Leptin regulation of bone resorption by the sympathetic nervous system and CART

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Bone remodelling, the mechanism by which vertebrates regulate bone mass, comprises two phases, namely resorption by osteoclasts and formation by osteoblasts; osteoblasts are multifunctional cells also controlling osteoclast differentiation. Sympathetic signalling via β2-adrenergic receptors (Adrb2) present on osteoblasts controls bone formation downstream of leptin[1]. Here we show, by analysing *Adrb2*-deficient mice, that the sympathetic nervous system favours bone resorption by increasing expression in osteoblast progenitor cells of the osteoclast differentiation factor *Rankl*. This sympathetic function requires phosphorylation (by protein kinase A) of ATF4, a cell-specific CREB-related transcription factor essential for osteoblast differentiation and function[2]. That bone resorption cannot increase in gonadectomized *Adrb2*-deficient mice highlights the biological importance of this regulation, but also contrasts sharply with the increase in bone resorption characterizing another hypogonadic mouse with low sympathetic tone, the *ob*/*ob* mouse[3]. This discrepancy is explained, in part, by the fact that CART ('cocaine amphetamine regulated transcript'), a neuropeptide whose expression is controlled by leptin and nearly abolished in *ob*/*ob* mice[4], inhibits bone resorption by modulating *Rankl* expression. Our study establishes that leptin-regulated neural pathways control both aspects of bone remodelling, and demonstrates that integrity of sympathetic signalling is necessary for the increase in bone resorption caused by gonadal failure.

Leptin antiosteogenic function is mediated by the sympathetic nervous system (SNS) acting through Adrb2, the only adrenergic receptor expressed in osteoblasts[1] (Supplementary Fig. 1). If both arms of bone remodelling are regulated by similar mechanisms, these results imply that bone resorption (BR) is controlled by neural means. To test this hypothesis, we used mutant mice in which pathways acting downstream of leptin signalling were disrupted.

Adrb2$^{-/-}$ mice have normal body weight and fat pad weight[5], and none of the endocrine abnormalities observed in mice lacking leptin (*ob*/*ob*) or noradrenaline (*Dopamine-β-hydroxylase (Dbh)*$^{-/-}$ mice, Fig. 1a)[6,7]. Analyses of vertebrae and long bones revealed two unanticipated features in 6-month-old male and female *Adrb2*$^{-/-}$ mice. First, *Adrb2*$^{-/-}$ mice had a more severe high bone mass phenotype (HBM) than *ob*/*ob* or wild-type (WT) mice receiving β-blockers[1,3] (Fig. 1b, c). Illustrating the importance of sympathetic signalling in bone remodelling, this HBM also affected *Adrb2*$^{+/-}$ mice yet *Adrb2*$^{+/-}$ osteoblasts do transduce a signal through Adrb2 following treatment with isoproterenol (ISO), a surrogate of sympathetic signalling (Fig. 1b, d). This HBM was not observed in mice lacking *Adrb1* (Supplementary Fig. 2), indicating that *Adrb2*$^{-/-}$ mice are the best model to elucidate how sympathetic signalling in bone cells regulates bone mass. That long-term leptin intracerebroventricular (ICV) infusion did not reduce bone mass of *Adrb2*$^{-/-}$ mice established that SNS integrity is necessary for leptin antiosteogenic function (Fig. 1e).

To determine whether *Adrb2*$^{-/-}$ mice HBM involves bone cell-autonomous mechanisms, we performed transplantation of non-adherent bone marrow cells (BMCs)[8]. Transplantation of WT BMC into γ-irradiated *Adrb2*$^{-/-}$ mice normalized bone formation parameters; conversely, transplantation of *Adrb2*$^{-/-}$ BMC into γ-irradiated WT mice significantly increased bone formation (Fig. 1f). Polymerase chain reaction (PCR) analysis of *in vitro* differentiated osteoblastic colonies showed the presence of WT or *Adrb2*$^{-/-}$ osteoblasts in *Adrb2*$^{-/-}$ or WT γ-irradiated mice, respectively (Fig. 1g and data not shown). Immunocytochemistry analysis showed Neomycin^{+}, Runx2^{+} osteoblasts in WT mice transplanted with *Adrb2*$^{-/-}$ BMCs and *Adrb2*$^{+}$, Runx2^{+}, Neomycin^{-} osteoblasts in *Adrb2*$^{-/-}$ mice transplanted with WT BMCs (Fig. 1h). Thus the SNS controls bone mass by acting, at least in part, on cells of the osteoblast lineage.

