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Novel leptin receptor signaling mutants identify location and sex-dependent modulation of bone density, adiposity, and growth

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Abstract

Leptin, a hormone primarily produced by adipocytes, contributes to the regulation of bone health by modulating bone density, growth and adiposity. Upon leptin binding, multiple sites of the long form of the leptin receptor (LepRb) are phosphorylated to trigger activation of downstream signaling pathways. To address the role of LepRb-signaling pathways in bone health, we compared the effects of three LepRb mutations on bone density, adiposity, and growth in male and female mice. The Δ65 mutation, which lacks the known tyrosine phosphorylation sites, caused obesity and the most dramatic bone phenotype marked by excessive bone adiposity, osteoporosis, and decreased growth, consistent with the phenotype of db/db and ob/ob mice that fully lack leptin receptor signaling. Mutation of LepRb Tyr_{1138}, which results in an inability to recruit and phosphorylate signal transducer and activator of transcription 3, also caused obesity, but bone loss and adiposity were more dominant in male mice and no growth defect was observed. In contrast, mutation of LepRb Tyr_{985}, which blocks SHP2/SOCS3 recruitment to LepRb and contributes to leptin hypersensitivity, promoted increased femur bone density only in male mice, while marrow adiposity and bone growth were not affected. Additional analyses of vertebral trabecular bone volume indicate that only the Tyr_{1138} mutant mice exhibit bone loss in vertebrae. Together, our findings suggest that the phosphorylation status of specific sites of the LepRb contribute to the sex- and location-dependent bone responses to leptin. Unraveling the mechanisms by which leptin responses are sex- and location-dependent can contribute to the development of uniquely targeted osteoporosis therapies.

KEYWORDS

adipocytes, bone density, bone volume, female, leptin receptor, male, marrow adiposity, obesity, osteoporosis

Ian C. McCabe and Alyssa Fedorko contributed equally to this work.

1 | INTRODUCTION

Leptin is a 16 kDa hormone/adipokine produced primarily by adipocytes and is involved in the regulation of body mass and bone density.[1-7] Serum leptin levels are positively linked with body fat stores. In the brain, leptin signals through neurons expressing the long form of the leptin receptor (LepRb) to communicate the status of the body's energy reserves.[8,9] When adipose tissue is abundant, increased serum leptin levels lead to increased energy expenditure, decreased food intake, and weight loss. Conversely, the absence of leptin causes a reduction in energy expenditure, increased food intake and weight gain. Leptin also directly regulates peripheral cell function by binding to leptin receptors (LRs) expressed on a variety of cell types including mesenchymal stem/stromal cells (MSCs), which can mature into osteoblasts or adipocytes.[10-14]

The effects of leptin on bone are complex. Leptin can stimulate or inhibit bone formation depending upon bone location and whether leptin is acting directly via receptors on MSCs and/or osteoblasts[10,13,14] or indirectly through LepRb-expressing hypothalamic neurons that can polysynaptically regulate bone.[1,14,15] Mice that are completely leptin-deficient (ob/ob mice) have age-dependent, site-specific phenotypes including lower femoral bone mineral content, cortical thickness, bone mineral density (BMD), trabecular bone volume, and decreased bone length.[1,2,16-18] Similarly, leptin receptor deficiency (db/db mice) causes decreased tibial trabecular bone volume, bone length, and cortical thickness.[18,19] Absence of leptin or LepRb in mice also increases bone marrow adiposity.[1,17,20] The receptor-mediated mechanisms underlying these skeletal and marrow adiposity phenotypes remain unknown.

The LR belongs to the class I cytokine receptor superfamily and is encoded by a single gene (*Lepr*). Several alternatively spliced LR isoforms exist and can be divided into three classes: secreted, long, and short.[21] Leptin initiates its action through binding to the single long form of the leptin receptor (LepRb), which leads to the autophosphorylation and activation of the LepRb-associated Jak2 protein. Jak2, in turn, phosphorylates three critical LepRb tyrosine residues (Tyr_{985}, Tyr_{1077}, and Tyr_{1138}) located in an intracellular domain that is composed of ~300 residues. Each of the phosphorylation sites can recruit specific Src homology 2 (SH2) domain-containing proteins. Specifically, phosphorylation of the first site, Tyr_{985}, recruits Src homology phosphatase-2 (SHP-2) as well as a suppressor of cytokine signaling 3 (SOCS3), which leads to an attenuation of LepRb signaling.[21] Phosphorylation of the second site, Tyr_{1077}, recruits the signal transducer, latent transcription factor, and signal transducer and activator of transcription 5 (STAT5).[22] Phosphorylation of Tyr_{1138} results in the recruitment of STAT3.[23,24] Thus, each phosphorylation site activates a unique signaling cascade to mediate distinct aspects of leptin action.

