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The Molecular Clock Mediates Leptin-Regulated Bone Formation

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Summary

The hormone leptin is a regulator of bone remodeling, a homeostatic function maintaining bone mass constant. Mice lacking molecular-clock components (*Per* and *Cry*), or lacking *Per* genes in osteoblasts, display high bone mass, suggesting that bone remodeling may also be subject to circadian regulation. Moreover, *Per*-deficient mice experience a paradoxical increase in bone mass following leptin intracerebroventricular infusion. Thus, clock genes may mediate the leptin-dependent sympathetic regulation of bone formation. We show that expression of clock genes in osteoblasts is regulated by the sympathetic nervous system and leptin. Clock genes mediate the antiproliferative function of sympathetic signaling by inhibiting *G1 cyclin* expression. Partially antagonizing this inhibitory loop, leptin also upregulates AP-1 gene expression, which promotes *cyclin D1* expression, osteoblast proliferation, and bone formation. Thus, leptin determines the extent of bone formation by modulating, via sympathetic signaling, osteoblast proliferation through two antagonistic pathways, one of which involves the molecular clock.

Introduction

Bone remodeling, the physiological process whereby bone mass is maintained constant during adulthood, is comprised of two phases. There is first a rapid phase of resorption of preexisting mineralized bone by osteoclasts, the bone-resorbing cells, followed by a relatively slow phase of de novo bone formation by osteoblasts, the bone-forming cells (Rodan and Martin, 2000). Osteoblasts have another function during bone remodeling: they control osteoclast differentiation. Bone remodeling, which is only partially understood at the molecular level, is disrupted in several frequent skeletal degenerative diseases. That one of these diseases, osteoporosis, is the most prevalent degenerative disease in developed countries (Riggs and Melton, 1986) explains the extent of the research effort underway to elucidate the molecular bases of this physiological function.

As expected for such a dynamic and complex process, bone remodeling is tightly controlled through two types of regulation (Chien and Karsenty, 2005). The first is exerted locally by factors secreted by either osteoblasts or osteoclasts. These include, but are not limited to, RANK ligand and osteoprotegerin, which control osteoclast differentiation, and insulin-like growth factors and other mitogens that affect bone formation. The second level of regulation is exerted systemically by hormones, such as sex steroids, parathyroid hormone, and leptin, and by neural inputs. This systemic regulation often impinges upon the expression of local regulators of bone mass. Among the many systemic regulators, leptin is nearly unique, as it affects profoundly both arms of bone remodeling. After binding to its receptor on hypothalamic neurons, leptin regulates, by targeting only one cell type, the osteoblast, bone formation (via the sympathetic nervous system [SNS]), and osteoclast differentiation (via the SNS and cocaine- and amphetamine-regulated transcript, or CART) (Ducy et al., 2000; Takeda et al., 2002; Elefteriou et al., 2005). A remarkable aspect of leptin's regulation of bone mass is that its mediators do not mediate leptin's other functions in animals that are unchallenged, i.e., fed with a normal diet. Indeed, mice unable to produce norepinephrine or mediate sympathetic signaling through the β2-adrenergic receptor (Adrβ2) have a high-bone-mass phenotype but are lean and fertile (Takeda et al., 2002; Elefteriou et al., 2005). Likewise, mice lacking CART are osteoporotic but are lean on a normal diet and fertile (Elefteriou et al., 2005). Conceivably, other molecules involved in the leptin-dependent sympathetic regulation of bone mass may not affect either body weight or reproduction.

To maintain bone mass constant, bone resorption and bone formation occur sequentially and in a balanced manner. Thus, bone remodeling is a homeostatic function (Rodan and Martin, 2000; Boyle et al., 2003). Since most homeostatic functions occur in a circadian manner (Lowrey and Takahashi, 2004; Perreau-Lenz et al., 2004), this feature suggests that the circadian clock could control bone mass. The diurnal variation in the synthesis of type I collagen and osteocalcin, the two main biosynthetic products of osteoblasts, supports this hypothesis (Simmons and Nichols, 1966; Gundberg et al., 1985).

Most physiological processes in mammals display circadian rhythms that are driven by the endogenous circadian clock. This clock comprises a central component located in the hypothalamic suprachiasmatic nucleus and subordinate clocks in peripheral tissues. Both central and peripheral clocks are operated by positive- and negative-feedback loops of circadian genes (Reppert and Weaver, 2002; Schibler and Sassone-Corsi, 2002; Fu and Lee, 2003). The positive-feedback loop is driven by the basic helix-loop-helix-PAS

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transcription factors, BMAL1 and CLOCK, that heterodimerize to stimulate the expression of other core circadian genes, such as *Period* (*Per1*, *2*, and *3*), *Cryptochrome* (*Cry1* and *2*), and *Rev-Erb*α. In the negative-feedback loop, PER and CRY proteins form a heteromultimeric complex with another core circadian regulator, casein kinase Iϵ, translocate into the nucleus, and suppress *Per*, *Cry*, and *Rev-Erb*α transcription by directly inhibiting the activity of BMAL1/CLOCK heterodimers. The positive and negative loops are connected by REV-ERBα, which inhibits *Bmal1* transcription through a retinoic-acid-related-orphan-receptor response element in the *Bmal1* promoter. Importantly for our purpose, mutations in the genes mentioned above (hereafter collectively referred to as clock genes) affect circadian behavioral rhythmicity in mice without resulting, in most cases, in sterility or obesity (Emery and Reppert, 2004).

In search of regulators of leptin functions in bone, we analyzed mice lacking clock components such as *Per1* and *2* or *Cry1* and *2* (Vitaterna et al., 1999; Zheng et al., 2001). Although these mutant mice developed a high-bone-mass phenotype due to an increase in bone formation, there was no evidence that clock genes act upstream of leptin to inhibit bone formation. Instead, and through their expression in osteoblasts, clock genes mediate the leptin-dependent sympathetic inhibition of bone formation by suppressing the expression of *G1 cyclins* and osteoblast proliferation. Removal of molecular-clock function uncovered that leptin and sympathetic signaling exert a countervailing and stimulatory effect on osteoblast proliferation through the AP-1 family of transcription factors. Thus, regulation of bone formation by leptin comprises two antagonistic pathways whose combined actions determine the rate of osteoblast proliferation.

Results

High Bone Mass in Mice Lacking *Per* or *Cry* Genes

To determine whether clock genes regulate bone mass, we studied *Per1*- and *2*- and *Cry1*- and *2*-deficient mice (Vitaterna et al., 1999; Zheng et al., 2001). Although these mutant mice showed disrupted circadian behavioral rhythms, they have, unlike *Clock*-deficient mice (Turek et al., 2005), normal body weight, fat-pad weight, food intake, fertility, and endocrine parameters (Figure 1A and Figure S1 in the Supplemental Data available with this article online). Thus, they could be used to determine whether clock genes participate in the regulation of bone formation by leptin.

Mice lacking either *Per* gene ($Per1^{-/-}$ or $Per2^{-/-}$) or only the *Per2* PAS domain ($Per2^{m/m}$) had a normal bone mass as determined by histology and microcomputed tomography (Figure 1B and data not shown). In contrast, starting at 6 weeks of age, $Per1^{-/-};Per2^{m/m}$ mice showed a significant increase in bone mass (HBM) that affected both vertebrae and long bones, a phenotype that worsened over time (Figures 1B–1D). A HBM of similar severity was also observed in double mutant mice lacking the entire *Per2* gene ($Per1^{-/-};Per2^{-/-}$), indicating that HBM in these mice was a loss-of-function phenotype (Figures 1B and 1C). Mice lacking *Cry1* and *2* ($Cry1^{-/-};Cry2^{-/-}$), two other components of the molecular clock, exhibited the same HBM as seen in $Per1^{-/-};Per2^{m/m}$ mice, suggesting that dysfunction of the molecular clock leads to HBM (Figure 1E).

To identify which arm of bone remodeling was affected by the loss of clock function, we performed biochemical and histomorphometric analyses. Urinary elimination of deoxypyridinoline crosslinks, a by-product of collagen degradation, was similar in wild-type (wt) and $Per1^{-/-};Per2^{m/m}$ mice, indicating that bone resorption was not overtly affected in these mice (Figure S2A). In contrast, there was a significant increase in the number of osteoblasts and, consequently, in bone-formation parameters (mineral apposition rate and bone-formation rate) in $Per1^{-/-};Per2^{m/m}$, $Per1^{-/-};Per2^{-/-}$, and $Cry1^{-/-};Cry2^{-/-}$ mice (Figure 1F). The same osteoblast abnormalities were also observed in $Bmal1^{-/-}$ mice that did not show HBM (Figure 1F). The absence of HBM in $Bmal1^{-/-}$ mice may be explained, at least in part, by their hypogonadism, a condition enhancing bone resorption (Figure S1B), although other mechanisms may contribute. That similar cellular abnormalities were observed in bones of multiple clock-gene-deficient mouse models supports the notion that bone formation is negatively regulated by the molecular clock.