Further cellular analysis of *Adrb2*$^{-/-}$ mice revealed a second unanticipated feature. Besides the expected increase in bone formation parameters[1] (Fig. 1b), there was, in all bones analysed, a significant decrease in BR parameters, including a decrease in number of tartrate-resistant acid phosphatase (TRAP)-positive multi-nucleated osteoclasts, indicative of a defect in osteoclast differentiation, and a decrease in urinary elimination of deoxypyridinoline (Dpd), a marker of osteoclast function (Fig. 2a). This was unexpected, because *ob*/*ob* or β-blocker-treated WT mice[1] have high and normal BR, respectively (Fig. 2b and data not shown). A decrease in Dpd urinary elimination was also observed in WT mice transplanted with *Adrb2*$^{-/-}$ BMC, while WT BMC transplantation into *Adrb2*$^{-/-}$ mice increased it (Fig. 1f). These BR abnormalities were not corrected by leptin ICV infusion, indicating that leptin signalling regulates BR via the SNS (Fig. 2b).

To determine whether sympathetic signalling acts on osteoclast, we cultured bone marrow macrophages (BMMs) in the presence of RANKL, an osteoclast differentiation factor, and M-CSF, an osteoclast proliferation factor[9,10]. WT or *Adrb2*$^{-/-}$ BMMs differentiated equally well into osteoclasts at each RANKL/M-CSF dose tested (Fig. 2c), and ISO did not hamper generation of osteoclasts (Supplementary Fig. 3). *Adrb2* inactivation did not alter the ability of osteoclasts to generate resorption pits on dentine slices, and ISO did not increase cAMP production in osteoclasts (Fig. 2d-e).

Next we asked whether the SNS affects BR by acting in osteoblasts, a cell type controlling osteoclast differentiation[9]. ISO enhanced generation of osteoclasts when WT, but not *Adrb2*$^{-/-}$, osteoblasts were co-cultured with WT BMMs (Fig. 2f). Moreover, ISO treatment of WT, but not *Adrb2*$^{-/-}$, osteoblasts increased expression of *Rankl* (a secreted osteoclast differentiation factor) to a greater extent than did parathyroid hormone (PTH), a hormone that upregulates *Rankl* expression in both WT[9] and *Adrb2*$^{-/-}$ osteoblasts (Fig. 2g). Accordingly, *Rankl* expression was decreased in *Adrb2*$^{-/-}$ osteoblasts (Fig. 2h). ISO did not affect expression of *Osteoprotegerin* (a *Rankl* decoy receptor) or of other cytokines (Supplementary Fig. 4).

ISO induction of *Rankl* was blunted by a PKA inhibitor, suggesting that CREB mediates it (Fig. 2i). A CREB-responsive element is present in *Rankl* promoter at −1097, and a protein–DNA complex formed upon incubation of osteoblast nuclear extracts with this element in electrophoretic mobility shift assay (EMSA) (Fig. 3a). Surprisingly, this protein–DNA complex was not affected by an antibody against CREB but was abolished by an antibody against ATF4, an osteoblast-specific CREB/ATF family member essential for osteoblast function[2]. Several lines of evidence

demonstrated that ATF4 mediates sympathetic regulation of *Rankl* expression in osteoblasts. First, in EMSA, this protein–DNA complex did not form when nuclear extracts of *Atf4*$^{-/-}$ osteoblasts were used as a source of proteins (Fig. 3b); second, in chromatin immunoprecipitation, ATF4 bound to this sequence while CREB did not, although it bound to the *c-Fos* promoter, a known target gene[11] (Fig. 3c). Third, in DNA cotransfection experiments, ATF4 transactivated a vector containing 6 copies of the WT but not of a mutant -1097^{Rankl} promoter element; CREB and other leucine zipper proteins did not (Fig. 3d); fourth, unlike PTH, isoproterenol did not upregulate *Rankl* expression in *Atf4*$^{-/-}$ osteoblasts (Fig. 3e); fifth, *Rankl* expression and osteoclast surface were decreased in *Atf4*$^{-/-}$ osteoblasts and bones, respectively (Fig. 3f, g).

ISO increased ATF4 transactivation function and triggered its phosphorylation, two events prevented by PKA inhibition (Fig. 3h, i). A consensus PKA phosphorylation site exists in ATF4 at serine 254. Mutating this highly conserved serine to alanine prevented PKA phosphorylation of ATF4 (Fig. 3j), and PKA forced expression enhanced ATF4 transactivation function (Fig. 3k). Conversely, ISO induced *Rankl* expression in osteoblasts lacking

a

	WT	*Adrb2*$^{-/-}$	WT	*Dbh*$^{-/-}$	WT	*ob/ob*
Corticosterone	203.8±39.1	192.3±24.4	118.7±43.3	238.5±46.8*	40.4±7.8	360.0±20.3*
Insulin	0.38±0.12	0.37±0.16	0.34±0.07	0.57±0.13	0.80±0.33	19.60±0.81*
Leptin	2.97±0.68	3.61±0.50	2.1±0.50	3.0±0.40	5.8±0.5	0.0
PTH	0.136±0.04	0.129±0.06	0.093±0.01	0.099±0.01	0.035.0±0.001	0.042±0.010

