ADDITIONS AND CORRECTIONS

VOLUME 280 (2005) PAGES 12799–12809

Interaction of the mammalian endosomal sorting complex required for transport (ESCRT) III protein hSnf7-1 with itself, membranes, and the AAA$^+$ ATPase SKD1.

Yuan Lin, Lisa A. Kimpler, Teresa V. Naismith, Joshua M. Lauer, and Phyllis I. Hanson

The plasmid used to express FLAG-hSnf7N (residues 1–116) in this paper had an unintended missense mutation changing serine at residue 2 to cysteine. We found that this cysteine was palmitoylated. Changing it back to serine decreased the amount of hSnf7N associated with membranes from all for the mutant fragment containing cysteine to approximately half for the wild-type fragment containing serine. The images of FLAG-hSnf7N in Figs. 7*B* and 8 represent average cells expressing mutant (Cys-2) hSnf7N, whereas for wild-type (Ser-2) hSnf7N, these images correspond to cells expressing high levels of protein. All other plasmids are as indicated, and the conclusions of the paper remain unchanged.

VOLUME 280 (2005) PAGES 28382–28387

Role of the N-terminal domain of the human DMC1 protein in octamer formation and DNA binding.

Takashi Kinebuchi, Wataru Kagawa, Hitoshi Kurumizaka, and Shigeyuki Yokoyama

PAGE 28385:

Due to an inadvertent error, the wrong image was presented in Fig. 4*C*. Fig. 4*C* should appear as shown below. The figure legend and text remain unchanged.
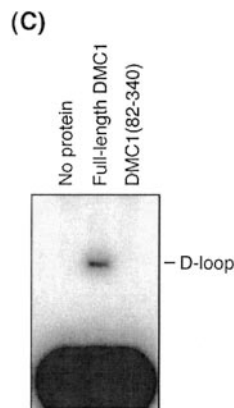

FIGURE 4*C*

VOLUME 281 (2006) PAGES 8888–8897

Dynamic changes in histone H3 lysine 9 methylations. IDENTIFICATION OF A MITOSIS-SPECIFIC FUNCTION FOR DYNAMIC METHYLATION IN CHROMOSOME CONGRESSION AND SEGREGATION.

Kirk J. McManus, Vincent L. Biron, Ryan Heit, D. Alan Underhill, and Michael J. Hendzel

The concentration of a drug that was employed, adenosine dialdehyde, was erroneously reported as 25 mM. The concentration that was employed was actually 250 μM.

VOLUME 281 (2006) PAGES 23436–23444

Conditional deletion of hypothalamic Y2 receptors reverts gonadectomy-induced bone loss in adult mice.

Susan J. Allison, Paul Baldock, Amanda Sainsbury, Ronaldo Enriquez, Nicola J. Lee, En-Ju Deborah Lin, Matthias Klugmann, Matthew During, John A. Eisman, Mei Li, Lydia C. Pan, Herbert Herzog, and Edith M. Gardiner

PAGE 23436:

Dr. Klugmann's name was misspelled in the author line. The correct spelling is shown above.

VOLUME 281 (2006) PAGES 31553–31561

The first structure from the SOUL/HBP family of heme-binding proteins, murine P22HBP.

Jorge S. Dias, Anjos L. Macedo, Gloria C. Ferreira, Francis C. Peterson, Brian F. Volkman, and Brian J. Goodfellow

PAGE 31554:

Column 2, first line: The concentrations should read "(4.0 μM) or hemin (3.5 μM). . ."

VOLUME 281 (2006) PAGES 11627–11636

PTP-PEST couples membrane protrusion and tail retraction via VAV2 and p190RhoGAP.

Sarita K. Sastry, Zenon Rajfur, Betty P. Liu, Jean-Francois Cote, Michel L. Tremblay, and Keith Burridge
PAGE 11632:

In Fig. 5, *panel D* was inadvertently omitted and is shown below. The figure legend is correct as it appears.

FIGURE 5

VOLUME 281 (2006) PAGES 24496–24505

Correction of pulmonary abnormalities in *Sftpd−/−* mice requires the collagenous domain of surfactant protein D.

Paul S. Kingma, Liqian Zhang, Machiko Ikegami, Kevan Hartshorn, Francis X. McCormack, and Jeffrey A. Whitsett
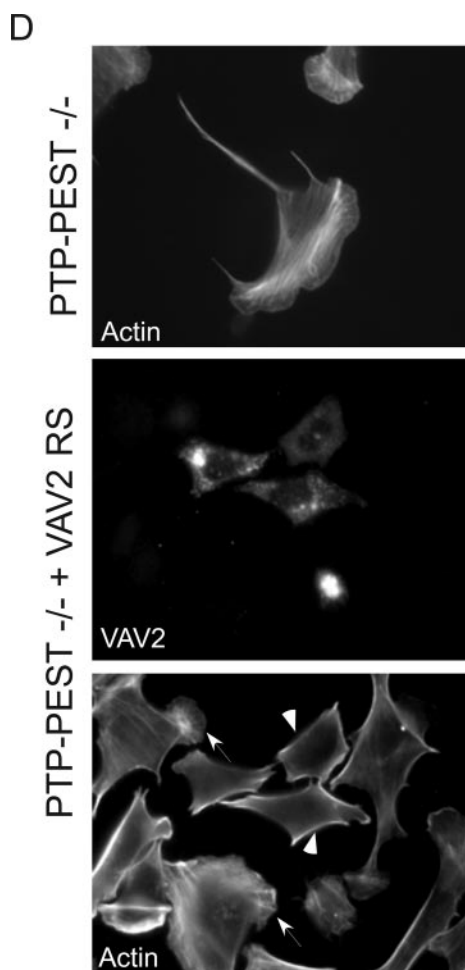**PAGE 24501:**

Fig. 5: An incorrect image was used for the *panel A inset*. The correct image is shown below.