Per Genes Mediate Leptin's Inhibition of Bone Formation

The resemblance between the bone phenotypes of $Per1^{-/-};Per2^{m/m}$, $Adr\beta2^{-/-}$, and *ob/ob* mice—i.e., increased osteoblast numbers, increased bone-formation parameters, and HBM—suggested that clock genes could either regulate or mediate the leptin-dependent sympathetic regulation of bone formation. To distinguish between these two possibilities, and given the similarities between the bone phenotypes of various clock-gene-deficient mice, we performed subsequent experiments in $Per1^{-/-};Per2^{m/m}$ mice.

Total or bound serum levels of leptin measured every 4 hr over a 24 hr period were elevated at most time points in $Per1^{-/-};Per2^{m/m}$ mice, although serum levels of free leptin, the biologically active form of leptin, were indistinguishable between wt and $Per1^{-/-};Per2^{m/m}$ mice (Figures 2A and 2B). Urinary elimination of catecholamines was normal over a 24 hr period in $Per1^{-/-};Per2^{m/m}$ mice, except for norepinephrine excretion that was significantly elevated at ZT22 (Figures 2C and 2D). The activation of the SNS, assessed by expression of uncoupling protein-1 (*Ucp1*), a direct target of sympathetic tone (Scarpace and Matheny, 1998), was also increased at ZT22 in $Per1^{-/-};Per2^{m/m}$ mice (Figure 2E). In addition, the increase in urinary elimination of norepinephrine induced by leptin intracerebroventricular (i.c.v.) infusion was of similar amplitude in $Per1^{-/-};Per2^{m/m}$ and wt mice (Figure 2F). Taken together, these data ruled out that the HBM of $Per1^{-/-};Per2^{m/m}$ mice was due to a mere decrease in either serum leptin or sympathetic tone (Takeda et al., 2002; Elefteriou et al., 2004).

Instead, the existence of HBM in $Per1^{-/-};Per2^{m/m}$ mice in the face of normal free leptin levels and slightly increased sympathetic tone suggested that the clock genes mediate the sympathetic regulation of bone formation. If this were the case, one would expect that leptin

A

Mouse strain	Body weight (g)	Fat pad weight (mg)	Food intake (g/day)	Uterus weight (mg)	Estradiol (pg/ml)	PTH (pg/ml)	Insulin (ng/ml)	Calcium (mg/dl)	Phosphorus (mg/dl)
Wt	**22.4** ± 0.3	**306** ± 21	**4.3** ± 0.1	**88** ± 7	**11.3** ± 2.2	**51** ± 11	**1.4** ± 0.4	**9.1** ± 0.5	**7.4** ± 0.7
Per1$^{-/-}$;*Per2*$^{m/m}$	**22.7** ± 0.2	**373** ± 29	**4.2** ± 0.1	**68** ± 5	**10.6** ± 0.7	**67** ± 17	**1.1** ± 0.2	**9.4** ± 0.4	**7.3** ± 0.8

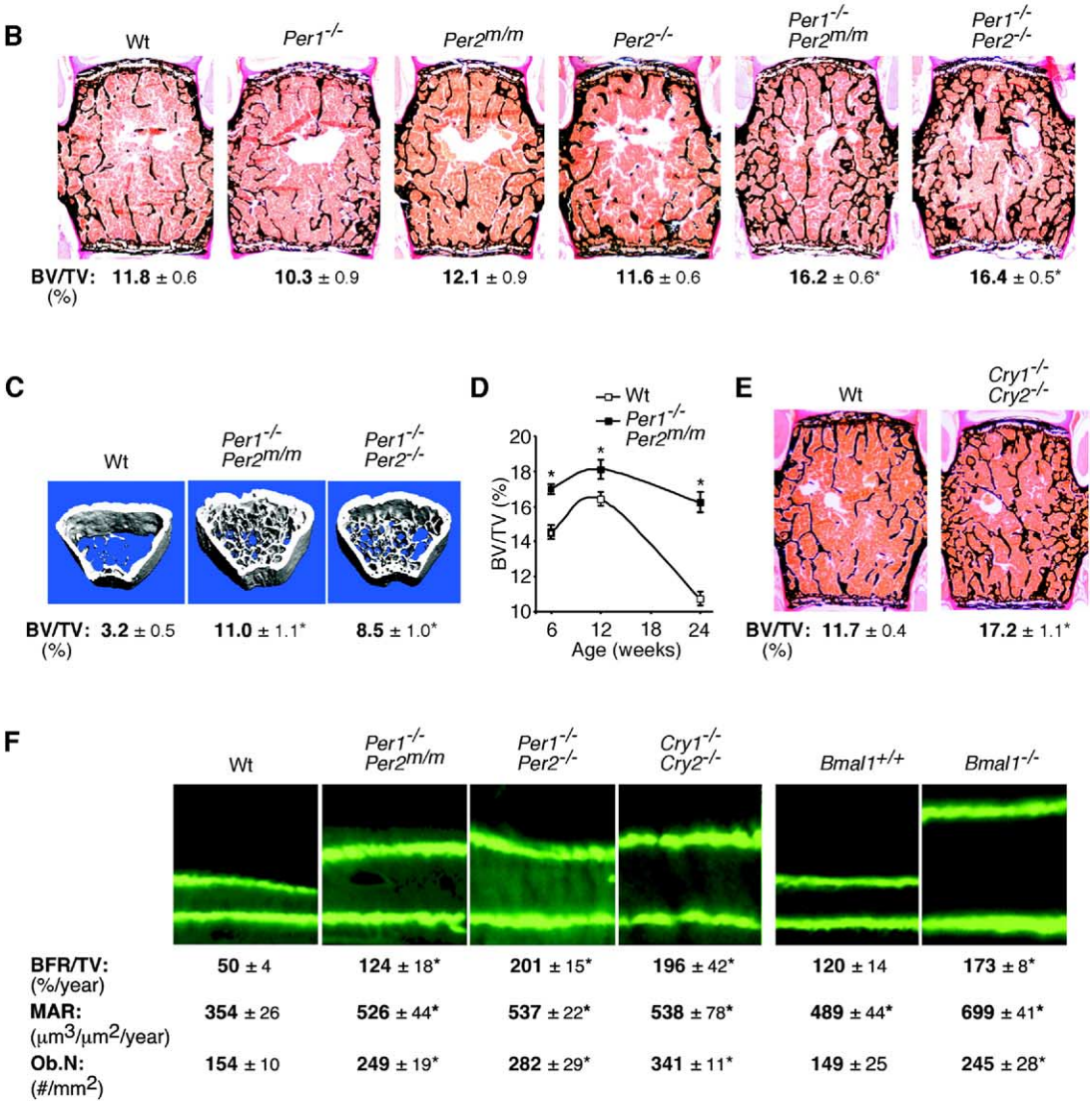

Figure 1. Clock Genes Inhibit Bone Formation

(A) Hormone and mineral ion measurements in 2-month-old wt and *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice.

(B and C) High bone mass (HBM) in vertebrae and distal femora of 6-month-old *Per1*$^{-/-}$;*Per2*$^{m/m}$ and *Per1*$^{-/-}$;*Per2*$^{-/-}$ mice.

(D) Worsening over time of the HBM. Error bars indicate standard error of the mean (±SEM).

(E) HBM in vertebrae of 6-month-old *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ mice.