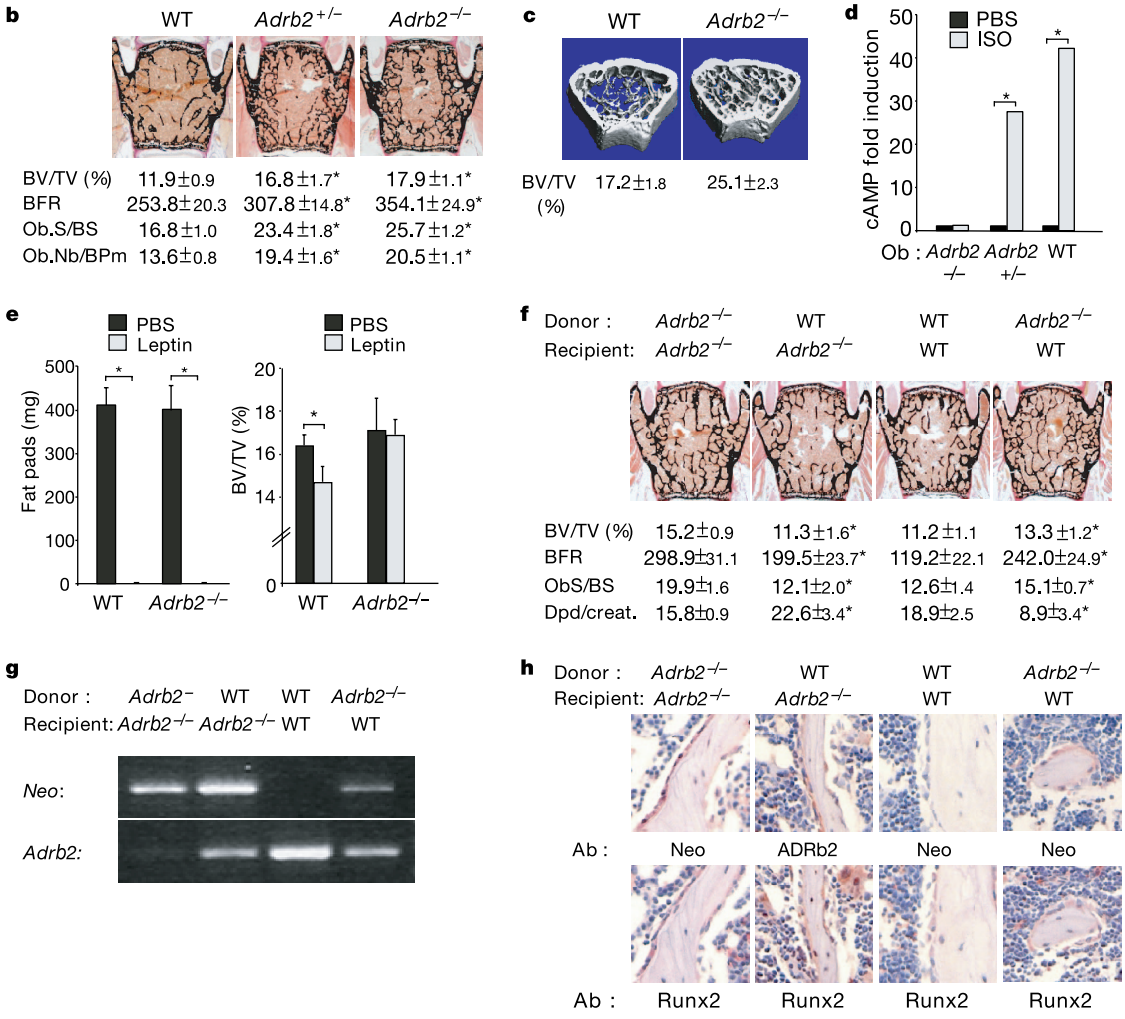

Figure 1 Increased bone formation in *Adrb2*-deficient mice. **a**, Hormonal measurements (ng ml^{-1}, mean + s.e.m) in 6- (*Adrb2*$^{-/-}$ and *Dbh*$^{-/-}$) and 3-month-old (*ob/ob*) mice. Differences in ages and genetic backgrounds explain different hormonal levels. **b**, Bone volume/tissue volume (BV/TV, %), bone formation rate (BFR, $\mu m^3\,\mu m^{-2}\,yr^{-1}$), osteoblast surface/bone surface (Ob.S/BS) and osteoblast number/bone perimeter (Ob.Nb/BPm) in 6-month-old mice (mean + s.e.m). **c**, μCT analysis of 6-month-old distal femurs. **d**, cAMP production in phosphate buffered saline (PBS) and isoproterenol (ISO)-treated osteoblasts (Ob). **e**, Fat pad weight and BV/TV following leptin ICV infusion. **f**, Formation and resorption parameters following transplantations. Comparison is between the same recipient genotype (mean + s.e.m). **g**, PCR analysis of transplantation efficiency using Neomycin (Neo) and *Adrb2*-specific primers. **h**, Immunocytochemical detection of Neomycin$^+$, Runx2$^+$ and Adrb2$^+$ osteoblasts in WT and *Adrb2*$^{-/-}$ bones following transplantation. Error bars, mean + s.e.m. *Statistically significant.