Genetically modified mice containing mutations in the LepRb can be used to determine the role of each of the three key phosphorylation sites on mouse physiology. Mice were generated by replacing the LepRb-specific exon 18b of *Lepr* with a mutant exon 18b. This gene-targeting strategy expresses mutant LepRb molecules from the genomic context of endogenous LepRb so that expression patterns and levels of mutant LepRb mirror those of wild-type (WT) LepRb.[25] Mice expressing a mutation in Tyr_{985} (termed LL mice) have abrogated phosphorylation of the site and blocked SHP2/SOCS3 recruitment. LL mice are lean and display leptin hypersensitivity. Conversely, mice expressing a mutation in Tyr_{1138} (termed SS mice), are unable to recruit STAT3 to LepRb and display an obese and hyperphagic phenotype marked by decreased energy expenditure with increased growth.[25] Finally, Δ65 animals lack LepRb signaling and are similar to db/db mice.[26] To understand the role of LepRb and its key phosphorylation sites on bone phenotypes, we carried out an exploratory study examining male and female skeletal parameters (trabecular and cortical; femur and vertebral) in the three LepRb mutant mouse lines and compared our findings to the corresponding WT littermates for each mutant line. Our findings support the complexity of LepRb signaling in the regulation of skeletal health.

2 | MATERIALS AND METHODS

2.1 | LepRb mutant mouse models

This study examined LepRb Δ65, LL, and SS mice on the C57/Bl6 background, as previously described.[22,25,26] Heterozygous mice were intercrossed to generate mice homozygous for each LepRb variant and littermate controls used for subsequent studies, which were identified by genotyping between 2 and 4 weeks of age. Study mice were group housed until 8 to 9 weeks of age, then they were anesthetized with a lethal dose of pentobarbital and transcardially perfused with 10% neutral buffered formalin. Bones were removed and postfixed in formalin for 24-hours before storage in 70% ethanol. All mice were bred and housed at the University of Michigan and maintained in a 12-hour light/dark cycle with ad libitum access to food and water. All procedures were approved by the University of Michigan University Committee on the Use and Care of Animals in accordance with Association

for Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health guidelines.

2.2 | Bone density measurement

Femurs and vertebrae (lumbar 3-4) were fixed in 10% formalin and imaged using a GE Explore Locus micro-computed tomography (μCT) system at a voxel of 20 μm obtained from 720 views. The beam angle increment was 0.5 and beam strength was set at 80 peak kV and 450 μA. Each run consisted of control and mutant mouse bones and a calibration phantom to standardize gray scale values and maintain consistency. Bone measurements were blinded. Maximum vertebral height was determined using GE Healthcare Microview Software (GE Healthcare, London, ON, Canada) and was consistent with previous reports.[27] Maximum femur length was determined as the distance between the most proximal region of the trochanter to the most distal region of the medial condyle. Distal femur trabecular bone analyses were performed in the metaphyseal region defined at 1% of the total length (~0.17 mm) proximal to the growth plate extending 2 mm toward the diaphysis excluding the outer cortical bone. Trabecular bone volume fraction (BV/TV) was computed by GE Healthcare MicroView software application using a threshold of 700. Cortical bone measurements were determined with a 2-mm^3 region of interest in the mid-diaphysis.

2.3 | Leptin serum measurements

Leptin concentration in female mouse serum was determined by enzyme-linked immunosorbent assay using the Mouse/Rat Leptin Quantikine Kit (R&D Systems, Minneapolis, MN).

2.4 | Adipocyte counts

Fixed bones were processed on an automated Thermo Electron Excesior tissue processor for dehydration, clearing, and infiltration using a routine overnight processing schedule. Samples were then embedded in Surgipath-embedding paraffin on a Sakura Tissue Tek II-Embedding Center (Sakura Finetek USA, Inc, Torrance, CA). Paraffin blocks were sectioned at 5 μm on a Reichert Jung 2030 rotary microtome (Leica Biosystems, Buffalo Grove, IL) and were haemotoxylin and eosin stained. Femur sections were examined by microscopy at ×4 optical zoom and digital images obtained. Images were examined blind to the section's condition. The marrow area starting at 170 μm from the growth plate and extending 2000 μm toward the diaphysis was measured, by outlining the region and quantifying the area using ImagePro Software (Media Cybernetics, Rockville, MD). Adipocytes greater than 30 μm in diameter were counted and expressed relative to the total marrow area. Analyses were done blinded to conditions.