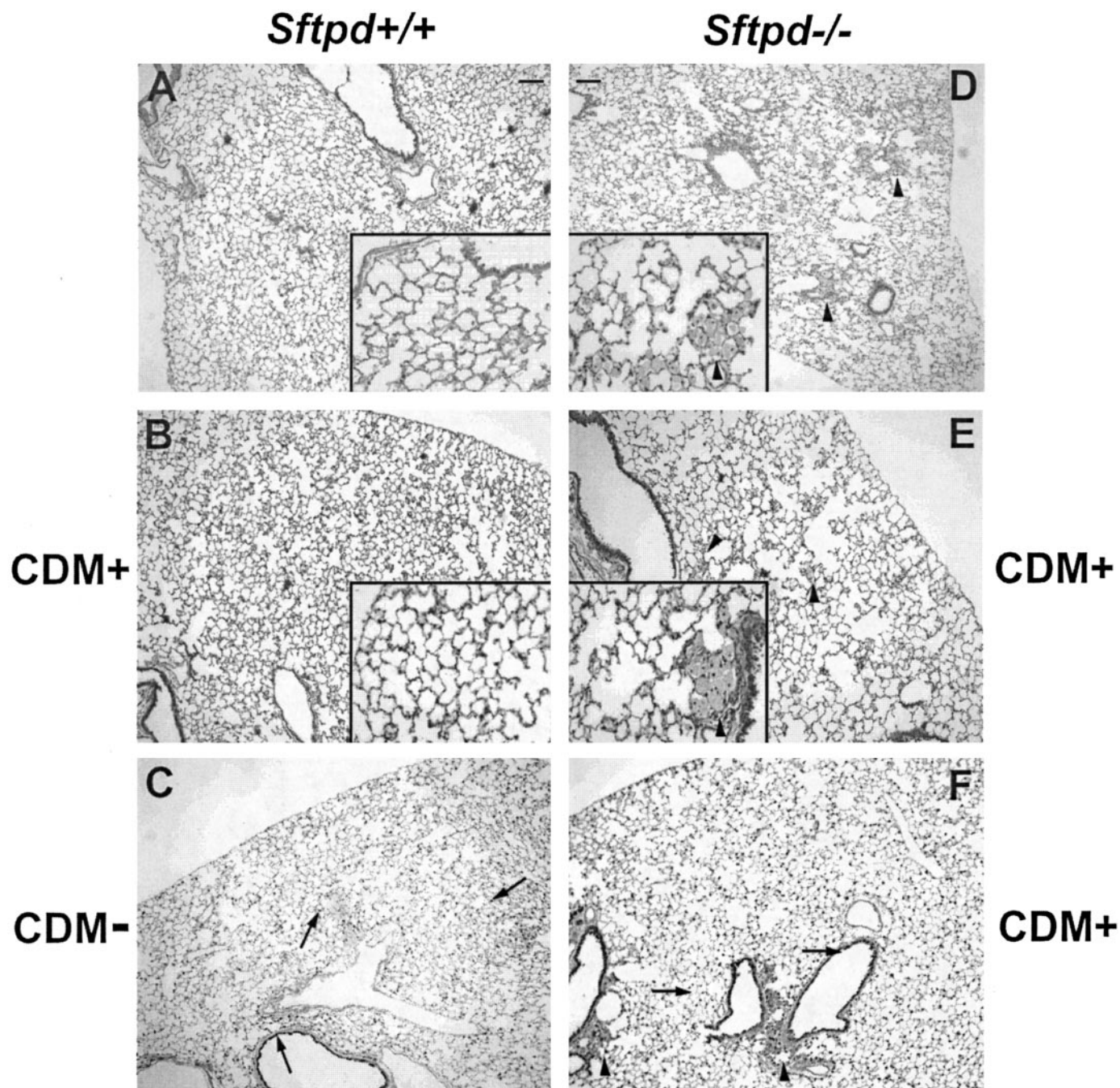FIGURE 5

Conditional Deletion of Hypothalamic Y2 Receptors Reverts Gonadectomy-induced Bone Loss in Adult Mice*

Susan J. Allison[‡], Paul Baldock[‡], Amanda Sainsbury[§], Ronaldo Enriquez[‡], Nicola J. Lee[§], En-Ju Deborah Lin[§], Matthias Klugman[¶], Matthew During[¶], John A. Eisman[‡], Mei Li[∥], Lydia C. Pan[∥], Herbert Herzog[§1,2], and Edith M. Gardiner**[1]

From the ‡Bone and Mineral Research Program and §Neuroscience Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, 384 Victoria Street, Darlinghurst, Sydney, New South Wales 2010, Australia, the ¶Department of Molecular Medicine and Pathology, University of Auckland, 85 Park Road, Grafton, Auckland, New Zealand, **University of Queensland School of Medicine, Princess Alexandra Hospital, Ipswich Road, Brisbane, Queensland 4120, Australia, and ∥Pfizer Global Research and Development, Inc., Groton, Connecticut 06340

Reduction in levels of sex hormones at menopause in women is associated with two common, major outcomes, the accumulation of white adipose tissue, and the progressive loss of bone because of excess osteoclastic bone resorption exceeding osteoblastic bone formation. Current antiresorptive therapies can reduce osteoclastic activity but have only limited capacity to stimulate osteoblastic bone formation and restore lost skeletal mass. Likewise, the availability of effective pharmacological weight loss treatments is currently limited. Here we demonstrate that conditional deletion of hypothalamic neuropeptide Y2 receptors can prevent ongoing bone loss in sex hormone-deficient adult male and female mice. This benefit is attributable solely to activation of an anabolic osteoblastic bone formation response that counterbalances persistent elevation of bone resorption, suggesting the Y2-mediated anabolic pathway to be independent of sex hormones. Furthermore, the increase in fat mass that typically occurs after ovariectomy is prevented by germ line deletion of Y2 receptors, whereas in male mice body weight and fat mass were consistently lower than wild-type regardless of sex hormone status. Therefore, this study indicates a role for Y2 receptors in the accumulation of adipose tissue in the hypogonadal state and demonstrates that hypothalamic Y2 receptors constitutively restrain osteoblastic activity even in the absence of sex hormones. The increase in bone formation after release of this tonic inhibition suggests a promising new avenue for osteoporosis treatment.

With age, changes in endocrine function can significantly affect physiological processes often with detrimental effects on health. One of the most substantial changes occurs with menopause in women, with cessation of estrogen production by the ovaries. This abrupt and dramatic decrease in estrogen production results in a number of acute and long term physiological effects, among the most notable of these being the loss of bone leading to the development of osteoporosis and increased deposition of white adipose tissue, contributing to an increased risk of heart disease and associated complications.

In the adult skeleton, bone mass and strength are determined by the multicellular process of bone remodeling, in which osteoclastic bone resorption is followed by osteoblastic bone formation. This turnover process is normally tightly coupled, such that the amount of bone resorbed equals the amount of bone formed, maintaining a constant bone mass. Multiple endocrine factors regulate turnover, in particular the sex hormones estrogen and testosterone, which protect bone by exerting anti-apoptotic effects on osteoblasts and pro-apoptotic effects on osteoclasts, in addition to suppressing osteoclast activity through both direct and indirect mechanisms (1, 2). Loss of sex hormones following menopause in women or in hypogonadal males results in increased osteoclast activity and loss of bone mass. Most current osteoporosis treatments inhibit bone resorption; however, although these treatments reduce further deterioration, they are unable to stimulate bone formation to replace bone that has already been lost, leaving osteoporotic patients with significantly weakened bones and at risk of further fragility fractures. Hence, there is a clear need to develop anabolic therapies, which can reverse bone loss by stimulating osteoblastic bone formation. Recent work demonstrated that disruption of a central regulatory circuit in skeletally mature mice by conditional deletion of hypothalamic neuropeptide Y2 receptors produced a rapid bone gain, associated with increased osteoblast activity and bone formation (3), identifying a novel central regulator of bone metabolism while providing persuasive evidence of the potential utility of this anabolic pathway for osteoporosis treatment.

The Y2 receptor also has an important role in the regulation of body weight, with Y2 receptor knock-out attenuating the obesity syndrome of hypogonadal, leptin-deficient *ob/ob* mice (4, 5). The increase in body fat mass following menopause contributes to an increased risk of cardiovascular and metabolic diseases (6, 7). This increase in adipose tissue is associated with a corresponding increase in levels of the Y2 receptor ligand

neuropeptide Y (NPY)[3] (8, 9). Thus Y2 receptors may play an important role in two of the major endocrine consequences of sex hormone deficiency, bone loss and adipose accumulation.

As there are no oral selective Y2 receptor antagonists available, the therapeutic potential of Y2 receptor ablation on body weight and bone cell function was investigated by using surgical gonadectomy to induce sex steroid-deficient bone loss in male and female mice in combination with germ line or hypothalamic deletion of Y2 receptors. Our results clearly demonstrate a protection against gonadectomy-induced changes in adipose mass and a significant protection against further bone loss induced by activation of the Y2-mediated central anabolic response.

EXPERIMENTAL PROCEDURES

Generation of Germ Line and Conditional Y2 Receptor Knockout Mice—Animal experiments were approved by the Garvan Institute of Medical Research Animal Research Authority and were conducted in accordance with relevant guidelines and regulations. Germ line and conditional knock-out mice were generated and genotyped as described previously (3, 10).

Gonadectomies—All mice were either sham-operated or gonadectomized at 8 weeks of age. To determine the effect of pre-existing Y2 receptor deletion on gonadectomy-induced bone loss, wild-type and germ line Y2 receptor knock-out ($Y2^{-/-}$) mice were collected 8 weeks after gonadectomy, at 16 weeks of age. To investigate the response to activation of the anabolic pathway following the development of bone loss, $Y2^{lox/lox}$ mice were injected with adeno-associated viral vectors (AAV-cre or AAV-empty) 8 weeks after gonadectomy, at 16 weeks of age. These mice were left for 6 weeks to allow the effect of Y2 deletion to develop and were collected at 22 weeks of age, a total of 14 weeks after gonadectomy.

Adeno-associated Virus Injection—16-Week-old $Y2^{lox/lox}$ mice were anesthetized with 100 mg/kg ketamine (Mavlab, Slacks Creek, Queensland, Australia) and 20 mg/kg xylazine (Ilium Veterinary Products, Smithfield, New South Wales, Australia) and injected with recombinant AAV vectors containing either the cre-recombinase gene or an empty cassette using a stereotaxic frame (Kopf Instruments, Tujunga, CA). Brain coordinates relative to bregma were posterior 2.3 mm, lateral ±0.4 mm, ventral 5.6 mm, corresponding to the arcuate nucleus. One microliter of virus (1×10^{14} genomic copies/ml) was injected bilaterally over 10 min using a 26-gauge guide cannula and a 33-gauge injector (Plastics One, Roanoke, VA) connected to a Hamilton syringe and a syringe infusion pump (World Precision Instruments, Waltham, MA). Mice were housed individually to monitor daily food intake and body weight.