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RSK2, another kinase regulating ATF4 function[2] (Fig. 3l), and failed to phosphorylate RSK2 (Supplementary Fig. 5). Thus, ATF4 phosphorylation by PKA but not by RSK2 is required for sympathetic regulation of *Rankl* expression.

ATF4 is required for sympathetic, but not PTH, regulation of *Rankl* expression, suggesting that sympathetic and PTH signalling target different subsets of osteoblasts. Indeed, *Adrb2* was expressed at its highest level in undifferentiated osteoblasts (day 0 of culture), when *Atf4* expression peaks, while *PTH receptor* expression peaked later in differentiated osteoblasts (Fig. 3m).

The biological importance of the sympathetic regulation of BR was addressed by ovariectomy of 1-month-old mice and histological analyses 4 and 12 weeks later. Ovariectomy increased osteoclast surface and Dpd urinary elimination in WT but not in *Adrb2*$^{-/-}$ mice. As a result, bone mass did not decrease in *Adrb2*$^{-/-}$ mice following gonadectomy (Fig. 4a–c). Although these results highlighted the importance of SNS integrity for the development of gonadal failure-induced bone loss, they were totally unexpected. Indeed, gonadectomized *Adrb2*$^{-/-}$ mice should be, in term of bone biology, a phenocopy of *ob*/*ob* mice that are hypogonadic and have a low sympathetic activity. Yet while *ob*/*ob* have an increase in BR, gonadectomized *Adrb2*$^{-/-}$ mice do not. This discrepancy implied that expression of gene(s) regulating BR is perturbed in *ob*/*ob* but not in gonadectomized *Adrb2*$^{-/-}$ mice.

To identify such inhibitors, we focused on genes whose expression is regulated by leptin but whose inactivation does not affect appetite or fertility. *Cart* encodes a neuropeptide whose expression in brain is increased by leptin, directly or indirectly, low in *ob*/*ob* mice[3] and normal in *Adrb2*$^{-/-}$ mice (Supplementary Fig. 6). *Cart*$^{-/-}$ mice have no overt phenotypic abnormalities[12] (Supplementary Fig. 7) but displayed, in both sexes, a low bone mass phenotype at 6 months of age (Fig. 4d-e and Supplementary Fig. 8). Osteoblast numbers and bone formation rates were normal while osteoclast surface and number was nearly doubled in *Cart*$^{-/-}$ bones (Fig. 4d). The significant increase in urinary Dpd elimination established that *Cart*$^{-/-}$ osteoclasts were functional.

To determine if leptin-dependent sympathetic regulation of bone mass occurred in the absence of CART, we performed leptin ICV