2.5 | Statistical analyses

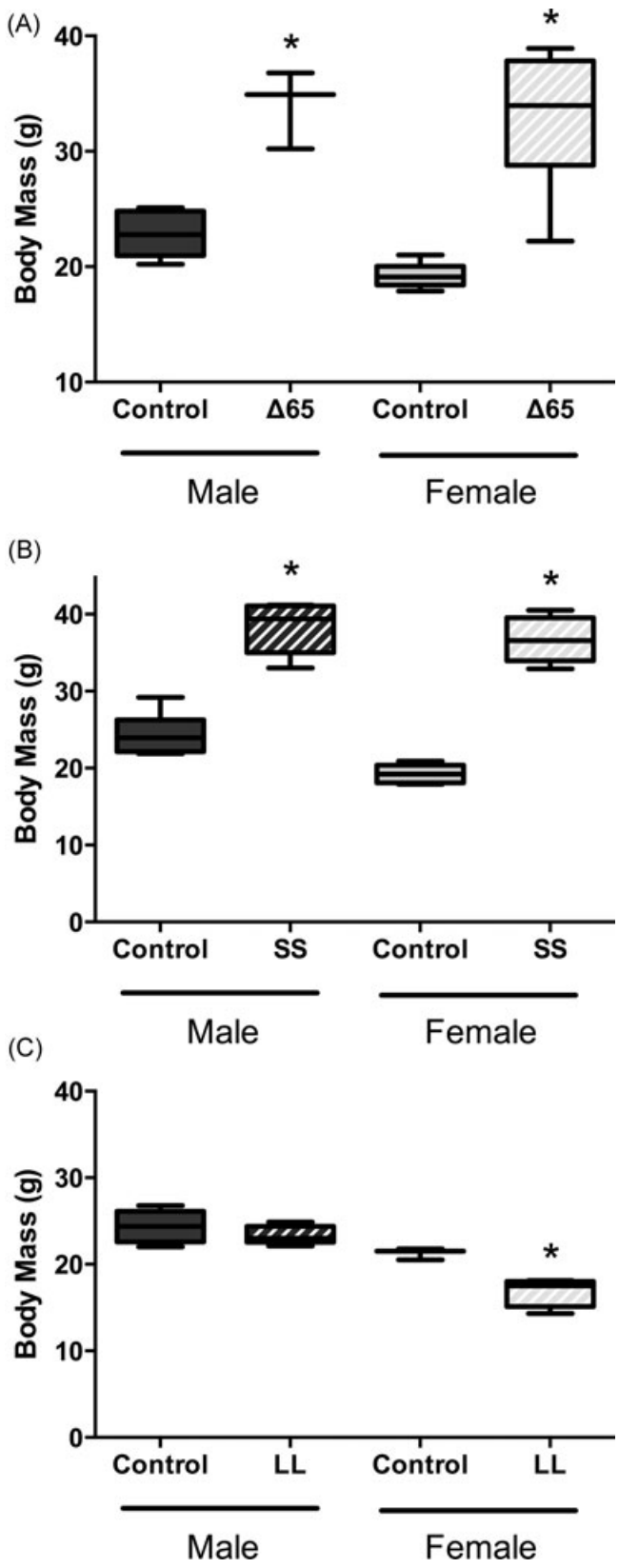
All measurements are presented as the mean ± SE. All groups contained at least five mice, except for the Δ65 male mouse group which has three mice. The Student two-tailed *t* tests were used to determine significance by comparing each genotype to its own littermate and sex-matched controls. Outliers were identified by ROUT and removed (only three identified). Statistical analyses used Student *t* tests that compared littermate WT to mutant mouse values for each sex. Analyses were performed using the GraphPad Prism Software version 6 (GraphPad, San Diego, CA). A $P \leq 0.05$ was considered significant.

3 | RESULTS

To determine the role of LepRb and its key phosphorylation sites on the male and female mouse skeleton, we examined 8- to 9-week-old mice that had one of the three LepRb mutations and compared findings to their corresponding sex- and age-matched littermates. We first assessed body mass (Figure 1), which is an important parameter that influences bone phenotype. Consistent with previous studies in db/db mice[9] which lack LepRb signaling, both male and female Δ65 mice had significantly greater body mass compared with their corresponding littermates, by 49% and 72% respectively. An even greater increase was seen in both the male and female SS mutant mice, which displayed significant increases in body mass that were 57% and 90% (respectively) greater than their corresponding littermate counterparts. In contrast, the LL mutant mice weighed less.[22] While the LL mutant males were on average only 4% less in weight, the female LL mutant mice exhibited a significant 27% decrease in body weight. This is consistent with the Tyr985 mutation promoting leanness specifically in female mice.[9,22] Consistent with changes in adiposity and body mass, serum leptin was significantly elevated in SS (148 ± 19 ng/mL) and decreased in LL (1.7 ± 0.45 ng/mL) compared with corresponding control female mice (2.8 ± 0.76 and 2.76 ± 0.48).

Next, we examined femur bone length to determine if the mutations had any impact on overall bone growth (Figure 2A-C). Both the male and female Δ65 mice displayed reduced femoral growth compared wtth littermate controls, by 9% and 12%, respectively. However, no differences were detected for the other genetically

modified mice, suggesting that signals beyond Tyr_{1138} induced Stat3 and Tyr_{985} recruited SHP2/SOCS3 mediate the control of bone length by leptin. We also examined vertebral bone height (L3-L4) but in contrast to another report[28] we did not observe differences in vertebral height between any of the conditions (data not shown).

