2011). Hcrtr1 and 2 signaling differentially regulates depression-like behaviors. *Behav. Brain Res.* **222**, 289–294.
- Sellayah, D., Bharaj, P., and Sikder, D. (2011). Orexin is required for brown adipose tissue development, differentiation, and function. *Cell Metab.* **14**, 478–490.
- Stone, K.L., Ewing, S.K., Lui, L.Y., Ensrud, K.E., Ancoli-Israel, S., Bauer, D.C., Cauley, J.A., Hillier, T.A., and Cummings, S.R. (2006). Self-reported sleep and nap habits and risk of falls and fractures in older women: the study of osteoporotic fractures. *J. Am. Geriatr. Soc.* **54**, 1177–1183.

- Sun, Y., Ahmed, S., and Smith, R.G. (2003). Deletion of ghrelin impairs neither growth nor appetite. *Mol. Cell. Biol.* *23*, 7973–7981.
- Wan, Y. (2013). Bone marrow mesenchymal stem cells: fat on and blast off by FGF21. *Int. J. Biochem. Cell Biol.* *45*, 546–549.
- Wan, Y., Chong, L.W., and Evans, R.M. (2007). PPAR-gamma regulates osteoclastogenesis in mice. *Nat. Med.* *13*, 1496–1503.
- Wei, W., Wang, X., Yang, M., Smith, L.C., Dechow, P.C., Sonoda, J., Evans, R.M., and Wan, Y. (2010). PGC1beta mediates PPARgamma activation of osteoclastogenesis and rosiglitazone-induced bone loss. *Cell Metab.* *11*, 503–516.
- Wei, W., Zeve, D., Suh, J.M., Wang, X., Du, Y., Zerwekh, J.E., Dechow, P.C., Graff, J.M., and Wan, Y. (2011a). Biphasic and dosage-dependent regulation of osteoclastogenesis by β -catenin. *Mol. Cell. Biol.* *31*, 4706–4719.
- Wei, W., Zeve, D., Wang, X., Du, Y., Tang, W., Dechow, P.C., Graff, J.M., and Wan, Y. (2011b). Osteoclast progenitors reside in the peroxisome proliferator-activated receptor γ -expressing bone marrow cell population. *Mol. Cell. Biol.* *31*, 4692–4705.
- Wei, W., Dutchak, P.A., Wang, X., Ding, X., Wang, X., Bookout, A.L., Goetz, R., Mohammadi, M., Gerard, R.D., Dechow, P.C., et al. (2012). Fibroblast growth factor 21 promotes bone loss by potentiating the effects of peroxisome proliferator-activated receptor γ . *Proc. Natl. Acad. Sci. USA* *109*, 3143–3148.
- Willie, J.T., Chemelli, R.M., Sinton, C.M., Tokita, S., Williams, S.C., Kisanuki, Y.Y., Marcus, J.N., Lee, C., Elmquist, J.K., Kohlmeier, K.A., et al. (2003). Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes. *Neuron* *38*, 715–730.
- Willyard, C. (2012). New class of sleep drugs may offer a safer night's rest. *Nat. Med.* *18*, 996.
- Wong, I.P., Nguyen, A.D., Khor, E.C., Enriquez, R.F., Eisman, J.A., Sainsbury, A., Herzog, H., and Baldock, P.A. (2013). Neuropeptide Y is a critical modulator of leptin's regulation of cortical bone. *J. Bone Miner. Res.* *28*, 886–898.
- Yirmiya, R., Goshen, I., Bajayo, A., Kreisel, T., Feldman, S., Tam, J., Trembovler, V., Csernus, V., Shohami, E., and Bab, I. (2006). Depression induces bone loss through stimulation of the sympathetic nervous system. *Proc. Natl. Acad. Sci. USA* *103*, 16876–16881.
- Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* *372*, 425–432.
- Zinman, B., Haffner, S.M., Herman, W.H., Holman, R.R., Lachin, J.M., Kravitz, B.G., Paul, G., Jones, N.P., Aftring, R.P., Viberti, G., and Kahn, S.E.; ADOPT Study Group (2010). Effect of rosiglitazone, metformin, and glyburide on bone biomarkers in patients with type 2 diabetes. *J. Clin. Endocrinol. Metab.* *95*, 134–142.
- Ziolkowska, A., Rucinski, M., Tyczewska, M., and Malendowicz, L.K. (2008). Orexin B inhibits proliferation and stimulates specialized function of cultured rat calvarial osteoblast-like cells. *Int. J. Mol. Med.* *22*, 749–755.