(F) Elevated bone-formation parameters (BFR, bone-formation rate; MAR, mineral apposition rate) and osteoblast number (Ob.N) in wt and clock-gene mutant mice at 6 months of age. *Bmal1*$^{-/-}$ and control mice were studied at 2 months of age before mobility becomes hampered in *Bmal1*$^{-/-}$ mice. Asterisks indicate statistically significant differences.

i.c.v. infusion in these mice would not decrease bone mass as it did in wt mice (Ducy et al., 2000). Indeed, while bone-formation parameters and bone mass decreased in wt mice following long-term leptin i.c.v. infusion, this treatment consistently increased bone-formation parameters in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice (Figure 2G). Three lines of experimental evidence ruled out that the failure of leptin i.c.v. infusion to decrease bone mass in

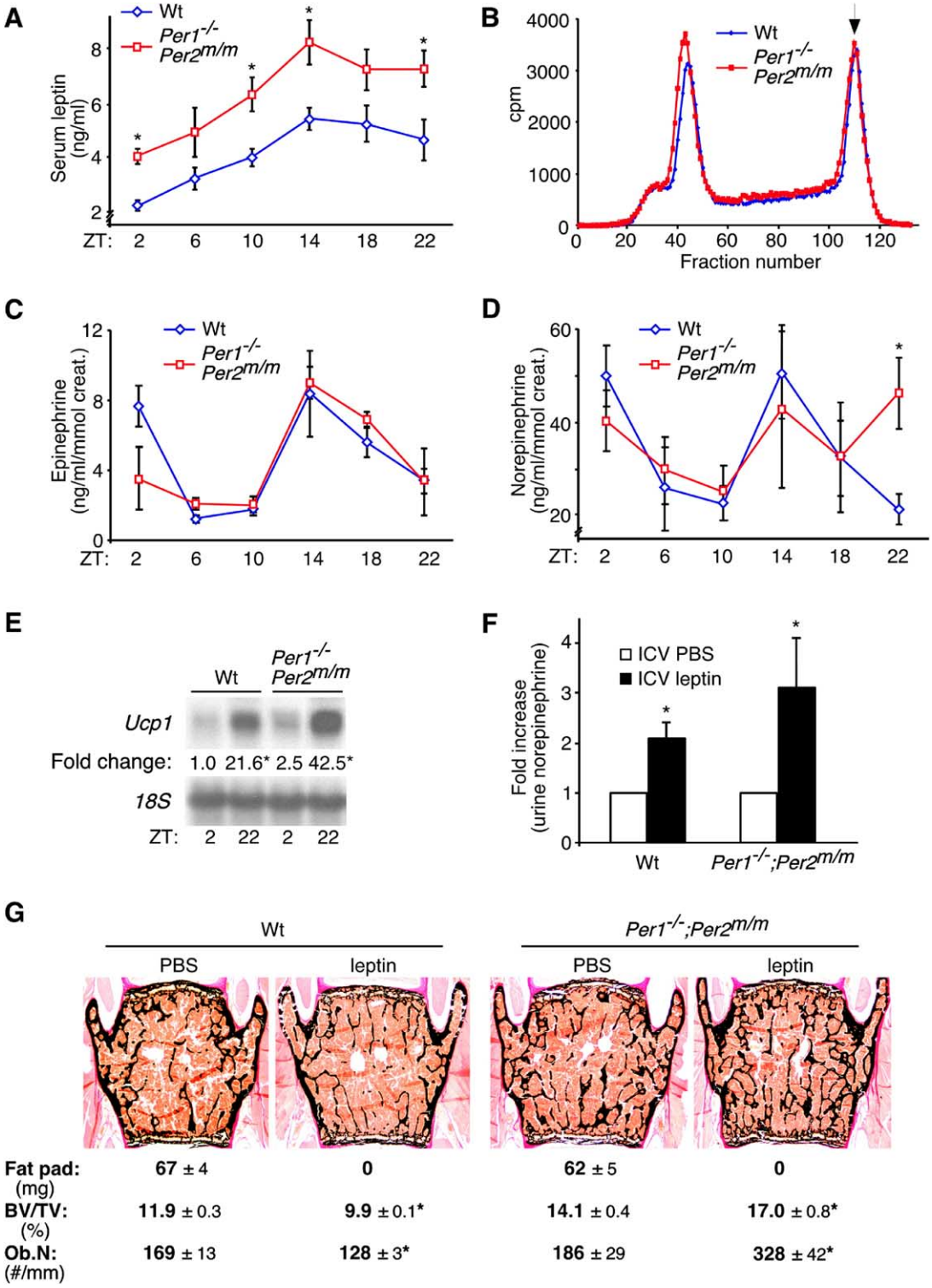

Figure 2. *Per* Genes Mediate Leptin's Regulation of Bone Formation

(A) Serum level of leptin is elevated in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice during a 24 hr period (n = 15/time point) (±SEM).

(B) Free serum leptin (arrow) is normal in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice as assessed by HPLC.

(C and D) Urinary catecholamines are normal or elevated in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice (±SEM).

(E) Sympathetic tone is normal or elevated in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice as assessed by *Ucp1* expression in brown fat.

(F) The induction of sympathetic tone by leptin is normal in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice, as indicated by a rise in urinary norepinephrine after leptin i.c.v. infusion (±SEM).

(G) Paradoxical effects of leptin i.c.v. infusion on bone mass and osteoblast number in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice. Asterisks indicate statistically significant differences.

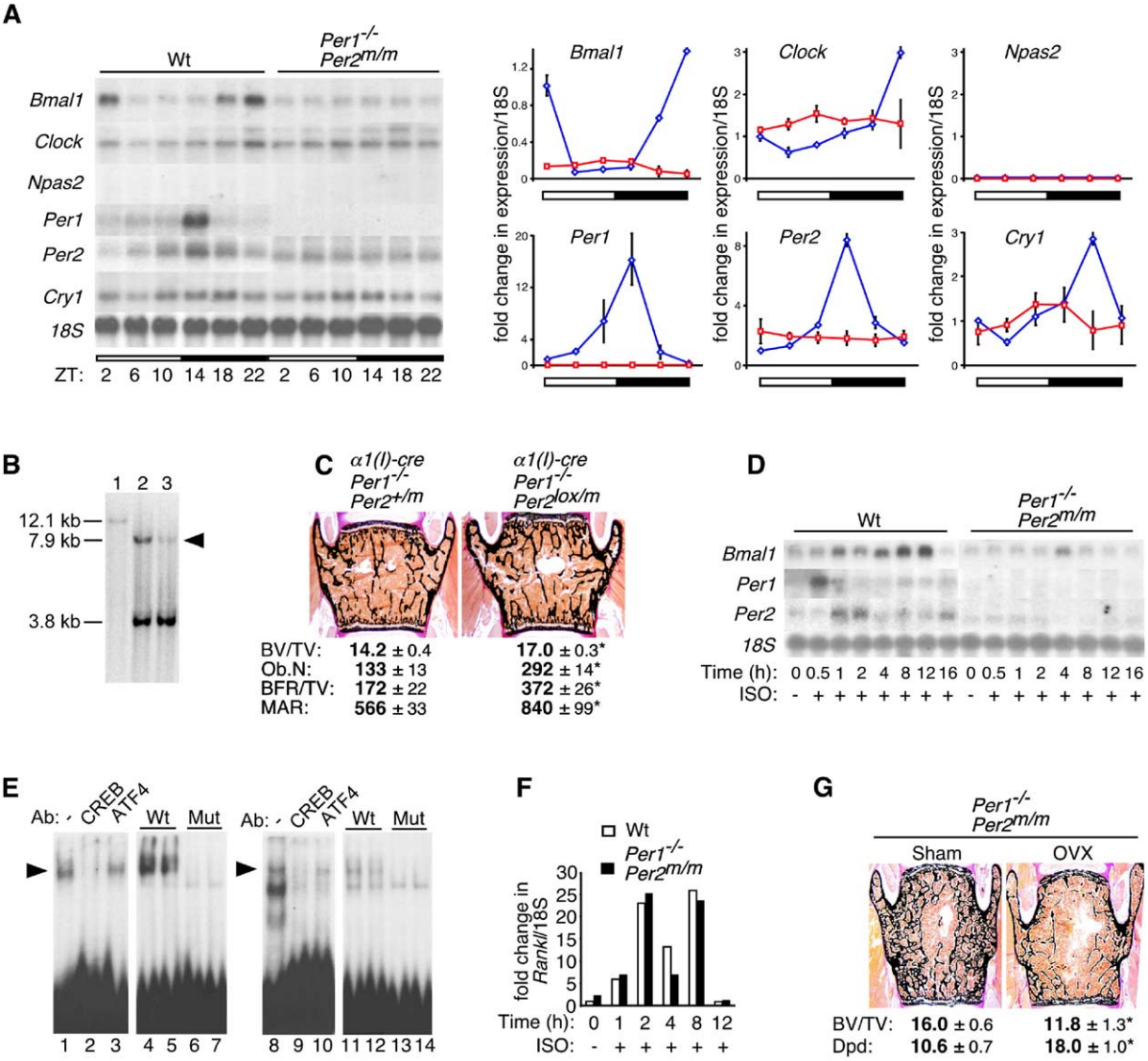

Figure 3. Clock Genes Respond to Sympathetic Signaling in Osteoblasts

(A) Clock genes are expressed and cycling in wt but not *Per1*$^{-/-}$;*Per2*$^{m/m}$ bones over a 24 hr period (Northern blots, n = 3). The mutant *Per2* mRNA is seen as a faster-migrating band due to an 87 amino acid in-frame deletion. Each RNA band was quantified using a PhosphorImager. After normalizing to 18S RNA, expression levels for each gene at ZT2 were arbitrarily set as 1. Error bars indicate standard deviation (±SD).