Tissue Collection and Analysis—Mice were injected with 15 mg/kg of the fluorescent tetracycline compound calcein (Sigma) 10 days and 3 days prior to collection. At either 16 (germ line Y2 receptor knock-outs) or 22 weeks of age (conditional Y2 receptor knock-outs), mice were killed by cervical dislocation, and trunk blood was collected for serum hormone

analysis. White adipose tissue (WAT) depots (right inguinal, right retroperitoneal, and mesenteric) were collected and weighed. The weight of these tissues was summed and expressed as total white adipose tissue mass. Interscapular brown adipose tissue (BAT) was also excised and weighed. Both femora were excised and bisected transversely at the shaft midpoint. The distal halves of the right femora together with lumbar vertebrae were fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded, undecalcified in methyl methacrylate (Medim-Medizinische Diagnostik, Giessen, Germany) (3). 5-μm sagittal sections were used to analyze and calculate mineralized trabecular bone volume, number, and thickness, osteoclast surface, and mineral apposition rate as described previously (3), using either BioQuant software (R & M Biometrics Inc., Nashville, TN) or Leica QWin analysis software (Leica Microsystems Ltd., Heerberg, Switzerland). Radioimmunoassay kits were used to determine serum concentrations of IGF-1 (Biclone Australia PTY. Ltd., Marrickville, Australia) and corticosterone (MP Biomedicals, Orangeburg, NY). Osteocalcin was measured using a mouse osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA).

Statistical Analysis—All data were assessed by factorial analysis of variance, followed by Fisher's post hoc tests, using StatView version 4.5 (Abacus Concepts, San Francisco). For all statistical analyses, $p < 0.05$ was accepted as being statistically significant.

RESULTS

Germ Line Y2 Receptor Deletion and Gonadectomy-induced Changes in Body Weight, Adipose, and Biochemical Parameters—To investigate whether deletion of Y2 receptors affects gonadectomy-induced changes in adipose tissue deposition, germ line $Y2^{-/-}$ mice were assessed for changes in body weight and adiposity at 16 weeks of age, 8 weeks following gonadectomy.

Body weights of female sham-operated wild-type and $Y2^{-/-}$ mice were similar, with an increase in body weight in wild-type but not $Y2^{-/-}$ females following ovariectomy (Fig. 1*A*), associated with increased inguinal, retroperitoneal, and total WAT mass (Fig. 1, *B*, *D*, and *E*), which was not increased in $Y2^{-/-}$ mice. Total WAT mass was significantly lower in ovariectomized $Y2^{-/-}$ mice compared with wild type (Fig. 1*E*), suggesting that germ line Y2 deletion provides resistance to the ovariectomy-associated increase in adipose mass.

Male germ line $Y2^{-/-}$ mice were significantly lighter than wild-type mice (Fig. 1*H*), with lighter inguinal, mesenteric, retroperitoneal, and total WAT (Fig. 1, *I*–*L*) in $Y2^{-/-}$ males. BAT mass was also significantly lower in $Y2^{-/-}$ sham-operated males compared with wild-type (Fig. 1*M*), suggesting increased thermogenesis that would also likely contribute to reduced body weight. Orchidectomized mice were lighter compared with sham-operated genotype-matched controls (Fig. 1*H*), demonstrating that although lighter than wild-type mice, germ line deletion of Y2 receptors does not significantly alter the body composition changes that occur in response to orchidectomy.

Serum concentrations of IGF-1 and osteocalcin were unaffected by gonadectomy or germ line deletion of Y2 receptors in both sexes. Serum corticosterone was significantly reduced in

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[3] The abbreviations used are: NPY, neuropeptide Y; WAT, white adipose tissue; BAT, brown adipose tissue; MS, mineralizing surface; BFR, bone formation rate; MAR, mineral apposition rate; AAV, adeno-associated virus.
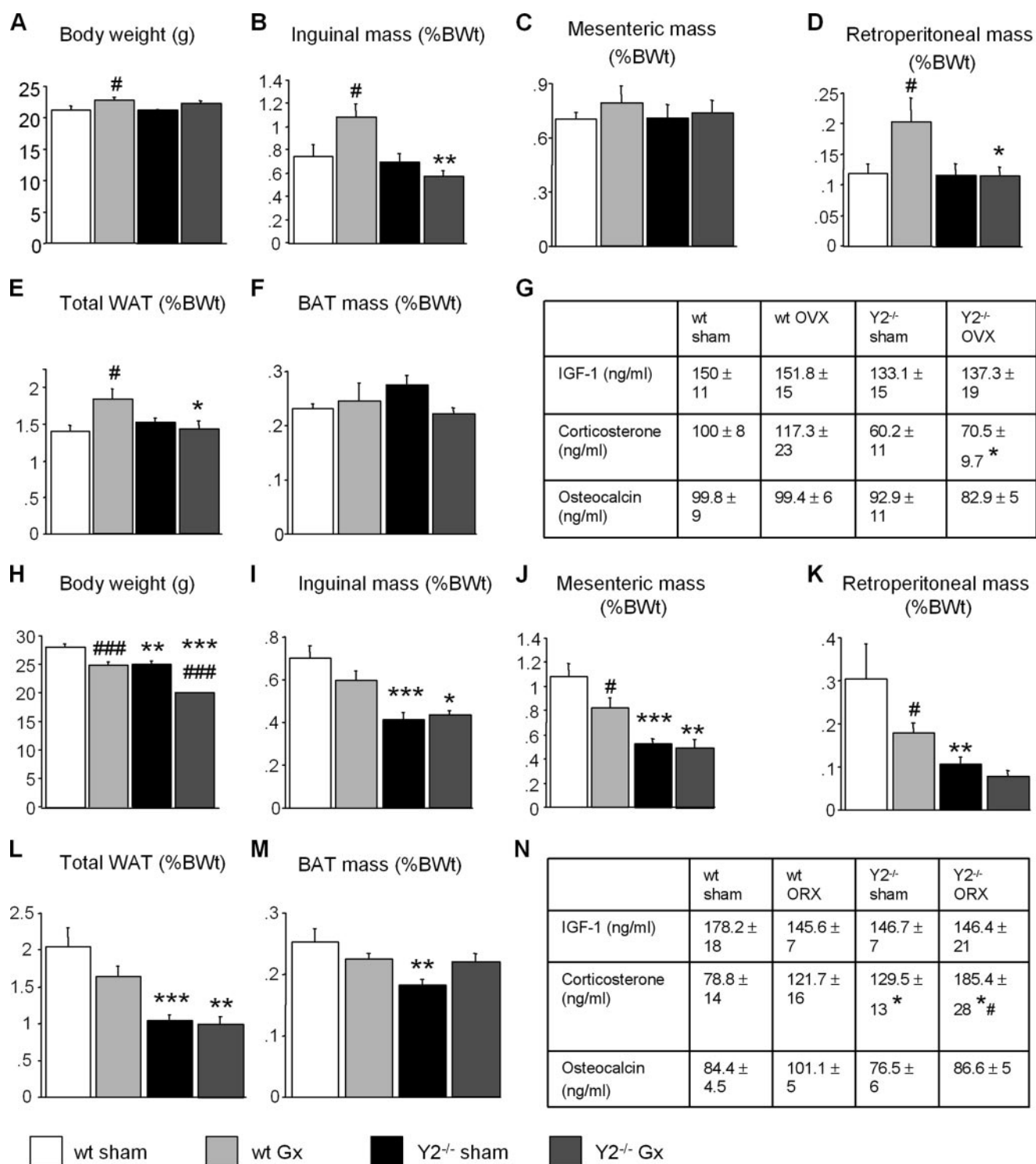
FIGURE 1. **Germ line Y2 knock-out reduces deposition of white adipose tissue.** *A–G,* female mice. Shown are the effects of ovariectomy (*OVX*) and germ line Y2 receptor deletion on body weight (*A*), inguinal (*B*), mesenteric (*C*), retroperitoneal (*D*), total white adipose tissue (*E*), brown adipose tissue (*F*), and serum levels of IGF-1, corticosterone, and osteocalcin (*G*). Deletion of Y2 receptors prevents increased body weight and white adipose tissue mass following ovariectomy. *H–N,* male mice. Shown are the effects of orchidectomy (*ORX*) and germ line Y2 receptor deletion on body weight (*H*), inguinal (*I*), mesenteric (*J*), retroperitoneal (*K*), total white adipose tissue (*L*), brown adipose tissue (*M*), and serum levels of IGF-1, corticosterone, and osteocalcin (*N*). Deletion of Y2 receptors reduces body weight and white adipose mass. *Asterisks* indicate statistically significant differences *versus* wild-type equivalent operation *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *Hatch marks* indicate significant differences *versus* sham within same genotype #, <0.05; ###, $p < 0.001$; *wt,* wild type; *BWt,* body weight.