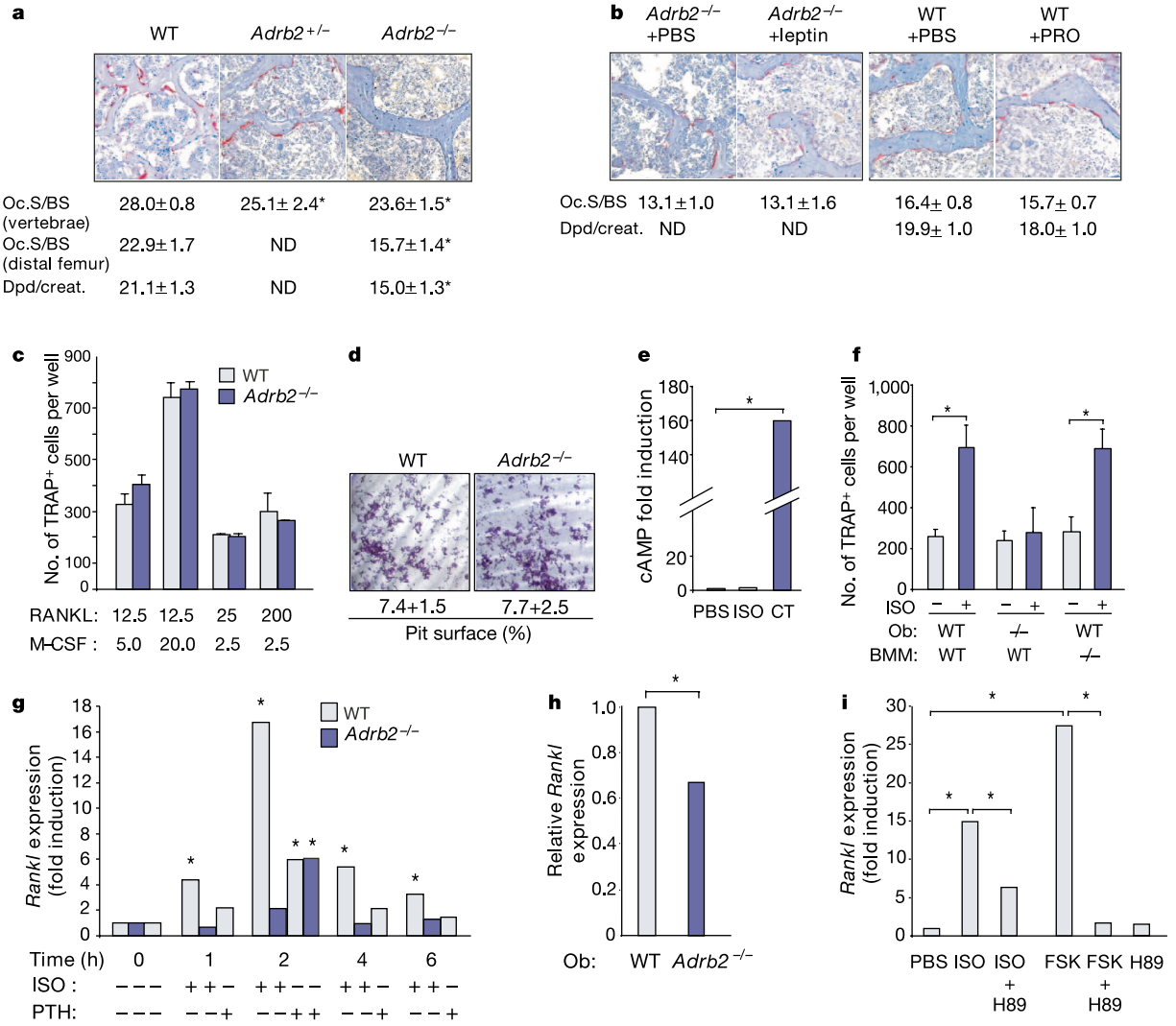

Figure 2 Sympathetic signalling in osteoblasts regulates BR. **a**,**b**, Osteoclast surface/bone surface and Dpd/creatinine in 6-month-old mice; **b**, following leptin ICV or propranolol (PRO) treatment. ND, not determined. **c**, *In vitro* differentiation of WT and *Adrb2*$^{-/-}$ BMMs with limiting amounts of RANKL and M-CSF (ng ml^{-1}). **d**, WT and *Adrb2*$^{-/-}$ osteoclasts generate resorption pits equally well. **e**, Calcitonin, not ISO, induces cAMP production in osteoclasts. **f**, Number of TRAP^{+} cells following co-culture of osteoblasts and BMMs in the presence of ISO. **g**–**i**, Real-time PCR. ISO induces *Rankl* expression in WT not *Adrb2*$^{-/-}$ osteoblasts. *Rankl* expression is decreased in *Adrb2*$^{-/-}$ osteoblasts and PKA is required for ISO induction of *Rankl* expression ($n = 3$ per experiment). Error bars, mean + s.e.m. *Statistically significant.