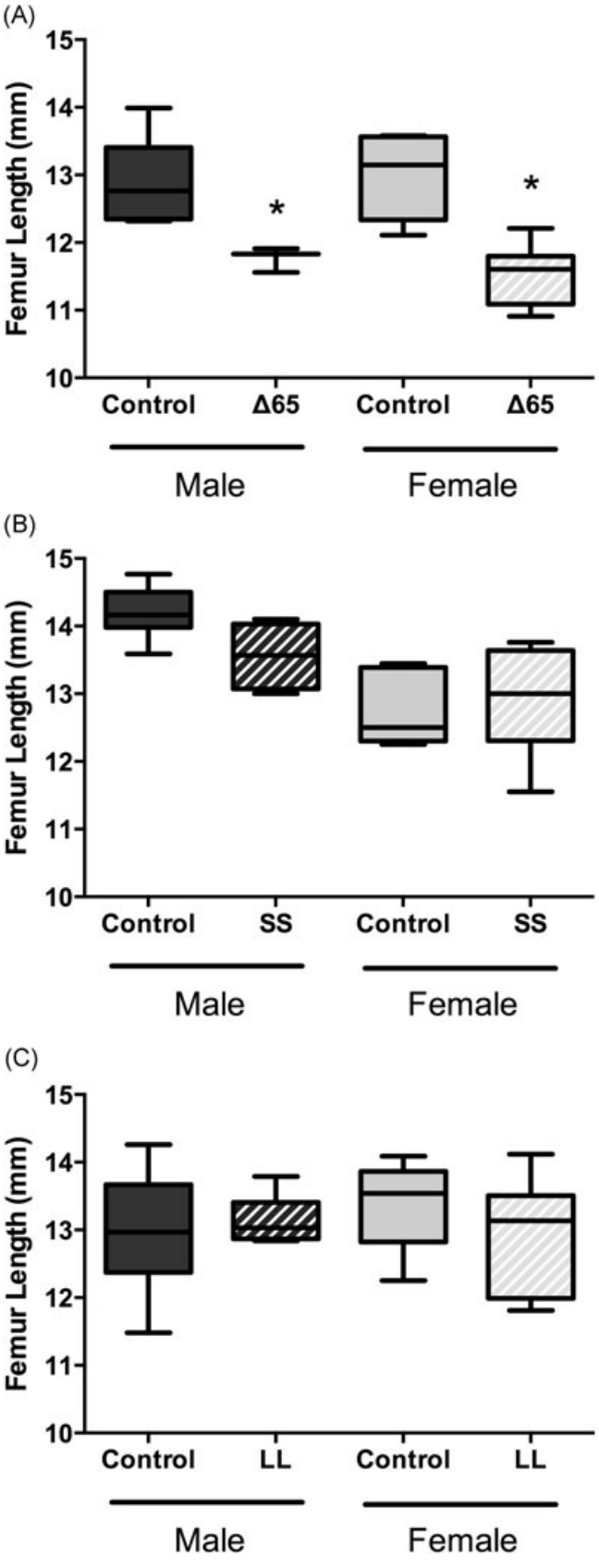


To determine if the LepRb mutations affected bone architecture, distal femur trabecular/cancellous bone was examined by microcomputed tomography. Analyses of BV/TV did not differ between conditions except for male LL mutant mice which displayed higher bone volume and greater trabecular thickness compared with controls (Table 1). Male and female Δ65 mice trended toward a 26% and 50% reduction in trabecular BV/TV. By contrast, SS mice, which were of similar body mass, had a trending, though nonsignificant 14% decrease and 19% increase in trabecular bone volume fraction. Bone volume was further analyzed relative to body weight since the mice displayed broad differences in body weight (as shown in Figure 1). When corrected for body weight, both male and female Δ65 mice had significantly reduced femur trabecular bone volume when compared with their corresponding control littermates, by 51% and 74% respectively, consistent with previous reports.[1,29-31] The SS mutant mice showed a significant 53% decrease for male BV/TV, and a nonsignificant 43% decrease for females ($P = 0.08$). In contrast, the LL mutant mice increased bone volume with average significant increase of 52% for males and a nonsignificant 20% increase for females, relative to littermate controls.

Examination of vertebral BV/TV, without correction to body weight, show an increase in BV/TV caused by the Δ65 mutation in male (significant) and female (trend) mice (Table 2). The Δ65 mutation also caused a significant reduction in vertebral trabecular spacing in both males and females. The SS mutation did not cause any notable effects on vertebral trabecular parameters while the LL mutation induced a significant decrease in trabecular BV/TV in females only. Given the significant differences in body weight between the mouse groups, we also analyzed the BV/TV data relative to body weight and found that there was no longer a difference in BV/TV in the Δ65 and LL mutant mice compared with their corresponding controls. However, the SS mutant mice exhibited a 22% decrease in males and a significant 42% decrease in females (Figure 3E), suggesting that the SS mutant could identify a unique signaling pathway that regulates vertebral bone density (Figure 4).

FIGURE 1 LepRb mutations Δ65 and SS increase male and female mouse body weight, while the LL mutation causes weight loss in female mice. Body weights were obtained from male and female 8- to 9-week-old mice expressing LepRb mutants: Δ65, SS (Tyr_{1138}), or LL (Tyr_{985}). Mutant mouse data are graphed with data obtained from corresponding littermate control mice. Values represent averages ± SE, $n = 3$ to 7 per group, $^*P < 0.05$

Cortical bone parameters were also examined and revealed differences that were sex- and mutation-specific (Table 3). Male LL and SS mutant mice did not show significant cortical differences, however, the Δ65 mutation displayed less cortical thickness and cortical area compared with WT control mice. Female LepRb mutant mice, on the other hand, displayed several significant differences. Female Δ65 mice had significantly reduced cortical bone density. Female SS mutant mice had greater inner and outer cortical bone perimeter and marrow area as well as lower BMD compared with WT controls. Regarding the LL mice, females had a smaller cortical inner perimeter than controls, but this did not result in greater mean thickness or cortical area (Table 2).



Previously, our lab and others[10-12,14,32] demonstrated that leptin can influence marrow adiposity. Therefore, we further examined the effect of the LR mutations on marrow adiposity. Adiposity was markedly increased in the male Δ65 mice by a significant 10.3-fold (to an average of 72 adipocytes/mm^2). While adiposity was also significantly increased in the SS male mutants, the increase was modest, 3.5-fold (to an average between 3 and 4 adipocytes/mm^2), compared with the effect of Δ65 on marrow fat. The female Δ65 mice exhibited an average increase of 2.7-fold (to 51 adipocytes/mm^2) and the SS female mice displayed a 1.5-fold average increase (to ~5 adipocytes/mm^2) compared with littermate controls, though these results did not reach statistical significance. The LL mutation did not impact marrow adiposity in either the male or female mice.