ovariectomized $Y2^{-/-}$ compared with wild-type mice (Fig. 1*G*), but was significantly higher in male $Y2^{-/-}$ mice compared with wild type and in orchidectomized compared with sham-oper-ated germ line $Y2^{-/-}$ mice (Fig. 1*N*), suggesting a gender-specific change in the levels and gonadectomy-induced response of this hormone.
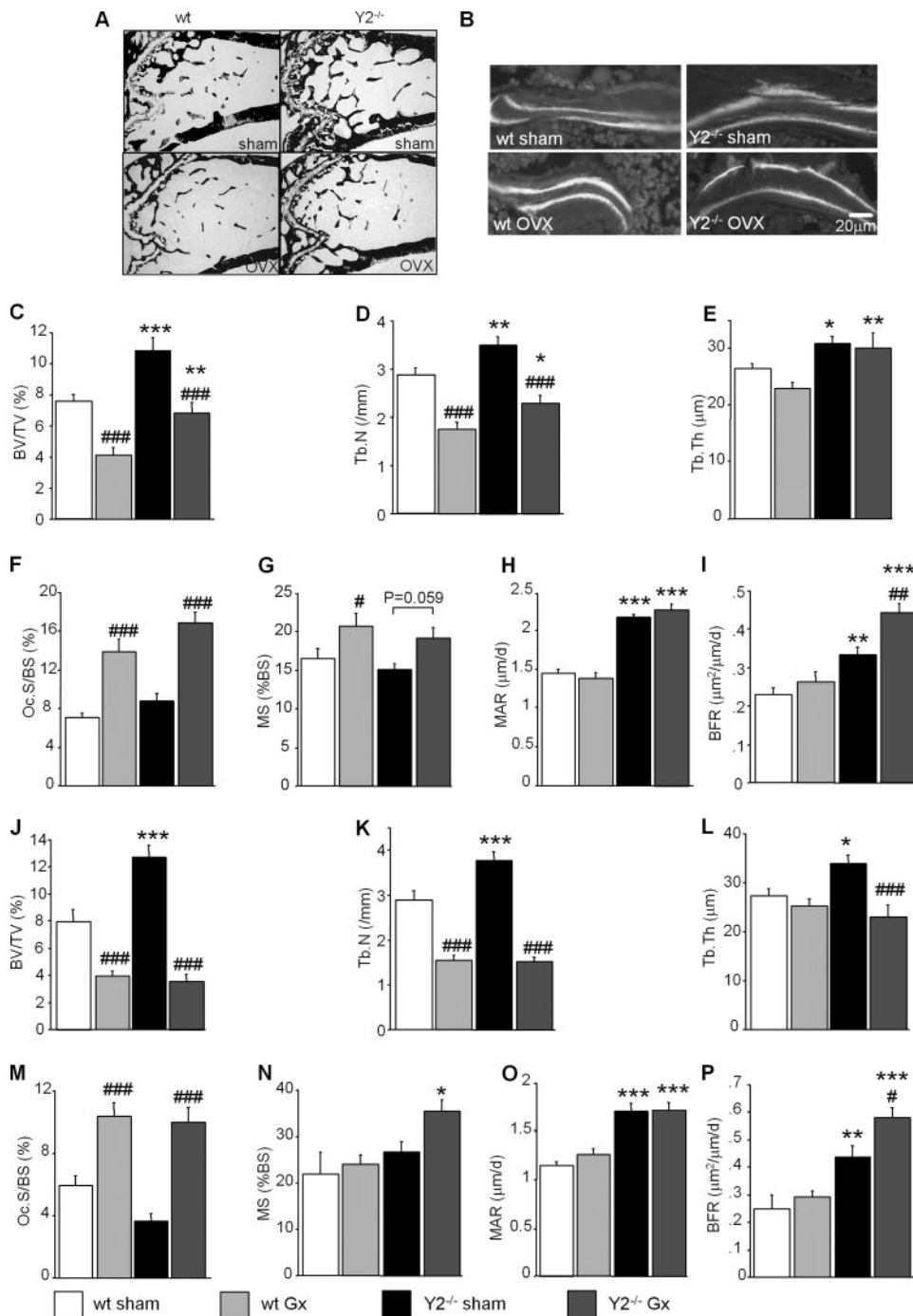
FIGURE 2. **Anabolic activity of germ line Y2 knock-out mice remains active following gonadectomy.** *A–I,* female mice. Shown are the histological analyses of distal femur from ovariectomized (*OVX*) wild-type and germ line Y2$^{-/-}$ mice (*A*), and measurements of trabecular bone volume (*BV/TV*) (*C*), trabecular number (*Tb.N*) (*D*), trabecular thickness (*Tb.Th*) (*E*), osteoclast surface (*Oc.S/BS*) (*F*), mineralizing surface (*MS*) (*G*), mineral apposition rate (*H*), and bone formation rate (*I*). Greater distance between calcein labels (*B*) demonstrates anabolic pathway is maintained in ovariectomized germ line Y2$^{-/-}$ mice and provides protection against ovariectomy-induced bone loss. *J–P,* male mice. Shown are the measurements of trabecular bone volume of distal femur from orchidectomized (*ORX*) wild-type and germ line Y2$^{-/-}$ mice (*J*), trabecular number (*K*), trabecular thickness (*L*), osteoclast surface (*M*), mineralizing surface (*N*), mineral apposition rate (*O*), and bone formation rate (*P*); the anabolic pathway is maintained in orchidectomized germ line Y2$^{-/-}$ mice. *Asterisks* indicate statistically significant differences *versus* wild-type equivalent operation: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *Hatch marks* indicate significant differences *versus* sham within same genotype #, <0.05; ##, $p < 0.01$; ###, $p < 0.001$; *wt,* wild type.

Germ Line Y2 Receptor Deletion and Gonadectomy-induced Bone Loss in the Distal Femur—16-Week-old male and female sham-operated germ line Y2$^{-/-}$ mice had significantly greater

trabecular bone volume at the distal femur than the sham-operated wild type (Fig. 2, *A*, *C,* and *J*). Gonadectomy at 8 weeks of age significantly reduced trabecular bone volume of both wild-type and Y2$^{-/-}$ mice (Fig. 2, *A*, *C,* and *J*). In females, however, despite a similar percentage reduction in bone volume in both ovariectomized Y2$^{-/-}$ and wild-type mice, the remaining bone volume in ovariectomized Y2$^{-/-}$ mice was comparable with wild-type sham levels and remained significantly greater than bone volume of ovariectomized wild-type mice (Fig. 2*C*). Similarly, trabecular number and thickness (Fig. 2, *D* and *E*) both remained significantly greater in ovariectomized Y2$^{-/-}$ compared with wild-type mice. Hence germ line deletion of Y2 receptors provided some protection against ovariectomy-induced bone loss and deterioration of bone microarchitecture in the distal femur.

In contrast to observations in ovariectomized female mice, orchidectomy of wild-type and Y2$^{-/-}$ male mice abolished the difference in trabecular bone volume between the two genotypes (Fig. 2*J*) because of reduced trabecular number and thickness (Fig. 2, *K* and *L*).