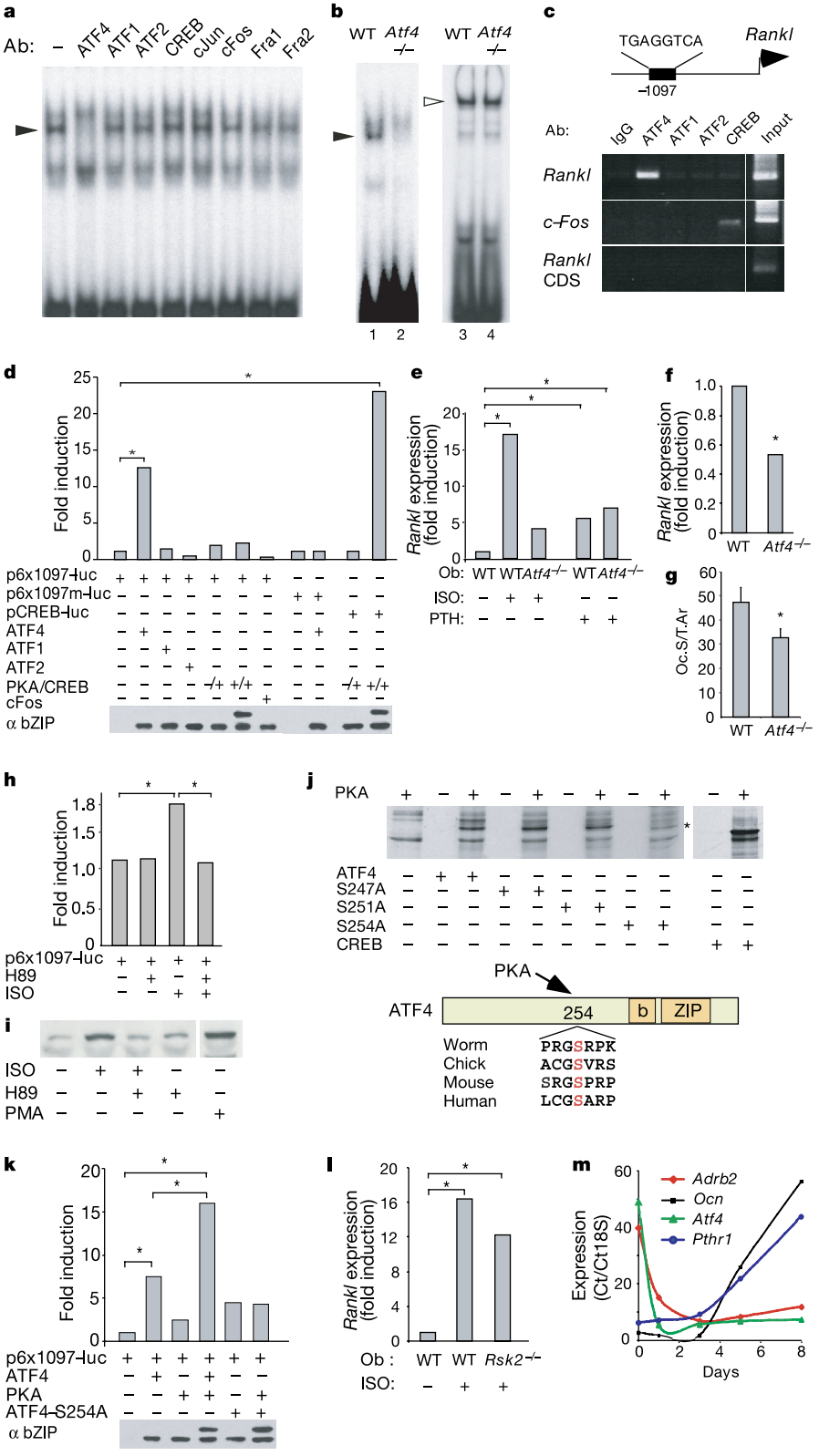

Figure 3 Sympathetic regulation of *Rankl* expression. **a**, EMSA: ATF4 antibody prevented formation of protein–DNA complex (filled arrow) on *Rankl* promoter CRE site. **b**, WT (1) but not *Atf4* $^{-/-}$ (2) osteoblast nuclear extracts bind to -1097^{Rankl}. Sp1 (open arrow) served as control (3, 4). **c**, ChIP: ATF4 binds to -1097^{Rankl} but not to *Rankl* coding sequence (CDS). **d**, DNA cotransfections in COS cells using multimers of WT or mutant -1097^{Rankl} (p6x1097-*luc*) or pCREB-luc and indicated expression vectors. **e**, Real-time PCR. *Rankl* induction by ISO but not by PTH is blunted in *Atf4* $^{-/-}$ osteoblasts. **f**, **g**, Decreased *Rankl* expression and Oc.S/TAr in absence of *Atf4* $^{-/-}$. **h**, ISO increased ATF4 transactivation activity, H89 inhibited it, in ROS 17/2.8 cells. **i**, Phospho-^{254}ATF4 western blot. H89 inhibits ATF4 phosphorylation following ISO treatment. **j**, *In vitro* kinase assay. ATF4 is phosphorylated by PKA on serine 254 (asterisk). CREB served as positive control. **k**, DNA cotransfection assays in COS cells. PKA increases ATF4 transactivation activity through serine 254. **l**, **m**, Real-time PCR. ISO induces *Rankl* expression in *Rsk2* $^{-/-}$ osteoblasts. *Adrb2, Pthr, Atf4* and *Osteocalcin* (*Ocn*) expression during osteoblast differentiation. Bottom line of **d** and **k** shows western blots of transfected proteins. *Statistically significant.

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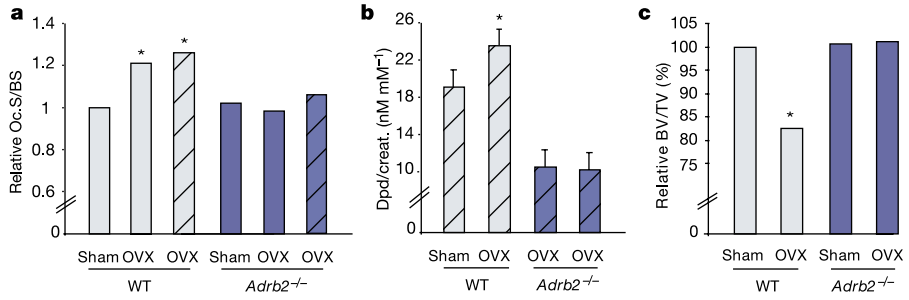
infusion in 1-month-old *Cart*$^{-/-}$ mice, a procedure increasing sympathetic signalling[13]. This infusion decreased bone mass and osteoclast surface more efficiently in *Cart*$^{-/-}$ mice than in WT littermates (Fig. 4f), demonstrating that leptin-mediated sympathetic regulation of bone mass is not impaired in the absence of CART. The more severe decrease of bone mass observed in *Cart*$^{-/-}$ mice following leptin ICV further established that CART is an inhibitor of BR. *Cart* is not expressed in bone cells (Fig. 4g), WT and *Cart*$^{-/-}$ BMMs differentiated equally well into osteoclasts, exogenous CART did not affect BMM differentiation into osteoclasts, and co-culture experiments failed to detect any *Cart*$^{-/-}$ cell-autonomous defect (Supplementary Fig. 9)—yet *Rankl* expression was upregulated in *Cart*$^{-/-}$ bones, suggesting that CART exerts its function by ultimately modulating *Rankl* signalling (Fig. 4h).