(B) Mean Cre-mediated excision frequency of 81%, assessed by using the 5′ loxP probe on HindIII-digested genomic DNA from osteoblast colony-forming units isolated from wt (lane 1), *Per1*$^{-/-}$;*Per2*$^{lox(ex10-12)/lox(ex10-12)}$ (lane 2), and α*1(I)-cre;Per1*$^{-/-}$;*Per2*$^{lox(ex10-12)/lox(ex10-12)}$ (lane 3) mice and quantifying the degree of loss of the 7.9 kb band.

(C) HBM with increased number of osteoblasts and bone-formation parameters (BV/TV, %; Ob.N, #/mm^2; BFR/TV, %/year; MAR, μm^3/μm^2/year) in α*1(I)-cre;Per1*$^{-/-}$;*Per2*$^{m/lox(ex10-12)}$ mice.

(D) Clock-gene expression is induced by isoproterenol (10 μM) in wt but not *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts.

(E) EMSA: CREB antibody (lane 2), not preimmune serum (lane 1) or ATF4 antibody (lane 3), prevented formation of a protein-DNA complex (filled arrow) on the *Per1* CRE site. A protein-DNA complex formed when nuclear extracts from wt (lane 4) or *Atf4*$^{-/-}$ (lane 5) osteoblasts were incubated with a wt *Per1* CRE but not a mutant *Per1* CRE (lanes 6 and 7). Similar results were obtained using the *Per2* CRE site (lanes 8–14).

(F) Induction of *Rankl* expression by isoproterenol (10 μM) is normal in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts, as measured by real-time PCR.

(G) Histology of 6-month-old *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice sham operated (Sham) or ovariectomized (OVX) at 2 months of age and Dpd (nmol/mmol creatinine) elevation demonstrated a normal osteoclastic response to gonadectomy in the absence of *Per* genes. Asterisks indicate statistically significant differences.

Per1$^{-/-}$;*Per2*$^{m/m}$ mice was due to leptin signaling defects. First, this infusion decreased fat-pad weight equally well in wt and *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice (Figure 2G); second, leptin receptor (*ObRb*) expression was normal in *Per1*$^{-/-}$;*Per2*$^{m/m}$ hypothalamus; and third, *Per* genes and *ObRb* were not coexpressed in hypothalamic neurons (data not shown). Thus, the most plausible explanation for the failure of leptin to lower bone mass in

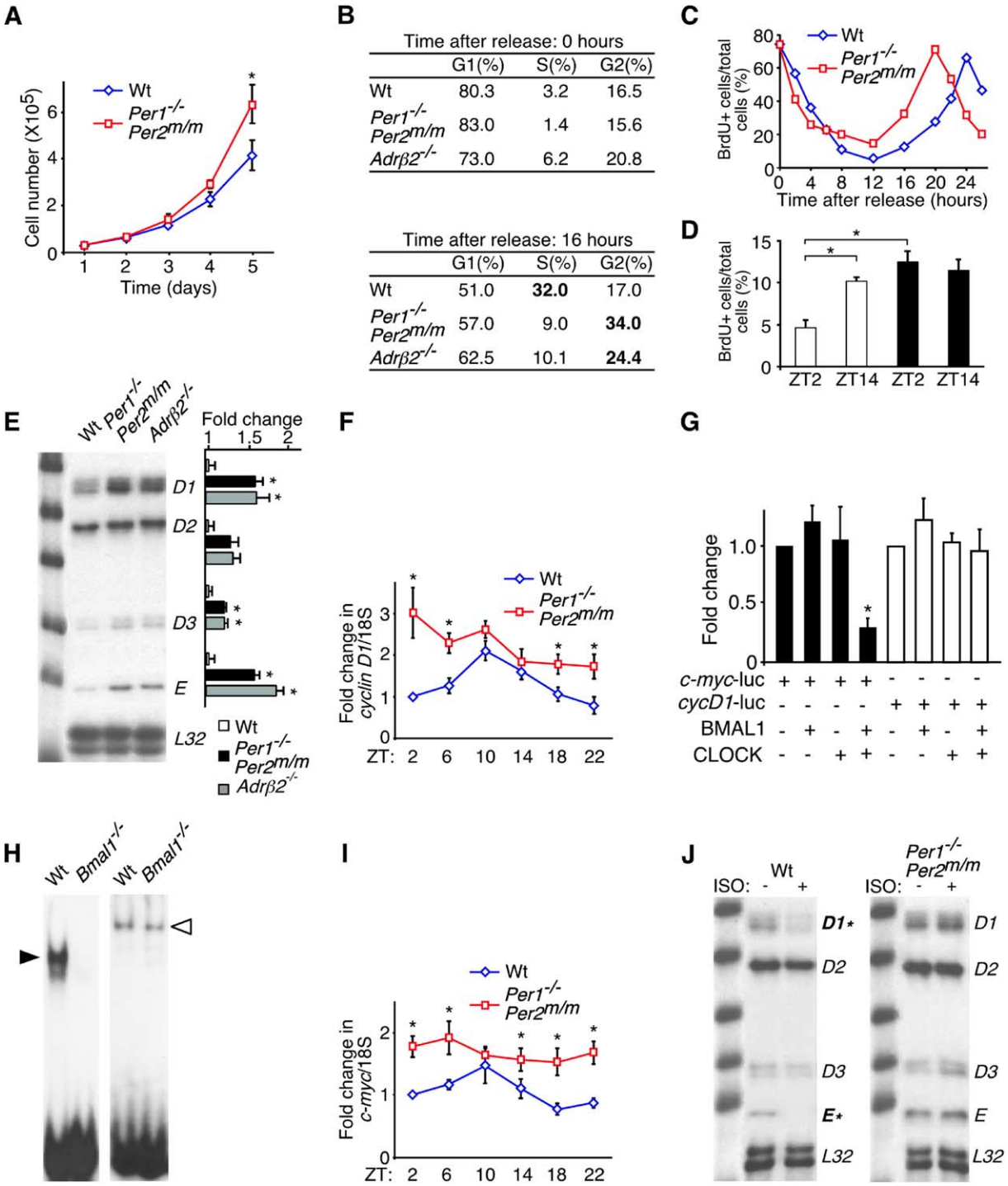

Figure 4. Control of Osteoblast Proliferation by Clock Genes

(A) Growth curves of wt and *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts over 5 days (±SD).

(B) The ratios of G1, S, and G2 phase cells for wt, *Per1*$^{-/-}$;*Per2*$^{m/m}$, and *Adr*β*2*$^{-/-}$ osteoblasts at 0 and 16 hr after releasing from serum starvation. Note the increase in G2 population of *Per1*$^{-/-}$;*Per2*$^{m/m}$ and *Adr*β*2*$^{-/-}$ osteoblasts 16 hr after release from G1 arrest, due to synchronized cells progressing into G2/M phase, but not G2/M arrest.

(C) The proportion of S phase cells in wt and *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts during a 26 hr period was determined by BrdU pulse chasing and flow cytometry. The time interval between the two S phase peaks was shorter in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts than in wt osteoblasts.

(D) Increased osteoblast proliferation in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice. Mitotic index of calvarial osteoblasts from 6-week-old wt (clear bars) and *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice (filled bars) (±SEM).

(E) The expression of *G1 cyclins* in wt, *Per1*$^{-/-}$;*Per2*$^{m/m}$, and *Adr*β*2*$^{-/-}$ osteoblasts. RNase-protection assay (RPA) shows upregulation of *G1 cyclins* in mutant osteoblasts. Three independent RPAs are summarized at right (±SD).

(F) Elevated *cyclin D1* expression in *Per1*$^{-/-}$;*Per2*$^{m/m}$ bones. PhosphorImager quantification of three independent Northern blots for *cyclin D1*

Per1$^{-/-}$;*Per2*$^{m/m}$ mice was that clock genes mediate, in osteoblasts, the leptin-dependent sympathetic inhibition of bone formation.