In females, the loss of trabecular bone volume following ovariectomy was associated with increased osteoclast and mineralizing surface (MS), consistent with the characteristic response to estrogen deficiency (11–14). The increased osteoclast surface in wild-type and Y2$^{-/-}$ mice was comparable and not affected by Y2 receptor deletion (Fig. 2*F*). MS, a measure of the extent of mineralization, was elevated in ovariectomized wild-type and Y2$^{-/-}$ mice, reaching statistical significance only in wild-type mice (Fig. 2*G*). Similarly, the loss of trabecular bone volume following orchidectomy in male mice was associated with significantly increased osteoclast surface in both genotypes (Fig. 2*M*). MS was elevated in orchidectomized Y2$^{-/-}$ but not wild-type mice (Fig. 2*N*).

Importantly, however, the elevated rate of mineral apposition (MAR), which confers the greater trabecular bone volume in nonoperated Y2$^{-/-}$ mice (3), was maintained following
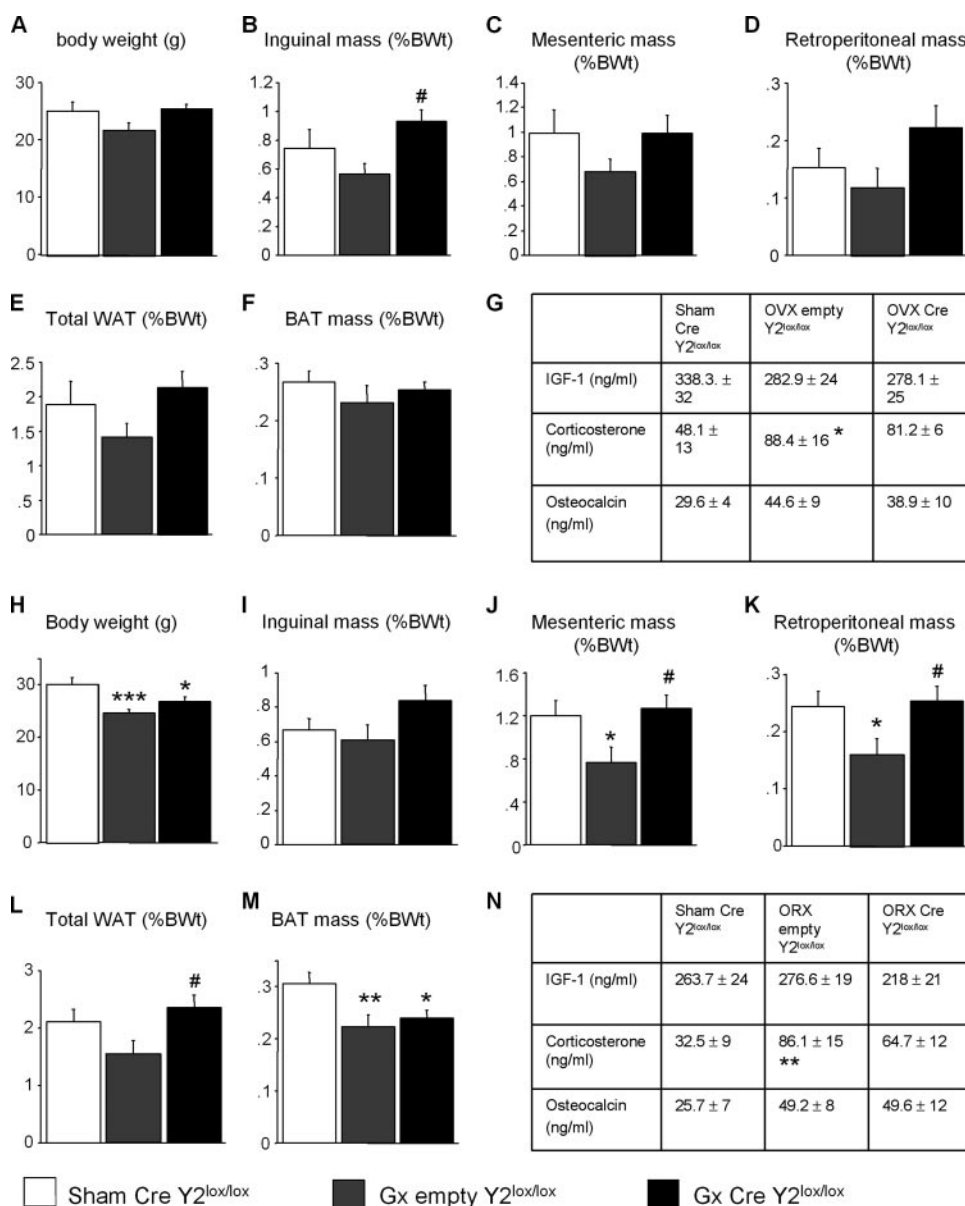
FIGURE 3. **Effect of hypothalamic Y2 receptor deletion on white adipose tissue deposition.** *A–G,* female mice. Shown are the effects of ovariectomy (*OVX*) and conditional deletion of hypothalamic Y2 receptors on body weight (*BWt*) (*A*), inguinal (*B*), mesenteric (*C*), retroperitoneal (*D*), total white adipose tissue (*E*), brown adipose tissue (*F*), and serum levels of IGF-1, corticosterone, and osteocalcin (*G*); deletion of hypothalamic Y2 receptors reduces white adipose tissue mass to sham levels. *H–N,* male mice. Shown are the effects of orchidectomy (*ORX*) and conditional deletion of hypothalamic Y2 receptors on body weight (*H*), inguinal (*I*), mesenteric (*J*), retroperitoneal (*K*), total white adipose tissue (*L*), brown adipose tissue (*M*), and serum levels of IGF-1, corticosterone, and osteocalcin (*N*); deletion of hypothalamic Y2 receptors does not affect body weight and increases white adipose mass compared with orchidectomized AAV-empty controls. *Asterisks* indicate statistically significant differences *versus* sham Cre Y2$^{lox/lox}$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *Hatch marks* indicate significant differences *versus* Gx Empty Y2$^{lox/lox}$; #, <0.05.

gonadectomy in both female and male mice (Fig. 2, *B*, *H*, and *O*). Furthermore, BFR, a measure of total osteoblast activity, was elevated in gonadectomized Y2$^{-/-}$ mice to a level even greater than sham-operated values (Fig. 2, *I* and *P*). Together, these results clearly demonstrate that the Y2-associated anabolic response persists in the absence of sex hormones in both males and females, and the preservation of bone micro-architecture post-ovariectomy in female Y2$^{-/-}$ mice was likely the result of Y2-dependent anabolic activity, demonstrating this anabolic pathway to be independent of the presence of sex hormones.

These results from germ line Y2$^{-/-}$ mice reveal that although the anabolic activity of the Y2 pathway remains elevated in the absence of sex hormones, this is not sufficient to provide resistance to gonadectomy-induced bone loss. In the clinic, however, an effective anabolic osteoporosis treatment must rebuild osteopenic bone. To assess whether activation of the Y2-mediated anabolic response can reverse bone loss that has already occurred, we utilized a conditional Y2 receptor knock-out model, using hypothalamic injection of recombinant adeno-associated viral vector containing the cre-recombinase gene (AAV-cre) to delete the Y2 receptor gene in adult Y2$^{lox/lox}$ mice. Hypothalamic Y2 receptors were deleted in 16-week-old Y2$^{lox/lox}$ mice, 8 weeks after gonadectomy, followed by a further 6 weeks to allow the consequences of Y2 gene deletion to develop. Control Y2$^{lox/lox}$ mice were injected with adeno-associated viral vector without the transgene (AAV-empty), therefore remaining genetically equivalent to wild-type mice. This model, in which the anabolic pathway is activated following the occurrence of bone loss, is more akin to the typical osteoporotic patient, in whom treatment is initiated after substantial bone has been lost.

Conditional Hypothalamic Y2 Receptor Deletion and Gonadectomy-induced Changes in Body Weight, Adipose, and Biochemical Parameters—Investigation of whether conditional activation of this central pathway would affect gonadectomy-induced changes in body weight revealed that hypothalamus-specific deletion of Y2 receptors had no significant effect on body weight or food intake in gonadectomized female or male mice (Fig. 3, *A* and *H*, and data not shown).

In females, fat pad weights from ovariectomized conditional Y2$^{-/-}$ mice were similar to sham-operated controls (Fig. 3, *B–F*). As WAT mass would be expected to increase following ovariectomy prior to the deletion of Y2 receptors, these findings suggest that similar to germ line deletion, hypothalamic deletion of Y2 receptors caused a decrease in WAT mass.