4 | DISCUSSION

Past studies demonstrate a role for leptin in the regulation of bone density and adiposity. Using mice expressing different LepRb-signaling mutations, we identified distinct roles for LR signaling pathways in the regulation of bone density, adiposity, or growth. By far the strongest phenotype we obtained was from the Δ65 mice, which lack LR signaling via characterized intracellular tyrosine residues. This mutation made both male and female mice obese while also decreasing bone density and growth and increasing marrow adiposity in mouse femurs. While the male mutant mouse number was underpowered ($n = 3$), our findings are consistent with reports on bone phenotypes caused by either leptin deficiency as seen in ob/ob mice or in db/db mice that lack LepRb.[1,29-31] The SS mutation of LepRb Tyr_{1138}, which prevents LepRb-

FIGURE 2 Only LepRb mutation Δ65 effected femur length/growth. Femur lengths were measured for male and female 8 to 9-week-old mice expressing LepRb containing mutations Δ65, SS, or LL. Mutant mouse data are graphed and analyzed relative to data obtained from corresponding littermate control mice. Values represent averages ± SE. Δ65 mice, $n \geq 3$ per group; SS and LL mice, $n = 6$ to 7 per group; $^*P < 0.05$

TABLE 1 Femoral trabecular bone parameters

	Male		Female	
	Control	**Mutant**	**Control**	**Mutant**
Δ65				
BV/TV	23.9 ± 3.6	17.7 ± 3.8	19.6 ± 4.1	9.8 ± 1.7
TbTh, mm	0.032 ± 0.003	0.029 ± 0.002	0.030 ± 0.002	0.026 ± 0.001
TbN, 1/mm	7.288 ± 0.847	5.957 ± 0.964	6.150 ± 0.873	3.651 ± 0.547
TbSp, mm	0.121 ± 0.029	0.164 ± 0.040	0.153 ± 0.031	0.315 ± 0.085
SS mutant				
BV/TV	52.9 ± 5.7	45.3 ± 9.5	24.3 ± 5.1	29.0 ± 1.8
TbTh, mm	0.064 ± 0.007	0.056 ± 0.011	0.029 ± 0.003	0.037 ± 0.001
TbN, 1/mm	8.308 ± 0.342	7.973 ± 0.839	5.805 ± 0.874	7.874 ± 0.199
TbSp, mm	0.058 ± 0.008	0.078 ± 0.022	0.165 ± 0.040	0.091 ± 0.005
LL mutant				
BV/TV	33.4 ± 0.7	48.2 ± 6.8*	32.6 ± 4.7	32.7 ± 4.3
TbTh, mm	0.038 ± 0.001	0.054 ± 0.009*	0.039 ± 0.003	0.039 ± 0.003
TbN, 1/mm	8.891 ± 0.509	8.862 ± 0.459	8.095 ± 0.576	8.157 ± 0.687
TbSp, mm	0.076 ± 0.004	0.059 ± 0.009	0.088 ± 0.0130	0.088 ± 0.014

Values represent the mean ± standard error ($n \geq 3$ per group).
*$P < 0.05$.

mediated phosphorylation of STAT3, made mice obese comparable to the Δ65 mice, however, the bone loss and marrow adiposity was not as great as observed in the Δ65 mice and no changes in growth were observed. In addition, the changes were more evident in the male SS mutant male mice compared with female mice. This suggests that the active signaling occurring in the SS is sufficient for growth and can maintain some normal bone phenotype in males and most of the normal phenotype in females. By contrast, the LL mutant mice did not gain weight and in the case of the female mice we observed reduced body weights. Male LL mutant mice were the only

TABLE 2 Vetebral trabecular bone parameters

	Male		Female	
	Control	**Mutant**	**Control**	**Mutant**
Δ65				
BV/TV	35.5 ± 4.0	57.9 ± 8.7*	40.5 ± 2.3	54.7 ± 5.5
TbTh, mm	0.039 ± 0.002	0.058 ± 0.016	0.044 ± 0.002	0.059 ± 0.008
TbN, 1/mm	8.923 ± 0.360	10.145 ± 1.272	9.069 ± 0.215	9.319 ± 0.312
TbSp, mm	0.073 ± 0.007	0.041 ± 0.004*	0.066 ± 0.004	0.049 ± 0.006*
SS mutant				
BV/TV	47.3 ± 5.7	54.5 ± 1.9	39.4 ± 3.5	43.4 ± 3.5
TbTh, mm	0.048 ± 0.004	0.055 ± 0.002	0.044 ± 0.003	0.044 ± 0.003
TbN, 1/mm	9.570 ± 0.414	9.944 ± 0.089	8,834 ± 0.290	9.706 ± 0.341
TbSp, mm	0.056 ± 0.008	0.046 ± 0.002	0.070 ± 0.006	0.059 ± 0.005
LL mutant				
BV/TV	39.6 ± 5.1	53.1 ± 4.6	44.8 ± 1.6	35.2 ± 2.3*
TbTh, mm	0.040 ± 0.003	0.051 ± 0.002*	0.048 ± 0.001	0.036 ± 0.002*
TbN, 1/mm	9.780 ± 0.433	10.287 ± 0.522	9.190 ± 0.257	9.658 ± 0.400
TbSp, mm	0.064 ± 0.007	0.048 ± 0.007	0.061 ± 0.003	0.069 ± 0.005

Bone volume fraction (bone volume/total volume, BV/TV), trabecular thickness (TbTh), trabecular number (TbN), trabecular spacing (TbSp). Values represent the mean ± standard error ($n \geq 3$ per group).
*$P < 0.05$.