Per Gene Expression, Function, and Regulation by Sympathetic Tone in Osteoblasts

Consistent with the aforementioned hypothesis, *Per1*, *Per2*, *Cry1*, *Bmal1*, and *Clock* all showed robust, rhythmic expression over a 24 hr period in wt bones, and their expression was decreased and arrhythmic in *Per1*$^{-/-}$;*Per2*$^{m/m}$ bones, indicating that bone has a peripheral clock (Figure 3A). The expression of all clock genes was also detected in osteoblasts (Figure S4A). In contrast, the expression of *Npas2*, whose deletion does not affect bone mass (M.S.P. and G.K., unpublished data), was not detected in bone or osteoblasts, although it was detected easily in liver (data not shown).

To determine whether *Per* genes regulate bone mass by acting in osteoblasts, we engineered an osteoblast-specific inactivation of *Per2* on a *Per1*$^{-/-}$ background since *Per1*$^{-/-}$ mice did not show a bone phenotype (Figure S3). To that end, we crossed *Per1*$^{-/-}$ mice carrying a floxed allele of *Per2* with α*1(I)* collagen-*cre* mice that express cre recombinase only in differentiating osteoblasts (Dacquin et al., 2002). Recombination at the *Per2* locus was effective in osteoblasts and absent in other cell types (Figure 3B and data not shown). As was the case for *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice, we observed HBM, increased osteoblast numbers, and increased bone-formation parameters in all α*1(I)-cre;* *Per1*$^{-/-}$;*Per2*$^{lox(ex10-12)/m}$ mice examined (Figure 3C). To ascertain that clock-gene expression in osteoblasts is regulated by sympathetic signaling, we treated wt and *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts with isoproterenol, a sympathomimetic. As shown in Figure 3D and Figure S4B, *Bmal1*, *Per1*, and *Per2* expression was upregulated by isoproterenol in wt but not *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts. Taken together, these observations establish that clock genes respond to sympathetic signaling in osteoblasts to inhibit bone formation.

Transcriptional Mediation of the Sympathetic Regulation of *Per* Expression

Adrβ2, the only adrenergic receptor expressed in osteoblasts, is a G-coupled protein receptor that most often uses the transcription factor CREB to regulate gene expression (Benovic et al., 1988). However, sympathetic signaling in osteoblasts regulates osteoclast differentiation by using ATF4, an osteoblast-specific member of the CREB family, as a transcriptional mediator (Yang et al., 2004; Elefteriou et al., 2005).

To test whether ATF4 also mediated the sympathetic regulation of bone formation, we first performed electrophoretic mobility shift assays (EMSA). A protein-DNA complex formed equally well upon incubation of the *Per1* or *Per2* cyclic AMP-response element (CRE) with wt or *Atf4*$^{-/-}$ osteoblast nuclear extracts. This complex could be supershifted by an anti-CREB antibody but not by an anti-ATF4 antibody (Figure 3E). Furthermore, isoproterenol induced expression of *Rankl,* a target gene of ATF4, to the same extent in wt and *Per1*$^{-/-}$; *Per2*$^{m/m}$ osteoblasts (Figure 3F) and enhanced *Per1* and *2* expression equally well in wt and *Atf4*$^{-/-}$ osteoblasts (Figure S4C). Thus, sympathetic signaling in osteoblasts uses different transcriptional mediators to regulate bone formation, a CREB-mediated process, and bone resorption, an ATF4-mediated process. That the integrity of the sympathetic ATF4 signaling pathway regulating osteoclast differentiation was preserved in mutant osteoblasts explained why gonadectomy of *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice resulted in increased osteoclast function and bone loss (Figure 3G).

Control of Osteoblast Proliferation by the Molecular Clock

The most striking feature observed in *Adr*β*2*$^{-/-}$, *Per1*$^{-/-}$; *Per2*$^{m/m}$, *Per1*$^{-/-}$;*Per2*$^{-/-}$, *Cry1*$^{-/-}$;*Cry2*$^{-/-}$, and *Bmal1*$^{-/-}$ mice was a large increase in osteoblast number. Furthermore, while leptin i.c.v. infusion decreased osteoblast number in wt mice, it increased it in *Per1*$^{-/-}$; *Per2*$^{m/m}$ mice. These observations suggested that the molecular clock inhibits bone formation by preventing osteoblast proliferation. Consistent with this hypothesis, *Per1*$^{-/-}$;*Per2*$^{m/m}$ bone marrow contained significantly greater numbers of stromal and osteoblast progenitor cells than wt controls (Figure S4D), and, when plated at the same density, *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts grew faster than wt osteoblasts, resulting in a larger cell population at the end of a 5-day culture period (Figure 4A).

To study cell-cycle progression in wt and *Per1*$^{-/-}$; *Per2*$^{m/m}$ osteoblasts, cells were synchronized in G1 phase by serum starvation, then refed with fresh medium to initiate cell proliferation. Sixteen hours after refeeding, synchronized *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts had a 2-fold increase in G2 population as determined by flow cytometry, whereas wt osteoblasts showed an increase in S but not G2 population (Figure 4B). Next, wt and *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts were synchronized in S phase, returned to normal growth conditions, and pulse-labeled with BrdU at various times during a 26 hr period, and the proportion of S phase cells was determined by flow cytometry. The two S phase peaks were separated by 24.0 ± 0.2 hr in wt osteoblasts but only by 21.0 ± 1.0 hr in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts ($p < 0.05$) (Figure 4C). In vivo BrdU labeling confirmed that the osteoblast mitotic index was elevated in *Per1*$^{-/-}$; *Per2*$^{m/m}$ mice (Figure 4D). At the molecular level, the

expression in wt and *Per1*$^{-/-}$;*Per2*$^{m/m}$ bones during a 24 hr period (±SD).

(G) DNA-cotransfection assays. BMAL1/CLOCK heterodimers inhibit *c-myc* promoter but not *cyclin D1* promoter (±SD).

(H) EMSA: a protein-DNA complex (filled arrowhead) formed when wt but not *Bmal1*$^{-/-}$ nuclear extracts were incubated with the *c-myc* –510 E box (left panel), in contrast to normal Sp1 site binding by both extracts (hollow arrowhead).

(I) Elevated *c-myc* expression in *Per1*$^{-/-}$;*Per2*$^{m/m}$ bones. PhosphorImager quantification of three independent Northern blots for *c-myc* expression in wt and *Per1*$^{-/-}$;*Per2*$^{m/m}$ bones during a 24 hr period (±SD).

(J) RPAs showing that isoproterenol (10 μM) inhibits *G1 cyclin*s expression in wt (left panel) but not *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts (right panel). Asterisks indicate statistically significant differences.

expression of *G1 cyclin*s, such as *cyclin D1* and *E,* was significantly elevated in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts (Figure 4E). Taken together, these different assays demonstrated that *Per* gene deficiency in osteoblasts results in elevated *G1 cyclin* expression that leads to shortening of the cell cycle and increased cell proliferation.

Given the central role of cyclin D1 in controlling G1/S phase transition (Fu et al., 2004), we focused our attention on this gene. When assessed over 24 hr, *cyclin D1* expression was arrhythmic and significantly elevated at most times in *Per1*$^{-/-}$;*Per2*$^{m/m}$ bones (Figure 4F), and cyclin D1 protein was more abundant in *Per1*$^{-/-}$;*Per2*$^{m/m}$ than in wt osteoblasts (Figure S4E). To determine whether *cyclin D1* was a direct target of the molecular clock, we performed DNA-cotransfection and DNA binding assays. In DNA-cotransfection experiments, BMAL1 and CLOCK did not affect *cyclin D1* promoter activity. In EMSA, no binding of osteoblast nuclear extracts was observed to the *cyclin D1* promoter E box (Figure 4G and data not shown). In contrast, BMAL1/CLOCK inhibited the promoter activity of *c-myc*, a critical regulator of *cyclin D1* (Figure 4G), and wt but not *Bmal1*$^{-/-}$ osteoblast nuclear extracts could bind to the proximal E box present in *c-myc* P1 promoter (Figures 4G and 4H). Consistent with these observations, *c-myc* expression was elevated in *Per1*$^{-/-}$;*Per2*$^{m/m}$ bones at most time points studied and in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts (Figure 4I and Figure S4F). Thus, one mechanism whereby clock genes inhibit osteoblast proliferation is the downregulation of *c-myc* expression, although other mechanisms may exist.