In males, BAT was similarly reduced in both orchidectomized models (Fig. 3*M*). However, in contrast to orchidecto-
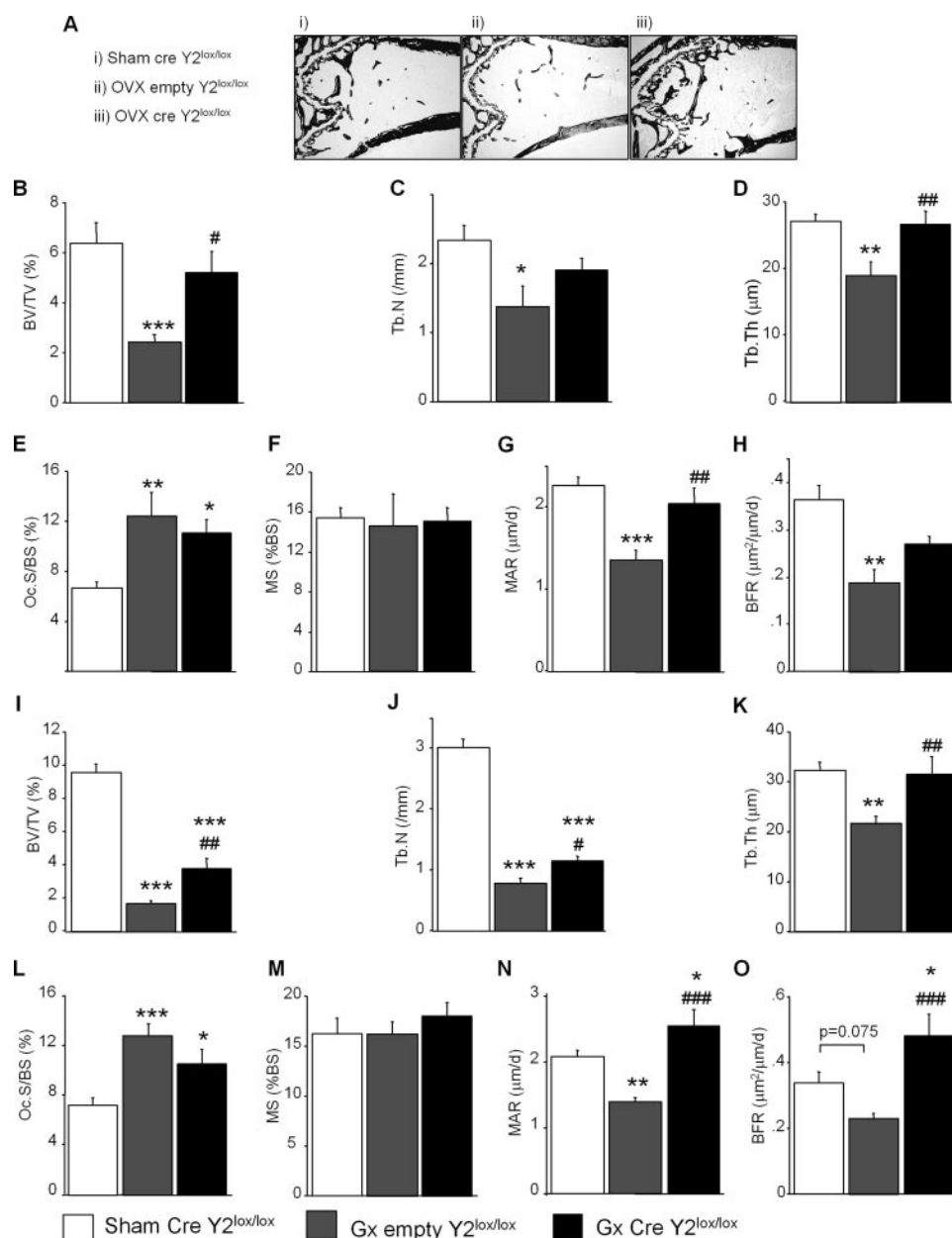
FIGURE 4. **Conditional deletion of hypothalamic Y2 receptors activates an anabolic response and prevents gonadectomy-induced bone loss in the distal femur.** *A–H,* female mice. Shown are the histological analyses of distal femur from ovariectomized (*OVX*) Y2^lox/lox mice receiving either AAV-empty or AAV-cre (*A*), and measurements of trabecular bone volume (*BV/TV*) (*B*), trabecular number (*Tb.N*) (*C*), trabecular thickness (*Tb.Th*) (*D*), osteoclast surface (*Oc.S/BS*) (*E*), mineralizing surface (*F*), mineral apposition rate (*G*), and bone formation rate (*H*); greater mineral apposition rate demonstrates the anabolic pathway is active and prevents further ovariectomy-induced bone loss despite elevated osteoclast surface. *I–O,* male mice. Shown are the measurements of trabecular bone volume of distal femur from orchidectomized Y2^lox/lox mice receiving either AAV-empty or AAV-cre (*I*), trabecular number (*J*), trabecular thickness (*K*), osteoclast surface (*L*), mineralizing surface (*M*), mineral apposition rate (*N*), and bone formation rate (*O*); greater mineral apposition rate and bone formation rate demonstrate the anabolic pathway is active and prevents further orchidectomy-induced bone loss despite elevated osteoclast surface. *Asterisks* indicate statistically significant differences *versus* sham Cre Y2^lox/lox: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *Hatch marks* indicate significant differences *versus* Gx Empty Y2^lox/lox: #, < 0.05; ##, $p < 0.01$; ###, $p < 0.001$.

mus, possibly peripheral, are important for sex hormone-regulated fat deposition in male mice.

Conditional deletion of Y2 receptors did not affect serum levels of IGF-1, corticosterone, or osteocalcin, although corticosterone was elevated in both gonadectomized groups compared with conditional $Y2^{-/-}$ sham-operated mice, with the difference reaching significance only in AAV-empty recipient mice (Fig. 3, *G* and *N*).

Conditional Hypothalamic Y2 Receptor Deletion and Gonadectomy-induced Bone Loss in the Distal Femur—Six weeks following conditional deletion of hypothalamic Y2 receptors in previously gonadectomized female and male Y2^lox/lox mice, trabecular bone volume at the distal femur was 2-fold greater compared with bone volume in gonadectomized Y2^lox/lox AAV-empty recipient mice (Fig. 4, *A, B,* and *I*). This difference in bone volume was due to a significantly greater trabecular number in male mice (Fig. 4*J*), with significantly greater trabecular thickness in both female and male mice (Fig. 4, *D* and *K*), demonstrating that conditional deletion of hypothalamic Y2 receptors can significantly improve outcome following gonadectomy-induced bone loss, with marked protection of bone mass and microarchitecture.

Osteoclast surface was significantly increased by gonadectomy in both groups (AAV-cre and AAV-empty) compared with sham-operated AAV-cre recipient mice (Fig. 4, *E* and *L*), indicating that as in the germ line Y2 knock-outs, conditional deletion of hypothalamic Y2 receptors did not inhibit gonadectomy-induced increases in bone resorption at this skeletal site. MS was unaffected by surgery or by conditional deletion of Y2 receptors (Fig. 4, *F* and *M*).