Mc4r$^{-/-}$ mice displayed an increase in hypothalamic *Cart* expression and, at 6 months of age, a HBM (Fig. 4i, j). While bone formation parameters were normal in *Mc4r*$^{-/-}$ mice, excluding the possibility that their HBM was secondary to a dysfunction of the leptin-dependent sympathetic regulatory loop, they displayed a marked reduction in osteoclast number. This feature provided an explanation for their HBM and was consistent with the increase in *Cart* expression. This finding suggested that the increased bone



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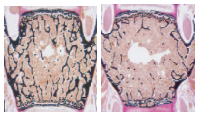
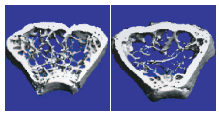


	WT	*Cart*$^{-/-}$
BV/TV (%)	12.3±0.7	9.3±0.9*
BFR	213.7±26.5	204.2±22.1
Ob.Nb/BPm	19.2±0.6	19.5±0.7
Oc.S/BS	9.9±0.5	19.8±1.0*
Dpd/creat.	9.1±0.8	12.4±1.6*

e



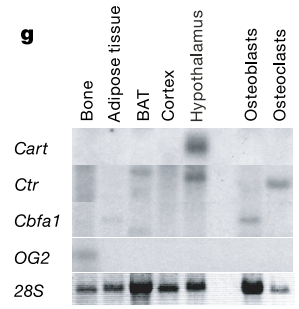
WT	*Cart*$^{-/-}$
10.1 ± 1.2	4.4 ± 1.8*

BV/TV (%)

f

	WT PBS	WT Leptin	*Cart*$^{-/-}$ PBS	*Cart*$^{-/-}$ Leptin
Fat pad (mg)	248.2±55.8	0* (−100%)	349.8±84.6	119.6±29.6* (−65.8%)
BV/TV (%)	14.3±1.1	11.9±0.7* (−16.8%)	13.1±0.7	9.9*±0.6* (−24.4%)
BFR	159.4±26.5	99.8±14.2* (−37.4%)	134.1±12.5	91.5±14.7* (−31.7%)
Ob.Nb/BPm	ND	ND	25.0±1.7	18.8±1.7* (−24.8%)
Oc.S/BS	17.6±1.5	19.4±1.2 (+10.1%)	16.9±0.8	19.2±0.8* (+13.8%)

g



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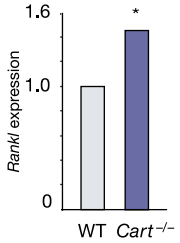

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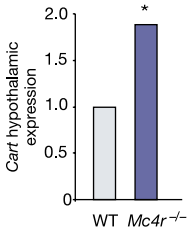

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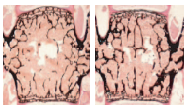

	WT	*Mc4r*$^{-/-}$
BV/TV (%)	14.1±0.5	18.6±1.2*
BFR	62.5±11.8	86.0±18.3
Ob.Nb/BPm	19.2±0.6	19.5±0.7
Oc.S/BS	13.1±0.3	10.8±1.1*

k

Genotype	WT	*Mc4r*$^{-/-}$ *1*	*Mc4r*$^{-/-}$ *2*
Age (years)	<8	3	8
Sex	M	M	M
BMI (kg m^{-2})	16.0	27.0	46.2
Dpd (nM)	6.13±0.20	5.86±0.5	4.43±0.4
CTX (μg l^{-1})	7.0	2.254±0.12	2.367±0.01

Figure 4 SNS and CART antagonistic functions. **a**-**c**, Relative Oc.S/BS, urinary Dpd and BV/TV in WT and *Adrb2*$^{-/-}$ mice ovariectomized (OVX) for 4 or 12 weeks (plain or hatched bars). *$P < 0.05$ versus WT sham littermates. **d**, BV/TV, formation and resorption parameters in 6-month-old *Cart*$^{-/-}$ mice. **e**, μCT analysis of 6 month-old femurs. **f**, Leptin ICV infusion in WT and *Cart*$^{-/-}$ mice. *Cart* deletion enhances leptin antiosteogenic and proresorptive functions. **g**, Northern blots. *Cart* is not expressed in bone cells. **h**,**i**, Real-time PCR. Increased *Rankl* and *Cart* expression in *Cart*$^{-/-}$ and *Mc4r*$^{-/-}$ bones and hypothalami. **j**, Increased BV/TV, normal bone formation, decreased bone resorption in 6-month-old *Mc4r*$^{-/-}$ mice. **k**, Serum Dpd and crosslaps CTX are decreased in MC4R-deficient patients. Error bars, mean + s.e.m. BMI, body mass index.