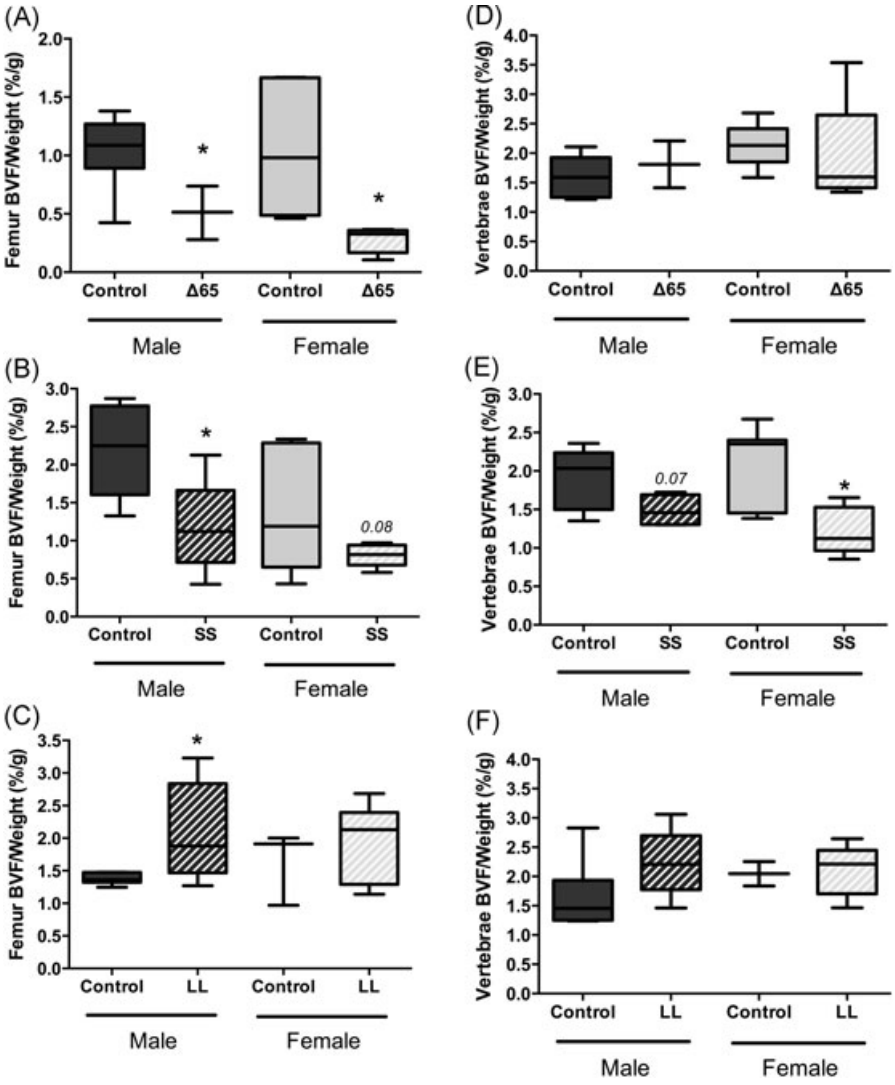


FIGURE 3 LepRb mutations have sex and location-dependent effects on bone volume fraction. Femur (A-C) and vertebral (D-E) bone volume fraction (BVF) was determined, in 8 to 9-week-old mice, by microcomputed tomography and expressed relative to mouse body weight. LepRb mutant mouse data (Δ65, SS, or LL) are grouped with data obtained from corresponding littermate control mice. Values represent averages ± SE. Δ65 mice, $n \geq 3$ per group; SS and LL mice, $n = 6$ to 7 per group; $^{*}P < 0.05$

group to show an increase in bone density, which is consistent with leptin hypersensitivity.

Interestingly, when corrected to body weight the Δ65 mutant mice did not experience vertebral bone loss. This is similar to previous reports showing significant bone loss in femur or tibia, with only mild changes in vertebrae, in db/db and ob/ob mice.[19,31] This is consistent with leptin having site-specific effects on bone.[27] Unexpectedly, we observed a moderate decrease in BVF/body weight in the SS mutant vertebrae. This suggests that a signaling pathway affected by the SS mutation, that is, STAT3, may contribute to leptin's location-dependent effects in femur but not vertebrae; thus, pathway inhibition allows a response in vertebrae. Future mechanistic studies are needed to better understand the underlying site-specific differences in the regulation of bone.