Importantly, and consistent with our findings in germ line $Y2^{-/-}$ mice, MAR was again significantly elevated in both sham-operated and gonadectomized mice lacking hypothalamic Y2 receptors, compared with AAV-empty controls (Fig. 4, *G* and *N*). A similar pattern was also observed for BFR (Fig. 4, *H* and *O*), and together they demonstrate that despite persistent high resorptive activity, condi-

mized germ line $Y2^{-/-}$ mice, which were significantly lighter and less fat than similarly operated wild-type mice, orchidectomized conditional $Y2^{-/-}$ mice had increased mesenteric, retroperitoneal, and total WAT mass compared with orchidectomized AAV-empty recipient mice (Fig. 3, *J–L*), suggesting that populations of Y2 receptors other than those in the hypothala-
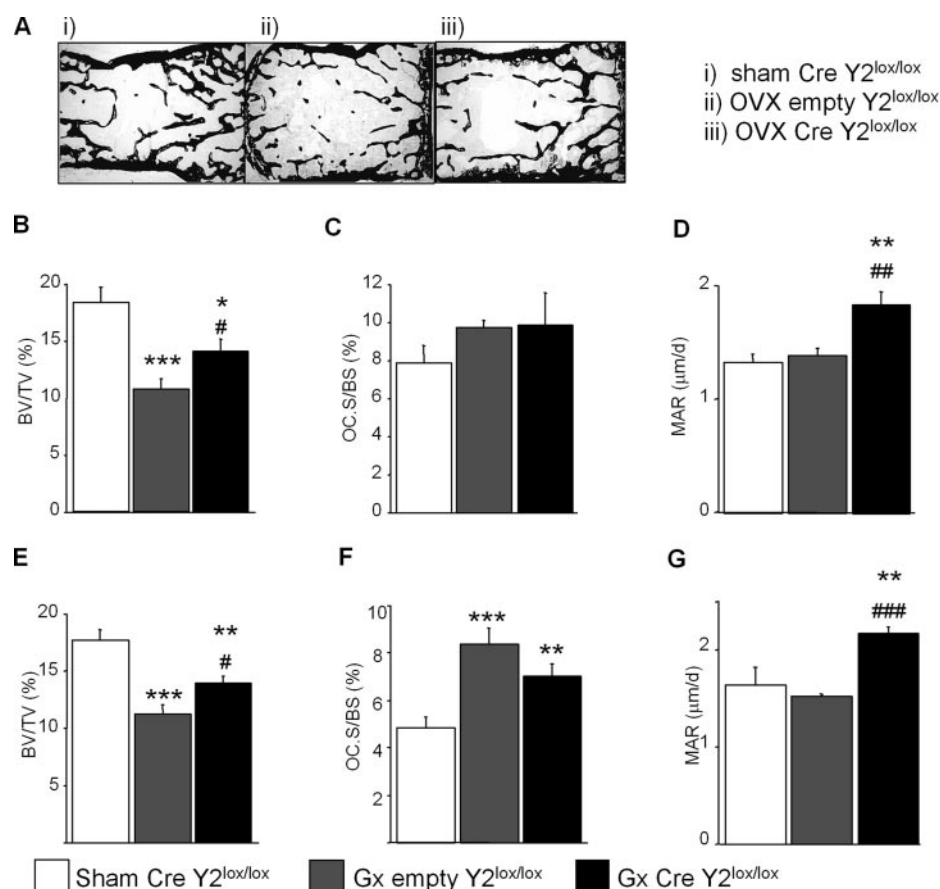
FIGURE 5. **Conditional deletion of hypothalamic Y2 receptors activates an anabolic response and prevents gonadectomy-induced bone loss in the vertebrae.** *A–D,* female mice. Shown are the histological analyses of lumbar vertebrae from ovariectomized (*OVX*) Y2^lox/lox mice receiving either AAV-empty or AAV-cre (*A*), and measurements of trabecular bone volume (*BV/TV*) (*B*), osteoclast surface (*Oc.S/BS*) (*C*), and mineral apposition rate (*D*); greater mineral apposition rate demonstrates anabolic pathway is active in the lumbar vertebrae and prevents further ovariectomy-induced bone loss. *E–G,* male mice. Shown are the measurements of trabecular bone volume of lumbar vertebrae from orchidectomized Y2^lox/lox mice receiving either AAV-empty or AAV-cre (*E*), osteoclast surface (*F*), and mineral apposition rate (*G*); greater mineral apposition rate demonstrates anabolic pathway is active in the lumbar vertebrae and prevents further orchidectomy-induced bone loss despite elevated osteoclast surface. *Asterisks* indicate statistically significant differences *versus* sham Cre Y2^lox/lox: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *Hatch marks* indicate significant differences *versus* Gx Empty Y2^lox/lox, #, < 0.05; ##, $p < 0.01$; ###, $p < 0.001$.

tional deletion of hypothalamic Y2 receptors can prevent further gonadectomy-induced bone loss in both female and male mice through activation of a central Y2-mediated bone anabolic pathway. Furthermore, this anabolic response was strong enough to produce a difference in bone mass compared with AAV-empty recipient mice within just 6 weeks, with substantial benefits to trabecular microarchitecture.

Conditional Hypothalamic Y2 Receptor Deletion and Gonadectomy-induced Bone Loss of the Vertebrae and Femur—Analysis of lumbar vertebrae revealed a pattern similar to the distal femur, with conditional deletion of hypothalamic Y2 receptors in gonadectomized Y2^lox/lox mice significantly elevating trabecular bone volume compared with AAV-empty controls (Fig. 5, *A*, *B*, and *E*). Osteoclast surface was significantly increased in gonadectomized compared with sham-operated male but not female mice regardless of knock-out status (Fig. 5, *C* and *F*). Importantly, as seen in the distal femur, the greater bone volume after hypothalamus-specific Y2 knock-out in gonadectomized mice could be attributed to a significantly greater MAR compared with gonadectomized AAV-empty recipient mice of

both genders (Fig. 5, *D* and *G*). This is the first demonstration that the bone anabolic response elicited by deletion of hypothalamic Y2 receptors is similar in different skeletal regions and indicates a generalized anabolic activity in bone.

Comparison with the effects of gonadectomy in wild-type mice establishes the consequences of deleting hypothalamic Y2 receptors in adult mice after the occurrence of significant gonadectomy-induced bone loss in the distal femur. In wild-type mice, trabecular bone volume was reduced by 50% in the distal femur 8 weeks after gonadectomy in either sex (Fig. 6, *A* and *D*). This reduction of bone volume continued to 14 weeks post-operation in AAV-empty recipient mice. Importantly, this latter phase of bone loss was blocked in mice in which hypothalamic Y2 receptors were deleted 6 weeks prior to collection, leaving these mice with twice the amount of bone volume compared with control AAV-empty recipient mice (Fig. 6, *A* and *D*). Osteoclast surface was elevated in all gonadectomy groups compared with sham-operated wild-type mice (Fig. 6, *B* and *E*). However, MAR was elevated only in mice deficient in hypothalamic Y2 receptors (Fig. 6, *C* and *F*), providing the first demonstration that the central Y2-associated anabolic response can overcome elevated bone resorption induced by sex hormone deficiency and effectively prevent further bone loss.

DISCUSSION

This study clearly demonstrates that hypothalamus-specific deletion of Y2 receptors prevents bone loss induced by deficiency of gonadal hormones in both male and female mice. Furthermore, our study demonstrates that germ line deletion of Y2 receptors prevents the elevation in WAT mass following ovariectomy, with differences between the regulation of WAT mass in orchidectomized male germ line and hypothalamic Y2$^{-/-}$ mice suggesting that Y2 receptors other than those located in the hypothalamus may be responsible for this effect. Together these findings are indicative of site specificity or alternative pathways regulated by Y2 receptors controlling bone formation and the deposition of WAT in the absence of sex hormones.