A Sympathetic-Signaling Clock-Gene Pathway Regulates Osteoblast Proliferation

If clock genes mediate the sympathetic inhibition of bone formation, then the cellular and molecular abnormalities observed in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts should be present in *Adr*β*2*$^{-/-}$ osteoblasts. Accordingly, *Adr*β*2*$^{-/-}$ osteoblasts showed increases in cell-cycle progression and *cyclin D1* expression similar to *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts (Figures 4B and 4E), and isoproterenol treatment decreased *cyclin D1* and *E* expression in wt but not *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts (Figure 4J). Thus, the sympathetic regulation of clock genes and *G1 cyclin*s in osteoblasts, the similarity in cell-cycle profile between *Adr*β*2*$^{-/-}$ and *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts, and the failure of leptin i.c.v. infusion to inhibit bone formation in *Adr*β*2*$^{-/-}$ and *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice all supported the contention that clock genes act downstream of sympathetic signaling in osteoblasts to inhibit their proliferation and thereby inhibit bone formation.

Sympathetic Signaling Also Favors Osteoblast Proliferation via AP-1

Many cellular and molecular features were identical between *Adr*β*2*$^{-/-}$ and *Per1*$^{-/-}$;*Per2*$^{m/m}$ bones, yet there was one major difference: leptin i.c.v. infusion paradoxically increased osteoblast number in *Per1*$^{-/-}$;*Per2*$^{m/m}$ but had no effect in *Adr*β*2*$^{-/-}$ mice. Such a glaring discrepancy between two models that should phenocopy each other could be best explained if sympathetic signaling exerts two regulatory actions on osteoblast proliferation, one negative through the clock genes and another positive through yet-to-be-identified genes.

In an effort to identify these genes, we studied the expression of transcription factors that affect osteoblast differentiation and/or proliferation. As shown in Figure 5A, among all the genes tested, *c-fos*, a gene that can induce osteoblast transformation (Grigoriadis et al., 1995), was most markedly overexpressed in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts. Extending the significance of this finding, expression of most AP-1 genes was increased in *Per1*$^{-/-}$;*Per2*$^{m/m}$ and *Adr*β*2*$^{-/-}$ osteoblasts (Figure 5B). That isoproterenol induced the expression of several AP-1 genes in wt osteoblasts further established that the AP-1 genes are regulated by sympathetic signaling (Figure 5C).

Since *c-fos*$^{-/-}$ bones showed a decrease in periosteal osteoblast number compared to wt bones (data not shown) and *c-fos* was the gene most robustly induced by isoproterenol, we focused on this AP-1 family member. When plated at the same density, *c-fos*$^{-/-}$ osteoblasts grew more slowly than wt osteoblasts (Figure 5D), indicating that c-Fos controls osteoblast proliferation. In addition, expression of clock-controlled cell-cycle genes, such as *c-myc* and *cyclin D1*, was decreased in *c-fos*$^{-/-}$ osteoblasts (Figure 5E). While no canonical AP-1 binding site could be found in the *cyclin D1* gene, there was an AP-1 binding site in both human and mouse *c-myc* promoters, and, in EMSA, a protein-DNA complex formed upon incubation of the mouse *c-myc* AP-1 site with wt but not with *c-fos*$^{-/-}$ osteoblast nuclear extracts, indicating that c-Fos is part of the AP-1 complex binding to the *c-myc* promoter (Figure 5F). In DNA-cotransfection experiments, AP-1 proteins activated a reporter construct containing four copies of the wt but not mutant *c-myc* AP-1 site (Figure 5G). Taken together, these data indicate that sympathetic signaling also exerts a positive influence on osteoblast proliferation via AP-1 proteins. This regulation occurs, at least in part, by favoring expression of the same cell-cycle regulators that are downregulated by the clock genes.

Inhibition of AP-1 by the Molecular Clock

If sympathetic signaling in osteoblasts triggers two pathways having opposite effects on cell proliferation, is there a functional hierarchy and/or crosstalk between them? While it is unlikely that c-Fos regulates clock-gene expression in osteoblasts since *c-fos* inactivation did not affect *Per2* induction by isoproterenol (Figure 6A), several lines of evidence identified the clock genes as primary regulators of osteoblast proliferation and AP-1 gene expression. First and foremost, osteoblast number decreases in wt mice following leptin i.c.v. infusion (Figure 2G). Second, AP-1 genes were overexpressed in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts (Figure 5B). Third, isoproterenol induced AP-1 gene expression to a higher level in *Per1*$^{-/-}$;*Per2*$^{m/m}$ than in wt osteoblasts (Figure 6B). Lastly, in EMSA, wt but not *Bmal1*$^{-/-}$ osteoblast nuclear extracts bound to the E box at −454 in the mouse *c-fos* promoter (Figure 6C). The presence of crosstalk between these two pathways suggests that the increased *c-fos* expression in *Adr*β*2*$^{-/-}$ osteoblasts (Figure 5B) might be the result of downregulation of the clock genes in these cells.

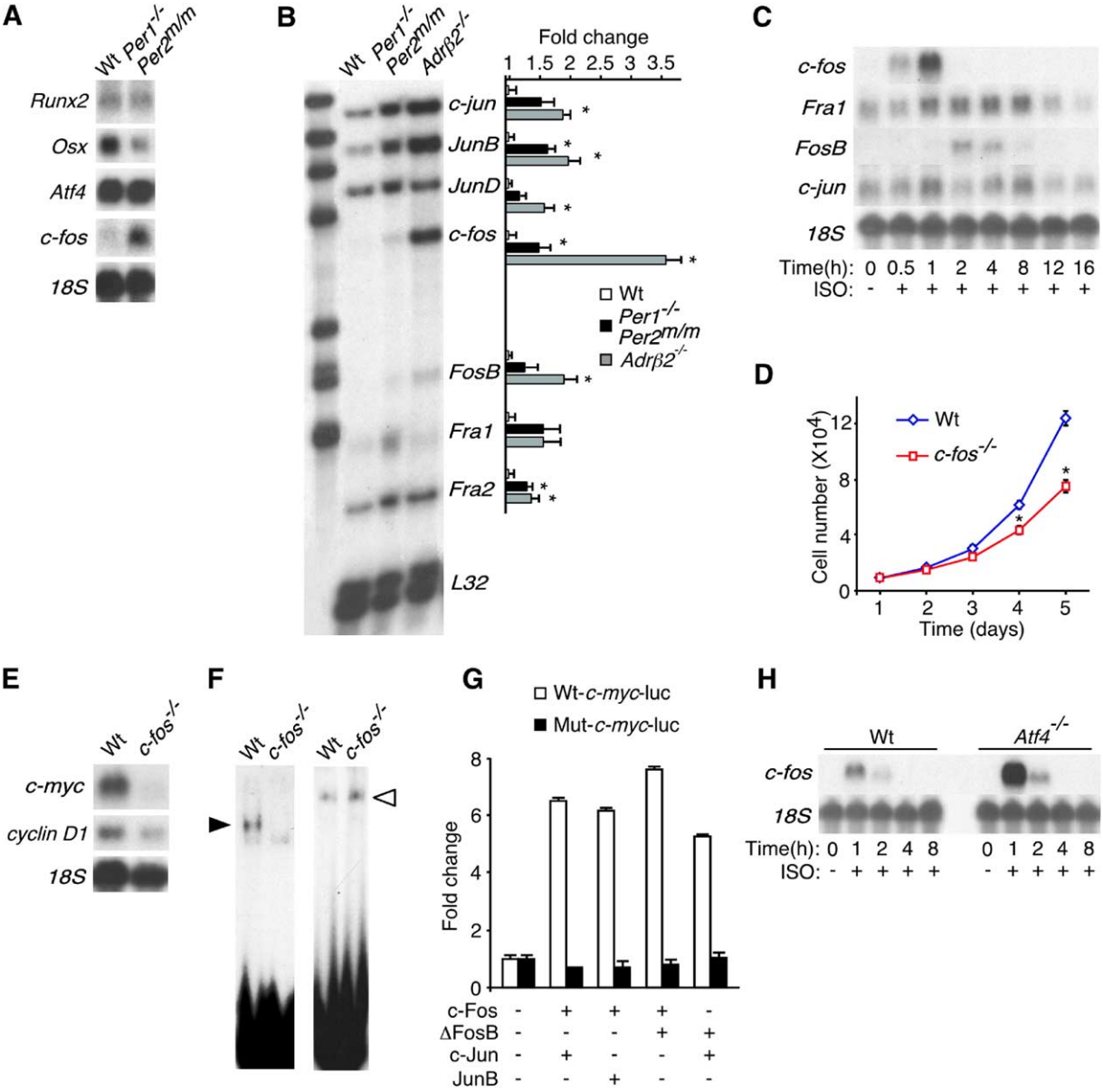

Figure 5. AP-1 Genes Are Targets of Sympathetic Signaling in Osteoblasts

(A) Expression of transcription factors affecting osteoblast differentiation and proliferation.