Analyses of cortical bone indicated that only the Δ65 mutation had an impact on male cortical bone parameters but not density. Whereas, the Δ65 and SS mutation affected female cortical bone density but only the SS mutant had significant negative effects on cortical bone parameters. The LL mutation did not have a major impact on cortical bone in either sex, but females did display a significant decrease in inner cortical perimeter. Thus, the LepRb mutations reveal a sex-dependent response. The Δ65 mutation affecting male cortical bone, while the SS (significant) and LL (trend) mutants affect cortical bone only in female mice and in opposite ways. This sex-dependent LepRb regulation of cortical parameters needs to be further dissected in the future larger study that identifies the downstream pathways that have opposing influences on cortical bone.

Leptin is a potent regulator of bone marrow adiposity. Increased LepRb signaling can decrease marrow adiposity,[10-12,14,33] whereas reduced leptin signaling promotes bone marrow adiposity.[1,17] The latter is most evident in ob/ob and db/db.[34,35] In our study, both the Δ65 mutant and SS mutants had increased femoral bone marrow

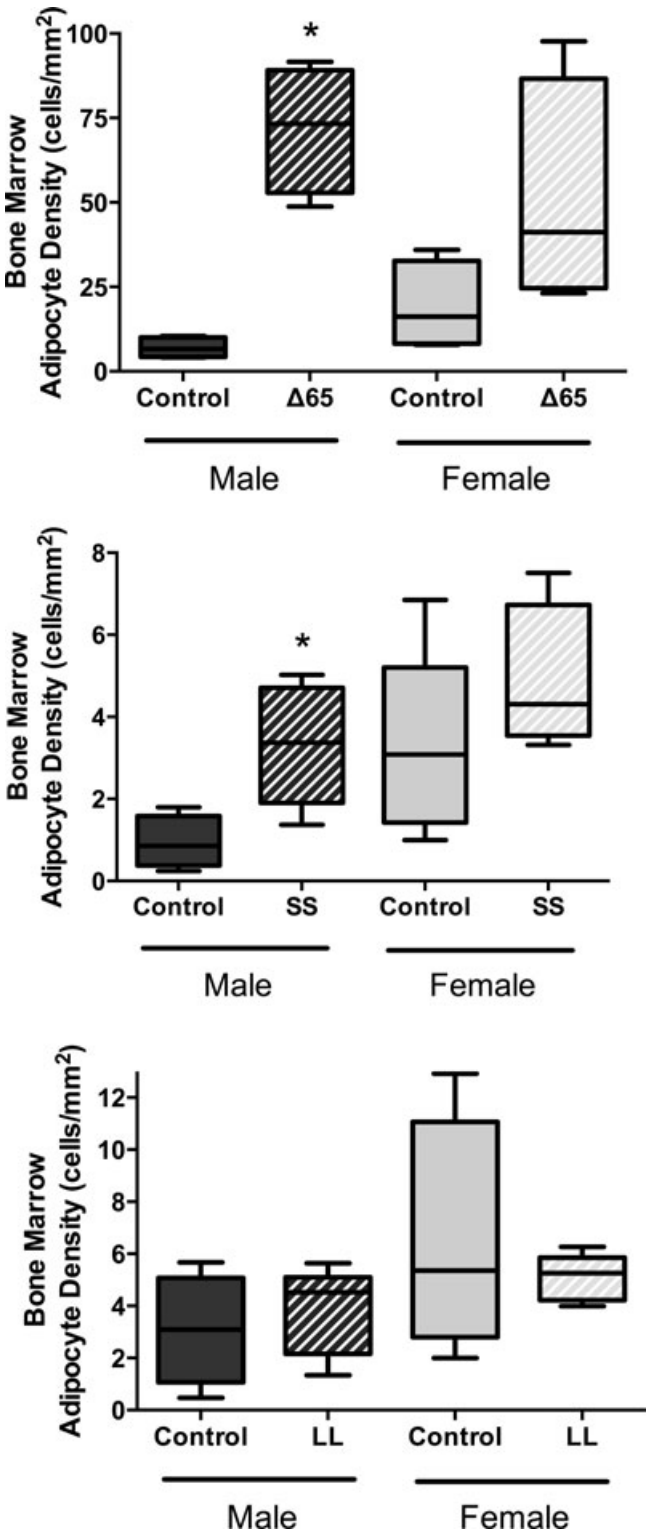


FIGURE 4 LepRb mutations have sex and location-dependent effects on bone marrow adipocyte density. Distal femur bone marrow, proximal to the growth plate, was examined for marrow adipocytes. Adipocytes were counted and expressed relative to the marrow area. LepRb mutant mouse data (Δ65, SS, or LL) are grouped with data obtained from corresponding littermate control mice. Values represent averages ± SE. $n \geq 4$ for all conditions; $^*P < 0.05$

adiposity. This was more prominent in males than females. In addition, the magnitude of the increase was much greater in Δ65 mutants than in SS, which lack only Tyr_{1138}/STAT3 signaling. The LL mutation had no effect on marrow adiposity. Mechanistically, LepRb signaling can regulate adiposity through its expression and signaling in mesenchymal stromal cells and on 94% of bone marrow-derived colonies that mature to make bone, cartilage, and adipocytes.[13] In vitro studies demonstrate that leptin promotes bone marrow stromal cell osteogenesis rather than adipogenesis.[10-12,14] Leptin treatment has been demonstrated to reduce marrow adiposity and corrects skeletal abnormalities in ob/ob mice, though minimal changes in bone occur in rodents capable of producing leptin.[2,33] Leptin treatment also prevents T1-diabetic–induced bone-marrow adiposity, but in this model, leptin treatment is unable to prevent T1-diabetic bone loss.[32]