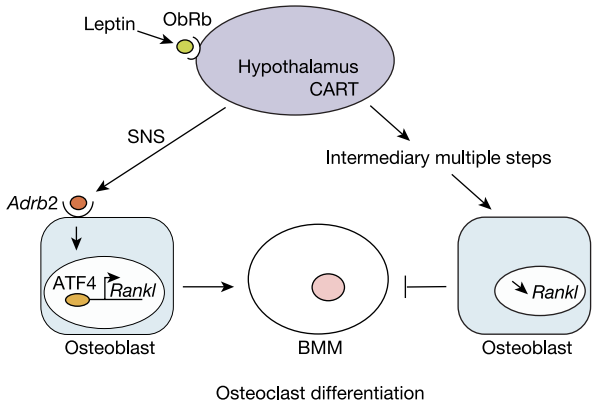

Figure 5 Model of the neuronal control of bone resorption.

density observed in *MC4R*-deficient patients[14] may be caused, at least in part, by a decrease in bone resorption. The decrease in serum levels of two BR markers, Dpd and CTX, in patients lacking MC4R verified this hypothesis (Fig. 4k).

Thus leptin controls BR through, at least, two distinct and antagonistic pathways. On one hand, sympathetic signalling via Adrb2 promotes osteoclast differentiation; on the other hand, CART inhibits it. Although both pathways regulate *Rankl* expression, the molecular bases of CART regulation of BR remain elusive in absence of a specific CART receptor (Fig. 5). That sympathetic regulation of bone mass occurs in the absence of CART suggests that CART uses other means to regulate bone resorption. This hypothesis is further supported by two suggestive lines of evidence: first, CART and sympathetic signalling have opposite effects on BR; second, CART (unlike sympathetic signalling) has no detectable effect on bone formation. From a biomedical perspective, the increase in bone formation and the decrease in BR characterizing *Adrb2*$^{-/-}$ mice add significant credence to the contention that an efficient, bone-specific, pharmacological blockade of Adrb2 signalling would be a great asset in the management of gonadal failure-induced bone loss. □

Methods

Animals, surgical procedures and histology

Mutant mice used in these studies have been described[15,16]. ICV infusions and propranolol treatment were performed as described[1] in 2- (*Adrb2*$^{-/-}$) or 1- (*Cart*$^{-/-}$) month-old mice. For bone marrow transplantations, 2-month-old mice lethally irradiated with 1,100 rad in a double dose were injected with 2×10^6 nucleated whole bone marrow cells and killed 4 months later ($n = 5$–8 per group). Bone marrow cells were then flushed from long bones, and differentiated *in vitro* in the presence of ascorbic acid (50 μg ml^{-1}) for 10 days. DNA extracted from osteoblasts was used for PCR genotyping. Immunocytochemistry studies were performed on decalcified sections using commercially available Neomycin and Adrb2 antibodies. Bone histology and histomorphometry analyses were performed as previously described[1,3]. Twelve-μm-resolution micro-computed tomography (μCT) measurements were performed on distal femurs. Five to ten mice were analysed for each group.

Cell and molecular studies

Osteoblasts were used[3] at day 0 for ISO induction. Osteoclasts were differentiated with RANKL (50 ng ml^{-1}) and M-CSF (30 ng ml^{-1}). Co-cultures of osteoblasts/osteoclasts were performed as described[17]. Following treatment, multinucleated TRAP$^+$ cells were counted in triplicate wells. For pit resorption analysis, BMMs were cultured for 3 days with M-CSF and RANKL, trypsinized, plated on dentine slices, resorption pits stained with haematoxylin and the resorbed area quantified. Gene expression was assessed by real-time PCR on osteoblasts or tissue RNA. Nuclear extracts were prepared and EMSAs were performed as described[18]. Chromatin immunoprecipitation assays (ChIP) were performed using primary osteoblasts. Phospho-^{254}ATF4 antibody was generated against NLPSPGGSRGSPPK peptide in which the underlined serine was phosphorylated. Cells were treated with ISO (10 μM), PTH(1-34)(10 nM), forskolin (10 μM), PMA (phorbol 12-myristate 13-acetate, 0.2 μg ml^{-1}) or H89 (30 μM, 30 min pre-treatment). *In vitro* kinase assays were performed as described[2]. COS cells were transfected with 100 ng of reporter, 30–100 ng of expression plasmids and 15 ng of RSV-β-gal reporter vector. ROS 17/2.8 were transfected as described[18]. Transfections were repeated at least 4 times in triplicates.

Biochemistry

cAMP, Dpd crosslinks, creatinine urinary values, carboxy-terminal telopeptides of type-I collagen (CTX), leptin, insulin and PTH serum levels were measured using commercial kits.

Statistical analyses

Data are expressed as mean ± s.e.m. Statistical significance was assessed by Student's test. Values were considered statistically significant at $P < 0.05$.

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