(B) AP-1 genes are overexpressed in *Per1*$^{-/-}$;*Per2*$^{m/m}$ and *Adr*β*2*$^{-/-}$ osteoblasts (RPA). Three independent RPAs are summarized at right (±SD).

(C) Induction of AP-1 gene expression in wt osteoblasts by isoproterenol (10 μM).

(D) Growth curves for wt and *c-fos*$^{-/-}$ osteoblasts over 5 days (±SD).

(E) Decreased expression of *c-myc* and *cyclin D1* in *c-fos*$^{-/-}$ osteoblasts.

(F) EMSA: a protein-DNA complex (filled arrowhead) formed when wt but not *c-fos*$^{-/-}$ osteoblast nuclear extracts were incubated with the *c-myc* AP-1 site, in contrast to normal Sp1 site binding by both extracts (hollow arrowhead).

(G) AP-1 transcription factors enhance the activity of a luciferase reporter construct containing four copies of a wt (clear bars) but not mutant (filled bars) *c-myc* AP-1 site (±SD).

(H) Normal induction of *c-fos* expression in *Atf4*$^{-/-}$ osteoblasts after isoproterenol treatment (10 μM). Asterisks indicate statistically significant differences.

Leptin Regulates the Expression of AP-1 and Clock Genes In Vivo

The last question we asked was whether the sympathetic regulation of osteoblast proliferation via the clock and AP-1 genes occurs under the control of leptin signaling. To that end, we used *ob/ob* mice, a mutant mouse strain that lacks leptin and responds to leptin i.c.v. infusion with dramatic phenotypic, cellular, and molecular consequences (Zhang et al., 1994). We transiently infused leptin in the third ventricle of these mice and, after confirming the expected increase in urine norepinephrine excretion (Figure S4G), studied gene

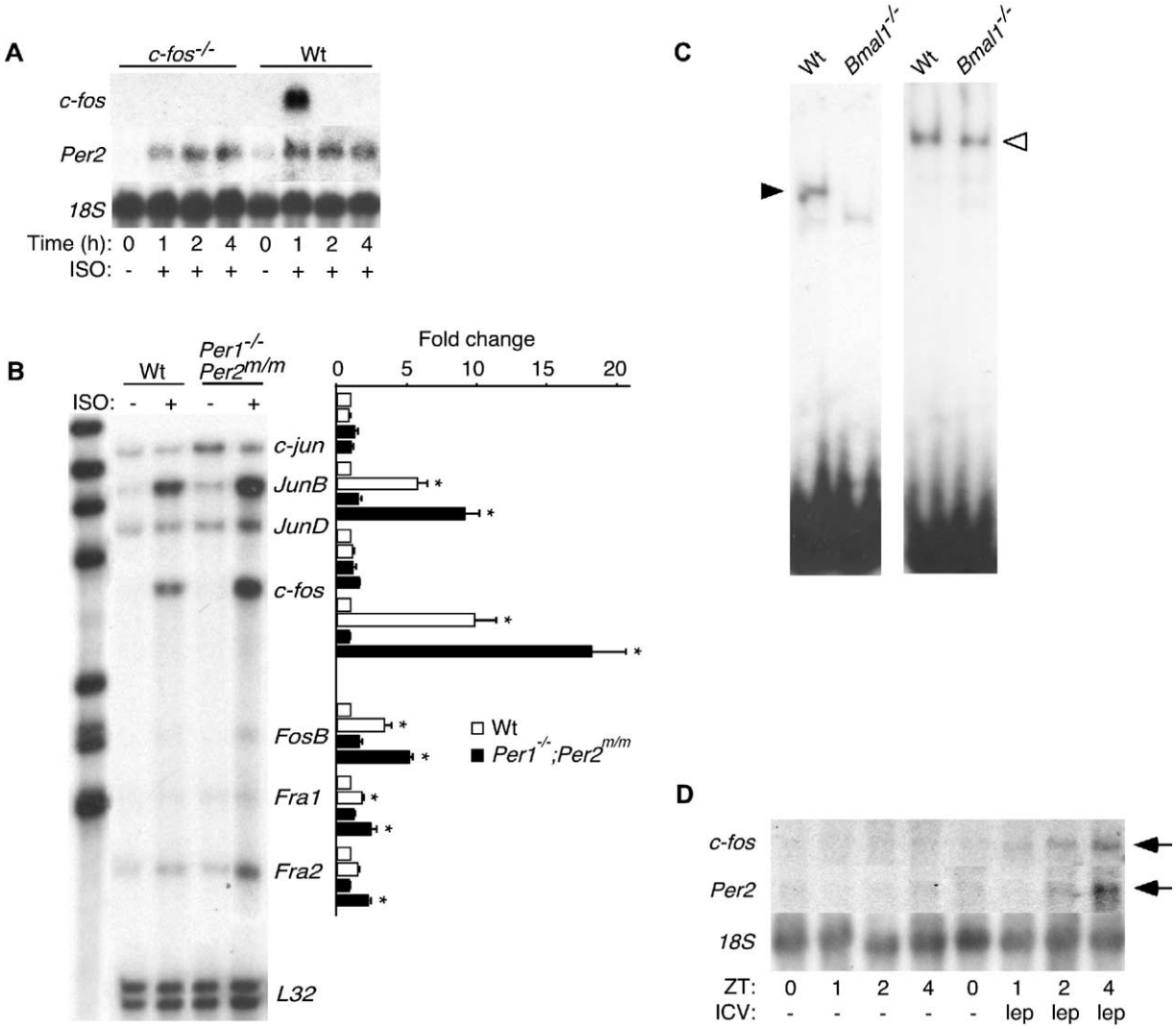

Figure 6. Crosstalk between Clock and AP-1 Genes

(A) Loss of *c-fos* function does not affect the induction of *Per2* by isoproterenol (10 μM) in osteoblasts.

(B) Most AP-1 family members are overinduced by isoproterenol (10 μM) in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts (RPA). Three independent RPAs are summarized at right (±SD).

(C) EMSA: a protein-DNA complex (filled arrowhead) formed when wt but not *Bmal1*$^{-/-}$ nuclear extracts were incubated with the *c-fos* −454 E box, in contrast to normal Sp1 site binding by both extracts (hollow arrowhead).

(D) Induction of *c-fos* and *Per2* in bone following a single i.c.v. injection of leptin into *ob/ob* mice. Asterisks indicate statistically significant differences.

expression in bone at various time points. As shown in Figure 6D, both *Per2* and *c-fos* expression increased significantly following this acute injection of leptin, and the same was true for *Clock* and additional AP-1 genes (data not shown). These results established that expression of these genes is controlled, in part, by leptin.

Discussion

This study demonstrates that clock genes in osteoblasts mediate the leptin-dependent sympathetic inhibition of bone formation. It also uncovers that leptin-dependent sympathetic signaling in osteoblasts promotes cell proliferation by activating AP-1 gene expression (Figure 7) a function of leptin not uncovered by the analysis of *ob/ob* mice. The regulation of bone formation by leptin through two antagonistic pathways is reminiscent of the dual mechanism that leptin uses to regulate bone resorption (Elefteriou et al., 2005).

A Candidate Gene Approach to Study Leptin's Antiosteogenic Function

Throughout the study of leptin's regulation of bone mass, we used a combination of two approaches: clinical observations for discovering a novel physiological pathway and reliance on candidate genes (Ducy et al.,

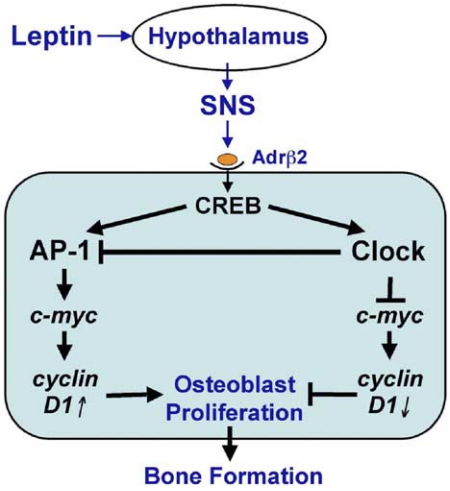

Figure 7. Current Model of the Leptin-Dependent Sympathetic Regulation of Bone Formation

Through CREB, sympathetic signaling activates the peripheral clock and AP-1 genes in osteoblasts. The clock genes inhibit osteoblast proliferation by inhibiting *c-myc*, which leads to *cyclin D1* downregulation. Counteracting these effects, AP-1 genes promote osteoblast proliferation by upregulating *c-myc* and thereby *cyclin D1*. The clock genes exert a dominant influence in part because they also inhibit AP-1 gene expression.