Sex hormones play an important role in regulating body fat, with levels of intra-abdominal fat increased up to 49% in post-menopausal compared with pre-menopausal women (15). Our
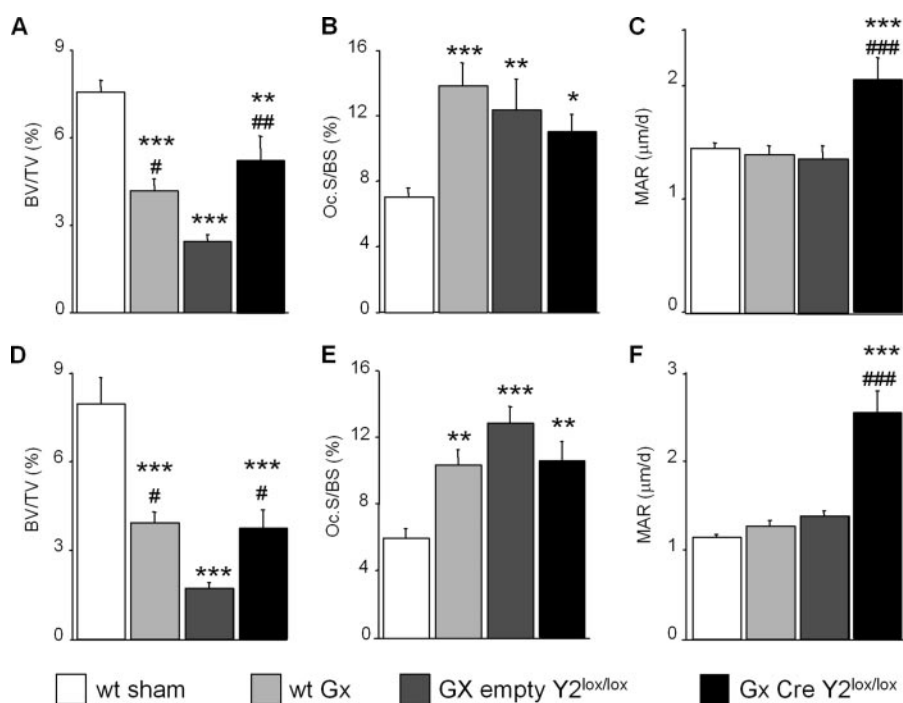
FIGURE 6. **Activation of central Y2 receptor-associated anabolic response halts further bone loss.** *A–C,* female mice; *D–F,* male mice. Shown are the comparisons of the effects of gonadectomy (*Gx*) after 8 and 14 weeks on bone loss in the distal femur of wild-type and conditional $Y2^{-/-}$ mice, respectively. Shown are the trabecular bone volumes (*BV/TV*) (*A* and *D*), osteoclast surfaces (*Oc.S/BS*) (*B* and *E*), and mineral apposition rates (*C* and *F*); conditional deletion of hypothalamic Y2 receptors 8 weeks after gonadectomy completely prevents further bone loss in both female and male mice, because of activation of a central anabolic response. *Asterisks* indicate statistically significant differences *versus* sham-operated wild type: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *Hatch marks* indicate significant differences *versus* gonadectomy Empty $Y2^{lox/lox}$: #, <0.05; ##, $p < 0.01$; ###, $p < 0.001$.

nerve fibers within bone tissue are under the control of synaptic transmission from the hypothalamus (17), providing a putative mechanism by which deletion of hypothalamic Y2 receptors can affect osteoblastic activity.

Notably, the marked improvement in bone volume seen in this study occurred within just 6 weeks of hypothalamic Y2 receptor deletion, a clear demonstration of the potency of this central anabolic pathway, providing crucial evidence that this pathway may be a suitable target to increase bone mass in osteoporotic patients, who are usually diagnosed only after significant bone loss has already occurred.

It is interesting to note that mice with a constitutively active bone formation response because of germ line Y2 knock-out were not more resistant to gonadectomy-induced bone loss, whereas activation of the Y2 anabolic response in adults prevented any further bone loss. There are a number of possible explanations for this finding. Osteoclast surface was reduced at the 22-week time point in ovariectomized conditional $Y2^{-/-}$ female mice compared with 16-week-old ovariectomized germ line $Y2^{-/-}$ mice. This finding is not unexpected as the gonadectomy-induced increase in resorption is an acute response, which rapidly diminishes (13, 14). MAR was also higher in orchidectomized conditional $Y2^{-/-}$ male mice compared with germ line knock-outs, suggesting that a greater osteoblastic response in the conditional knock-outs may be responsible for the protective effect on orchidectomy-induced bone loss. As the anabolic bone formation response was actually active in both male and female gonadectomized germ line $Y2^{-/-}$ mice, as evidenced by a greater MAR and BFR, it is possible that the only reason why a protective effect was observed in the conditional knock-outs and not in the germ lines was a longer time post-operation, allowing the anabolic response to overcome the acute increase in resorption.

Higher body weight is associated with higher bone mineral density in human studies (18). The data presented here, however, show that the increased bone volume of gonadectomized Y2 receptor knock-out mice was not because of increased body weight, as body weight and fat mass were actually reduced in germ line Y2 knock-out mice and unchanged in conditional knock-out mice. IGF-1 is known to affect bone growth and turnover (19), and Y2 receptors regulate serum IGF-1 concentrations under conditions of elevated NPY-ergic expression (4). However, serum IGF-1 concentrations were unaffected by Y2 receptor deletion. Elevated corticosterone decreases bone mass; however, serum corticosterone also cannot explain the

study also demonstrates a significant increase in WAT content in ovariectomized wild-type mice but not germ line $Y2^{-/-}$ mice. Studies in humans (16) and rodents (8, 9) have established a potential role for elevated NPY in the accumulation of WAT that occurs with menopause. Our findings are supportive of a role for the NPY system in the regulation of WAT content following the removal of gonadal hormones and strongly suggest a role for Y2 receptors in the control of this process.

These studies also demonstrate that whereas constitutive activation of the bone anabolic response in germ line $Y2^{-/-}$ mice does not entirely protect against sex hormone-deficient bone loss, selective activation of only the hypothalamic Y2-associated bone formation response following the occurrence of significant hypogonadal bone loss effectively prevents further bone loss. Of particular interest, this prevention of bone loss occurred despite elevated bone resorption and thus is specifically attributable to a Y2-associated anabolic response of osteoblastic cells. The end result was a doubling of trabecular bone volume in the distal femur and significantly increased bone mass in the lumbar vertebrae of gonadectomized mice lacking hypothalamic Y2 receptors compared with their counterparts with an intact Y2 receptor gene.

Interestingly, maximal activation of the bone formation pathway achieved equally by deletion of germ line and hypothalamic Y2 receptors, demonstrating this pathway to be centrally mediated. A recent study using pseudorabies virus-based transneuronal tracing to map trans-synaptically connected neurons from rat bone provided direct evidence that

bone differences observed in these mice. Ovariectomized wild-type mice had greater serum corticosterone compared with ovariectomized $Y2^{-/-}$ mice, which could possibly influence bone volume; however, corticosterone was unchanged in sham-operated $Y2^{-/-}$ compared with wild-type mice suggesting this is unlikely. Moreover, serum corticosterone was actually greater in germ line $Y2^{-/-}$ mice compared with wild-type, which would be expected to produce a decrease rather than the observed increase in bone volume. In conditional $Y2^{-/-}$ mice, serum corticosterone levels in both male and female mice were similar between both gonadectomy groups, again arguing against changes in corticosterone levels being responsible for the bone changes.

In osteoporosis, excess osteoclastic resorption results in deep resorption pits, causing trabecular perforation and loss of trabecular structures. A reduction in bone formation contributes to the deterioration of bone microarchitecture, as lost bone material is not adequately replaced (20–23). Anabolic treatments to balance out excess osteoclastic activity are therefore essential. In this study, conditional deletion of hypothalamic Y2 receptors in gonadectomized female and male adult mice resulted in significantly greater trabecular thickness compared with AAV-empty recipient mice and, most importantly, greater trabecular number in orchidectomized conditional knock-out mice compared with AAV-empty controls. A similar but non-significant trend was noted in female mice. These data suggest that the Y2 receptor-associated anabolic pathway not only improves total bone mass but also results in a beneficial microarchitectural outcome compared with gonadectomized wild-type mice.

These beneficial effects on bone volume and microarchitecture occurred in the face of elevated resorption. It is possible that even more bone mass could be replaced over a longer period of time after conditional deletion of hypothalamic Y2 receptors. It will also be important to investigate whether concurrent or sequential administration of an anti-resorptive treatment in combination with Y2 receptor deletion can allow a more effective anabolic response. Interestingly, recent studies of co-administration of the only available anabolic therapy, parathyroid hormone-(1–34) with anti-resorptive bisphosphonate, indicated that the anti-resorptive treatment may reduce the anabolic potential of parathyroid hormone (24, 25).

With the aging population, it is predicted that an increase in the incidence of osteoporotic fractures will be a major problem (26). The studies presented here demonstrate that inactivation of hypothalamic Y2 receptor signaling can prevent continued loss of bone by stimulating bone formation in gonadectomized adult mice. Furthermore, germ line deletion of Y2 receptors prevented the accumulation in WAT in ovariectomized female mice and reduced WAT mass in male mice. These results highlight the potential of this promising new avenue for the treatment of osteoporosis and obesity. Currently, there are no orally available Y2 receptor-specific antagonists. Our study emphasizes the need to develop such Y2 receptor-specific antagonists with the promising potential for treatment of excess fat deposition and bone degenerative conditions.

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