2000; Takeda et al., 2002). It is this latter approach that led us to test whether bone remodeling, like other homeostatic functions, is regulated by the molecular clock and resulted in the identification of the two molecular pathways summarized in Figure 7. Nevertheless, we are aware of the limitations of this strategy in studying a process as complex as the control of osteoblast proliferation. Indeed, this approach cannot be as comprehensive as a forward genetic approach; therefore, we cannot exclude that other mediators of clock-gene regulation of bone formation exist.

Leptin-Dependent Sympathetic Regulation of Clock-Gene Expression Restrains Osteoblast Proliferation

It has been proposed that the suprachiasmatic nucleus is required for the diurnal change in plasma leptin levels (Kalsbeek et al., 2001), a notion supported by the increase in serum leptin levels observed in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice. However, this increase in serum leptin levels does not result, as it should, in lower bone mass (Elefteriou et al., 2004). We show here that this is because clock genes are required in osteoblasts for the leptin-dependent sympathetic regulation of bone formation to take place. The evidence supporting this hypothesis includes: leptin i.c.v. infusion paradoxically increases bone mass in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice; clock-gene expression in osteoblasts is regulated by sympathetic signaling; and mice with an osteoblast-specific deletion of *Per2* showed increased bone formation, resulting in high bone mass on a *Per1*$^{-/-}$ background. Moreover, bone-marrow transplantation of *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblast precursors into wt recipients led to an increase in bone-formation parameters (data not shown). That leptin infusion activated *Per* expression in wt bone established that this regulation occurs in vivo.

A Molecular Blueprint for the Regulation of Osteoblast Proliferation by Clock Genes

Cell-based assays established that lack of clock genes favors osteoblast proliferation by accelerating G1/S phase transition. The molecular experiments suggested the following model to explain the increased cell proliferation in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts. In the absence of a functional molecular clock, the expression of *G1 cyclins*, in particular *cyclin D1*, is increased. The molecular clock controls *cyclin D1* expression through an indirect mechanism by inhibiting the expression of *c-myc*, a critical regulator of *cyclin D1* and a gene highly expressed in osteoblasts (Resnitzky and Reed, 1995; Perez-Roger et al., 1999). It has been shown previously that BMAL1/NPAS2 heterodimers inhibit *c-myc* promoter activity in vitro and that *c-myc* expression is increased in *Per2*$^{m/m}$ mouse liver (Fu et al., 2002). However, NPAS2 is not expressed in bone, and *Npas2*$^{-/-}$ mice have a normal bone mass (data not shown). On the other hand, CLOCK, another partner for BMAL1, is expressed in osteoblasts and, when cotransfected with BMAL1, could inhibit *c-myc* promoter activity in vitro.

We should emphasize that this model does not exclude the possibility that the molecular clock controls osteoblast proliferation through additional mechanisms. For instance, it has been shown that the molecular clock affects liver regeneration, presumably by directly controlling the Wee1-mediated G2/M checkpoint (Matsuo et al., 2003). Although, under normal growth conditions, we did not detect abnormal activation of G2/M checkpoint in *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts or a differential expression of Wee1 protein in wt and *Per1*$^{-/-}$;*Per2*$^{m/m}$ osteoblasts (data not shown), the possibility remains that Wee1 may regulate osteoblast proliferation under other physiological conditions.

Leptin-Dependent Sympathetic Signaling Favors Osteoblast Proliferation via AP-1

The increase in osteoblast number in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice following leptin i.c.v. infusion uncovered an unanticipated function of leptin for promoting osteoblast proliferation. Indeed, under the control of leptin, sympathetic signaling in osteoblasts favors AP-1 gene expression, which in turn induces expression of *c-myc* and *cyclin D1*. This leptin-dependent sympathetic regulation of AP-1 expression explains why the number of osteoblasts increases in *Per1*$^{-/-}$;*Per2*$^{m/m}$ mice following leptin i.c.v. infusion. These data are also consistent with a growing body of evidence obtained from both loss-of-function and gain-of-function studies that demonstrate that AP-1 plays a role in osteoblast proliferation and bone formation. These lines of evidence include the deficiency of cortical osteoblasts in *c-fos*$^{-/-}$ mice, the low bone mass seen in *Fra1*$^{-/-}$ mice, the osteopenia seen in *JunB*$^{\Delta/\Delta}$ mice (Kenner et al., 2004), and the increase in osteoblast proliferation following *Fra1* or Δ*FosB* overexpression (Jochum et al., 2000; Sabatakos et al., 2000).

There is a functional hierarchy between the two sympathetic-dependent antagonistic pathways in osteoblasts,

and two experimental arguments strongly suggest that the clock-mediated pathway plays the dominant role. First, increasing sympathetic tone by leptin i.c.v. infusion decreases osteoblast number in wt mice, a function ascribed here to the clock genes. Second, expression of multiple AP-1 genes is increased in the absence of *Per* genes, indicating that clock genes control directly and/or indirectly AP-1 expression (Figure 7).

Unexpected Specificity of the Mediators of Leptin's Regulation of Bone Mass

A feature progressively emerging from the molecular dissection of leptin's regulation of bone mass is that its mediators are not involved in a significant manner in mediating leptin's other cardinal functions, such as control of appetite and reproduction, in unchallenged animals. This specificity of action is surprising in the case of clock genes, AP-1 genes, and *Adr*β*2*, which are not osteoblast-specific genes. The narrow specificity of action of these mediators and the ability of leptin to regulate powerfully both aspects of bone remodeling, along with the fact that it first appeared during evolution in vertebrates, underscore the importance of leptin's regulation of bone mass and raise the prospect that this may have been the ancestral function of this hormone.

Experimental Procedures

Animal Maintenance

Mice were housed in 12 hr:12 hr light/dark cycles with light on at ZT0 and off at ZT12. Human leptin (Sigma; 8 ng/hr in PBS) or PBS was infused i.c.v. as described (Ducy et al., 2000) or transiently (3.5 μg in 2 μl over 6 min) using an infusion pump. Mice were injected with 25 mg/kg calcein 6 and 2 days prior to sacrifice, and histomorphometric analyses were performed as described (Kato et al., 2002). Mice were injected i.p. with 15 μl/g of 25 mM BrdU at ZT2 and ZT14 and were sacrificed 2 hr later, and BrdU was detected as previously described (Kato et al., 2002).

Cell Culture

Stromal-cell and osteoblast progenitors were cultured according to Kato et al. (2002). Primary osteoblasts were isolated as in Ducy et al. (1999) and cultured in αMEM with 10% FBS (Hyclone). Osteoblast growth curves were obtained by manual counting and by crystal violet staining. For cell-cycle studies, osteoblasts were collected after releasing from serum starvation or hydroxyurea (0.5 mM) block and pulse-chasing with BrdU (10 μM, 30 min) at various times and analyzed by a Becton Dickinson FACScan flow cytometer following standard procedures. Lipofectamine (Invitrogen) was used for transient transfection assays, with β-gal cotransfection serving as a control.

RNA and Protein Analyses

RNA was prepared from cells treated with isoproterenol (10 μM in PBS) for 20 min in αMEM/0.2% FBS or from bones using TriZOL. RPA was performed using the RiboQuant multiprobe RPA systems for mouse AP-1 and *cyclin* genes (BD Biosciences). Northern blots and RPAs were quantified using a Molecular Dynamics Storm 860 PhosphorImager/Fluorima. Real-time PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with probes and reagents supplied by the manufacturer. The level of expression for all mRNAs was normalized to that of 18S rRNA. Cyclin D1 was detected by Western blot using a cyclin D1-specific antibody (Santa Cruz Biotechnology) and an anti-β-actin antibody (Sigma) as a loading control. Electrophoretic mobility shift assays (EMSA) were performed following standard procedures.

Chemistry and Hormone Measurements

Leptin (Crystal Chem), estradiol (DSL), PTH (Immunotopics), insulin (Crystal Chem), calcium, and phosphate were measured using commercially available kits. Deoxypyridinoline crosslinks were measured in two consecutive morning urines (ZT3) per mouse (Metra Biosystems) in 6-month-old mice or 2 weeks after ovariectomy (OVX). Norepinephrine and epinephrine were measured in acidified spot urine samples by either TLC (Amersham) or RIA (BiCAT; Alpco) with similar results. Creatinine was used to standardize between urine samples.

Statistical Analyses

Statistical significance was assessed by Student's two-tailed t test. Values were considered statistically significant at $p < 0.05$.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/cgi/content/full/122/5/803/DC1/.

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