Leptin has previously been shown to regulate bone growth, in part by its ability to affect chondrocytes.[17,28,36] Leptin-deficient ob/ob mice have fragile growth plates with disturbed columnar structures and increased apoptosis, a phenotype abolished by treatment with leptin.[28] Interestingly, only the Δ65 mutation, lacking all tyrosine kinase signaling from LepRb, causes reduced bone length. Neither the LL or SS mutant mice had an observable growth defect in the femur, suggesting that full inhibition of LepRb-mediated tyrosine kinase signaling is required to obtain notable growth stunting. The lack of a growth effect in LL mice is consistent with a previous study that found no difference in snout-anus length in LL mutant versus WT mice.[22] In our study, we also found no change in vertebral growth as determined by vertebrae height measures. This is consistent with many of the leptin effects being targeted to long bones rather than axial bones.[27]

It should be noted that in contrast to total body LepRb signaling modulation, as in ob/ob, db/db and our studies, the targeted deletion of LepRb in bone marrow mesenchymal stromal cells causes a different phenotype characterized by increased osteogenesis and decreased adipogenesis and increased fracture healing.[11,37] These studies point out the complexity of LepRb signaling in the body, which encompasses effects on the brain, immune system, metabolism, and eating behavior,[8,9,16,18,20] all of which impact bone. Our study suggests that the modulation of whole body LepRb signaling may outweigh the contribution of MSC LepRb regulation of bone and thus lead to increased marrow adiposity and decreased bone density. While it is critical to understand the role of LepRb signaling within the bone as well as in other individual tissues such as brain, our studies provide important

TABLE 3 Cortical bone parameters

	Male		Female	
	Control	**Mutant**	**Control**	**Mutant**
Δ65				
Mean thickness, mm	0.180 ± 0.008	0.145 ± 0.003**	0.174 ± 0.004	0.169 ± 0.006
Inner, mm	3.764 ± 0.047	4.071 ± 0.195	3.759 ± 0.052	3.529 ± 0.181
Outer, mm	4.868 ± 0.076	4.934 ± 0.230	4.774 ± 0.065	4.533 ± 0.199
Marrow area, mm^2	0.995 ± 0.022	1.034 ± 0.035	0.994 ± 0.032	0.906 ± 0.056
Cortical area, mm^2	0.687 ± 0.041	0.468 ± 0.028**	0.588 ± 0.032	0.545 ± 0.042
BMD, mg/cc	937 ± 10	885 ± 10	983 ± 14	922 ± 19**
SS mutant				
Mean thickness, mm	0.245 ± 0.008	0.255 ± 0.010	0.200 ± 0.007	0.203 ± 0.007
Inner, mm	3.720 ± 0.082	3.619 ± 0.109	3.497 ± 0.056	3.775 ± 0.089*
Outer, mm	5.246 ± 0.091	5.232 ± 0.132	4.733 ± 0.055	5.007 ± 0.038*
Marrow area, mm^2	0.985 ± 0.043	0.942 ± 0.055	0.870 ± 0.025	0.989 ± 0.041*
Cortical area, mm^2	1.041 ± 0.039	1.072 ± 0.060	0.752 ± 0.040	0.772 ± 0.041
BMD, mg/cc	1076 ± 15	1081 ± 16	1023 ± 12	955 ± 18**
LL mutant				
Mean thickness, mm	0.211 ± 0.007	0.219 ± 0.006	0.205 ± 0.007	0.201 ± 0.009
Inner, mm	3.726 ± 0.042	3.780 ± 0.068	3.686 ± 0.070	3.513 ± 0.039 *
Outer, mm	5.029 ± 0.030	5.126 ± 0.067	4.948 ± 0.091	4.761 ± 0.072
Marrow area, mm^2	0.983 ± 0.021	1.001 ± 0.032	0.942 ± 0.026	0.895 ± 0.022
Cortical area, mm^2	0.853 ± 0.033	0.902 ± 0.038	0.780 ± 0.054	0.779 ± 0.052
BMD, mg/cc	979 ± 28	1026 ± 26	999 ± 19	994 ± 26

Abbreviation: BMC, bone mineral density.
Values represent the mean ± standard error ($n \geq 3$ per group).
*$P < 0.05$.
**$P < 0.01$ compared with corresponding wild-type control.

TABLE 4 Summary of findings

	Body weight		Femur BVF/wt		Marrow adiposity		Vertebrae BVF/wt		Cortical parameters		Bone growth	
Lepr mutants:	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Δ65	↑	↑	↓	↓	↑↑↑	↑↑	–	–	Δ	Δ	↓	↓
SS	↑	↑	↓	⇣	↑	↑	⇣	↓	–	Δ	–	–
LL	–	↓	↑	–	–	–	–	–	–	–	–	–

Abbreviations: ↑, significant increase; ↓significant decrease; ↑, trend; Δ, change; BVF, bone volume fraction.
Changes are relative to littermate sex and age-matched controls.

insight into potential systemic effects of leptin, which contribute bone density regulation.

5 | CONCLUSIONS

Similar to leptin-receptor deficient db/db mice, truncation of the signaling domain of LepRb in the Δ65 mutant led to increased body mass, decreased femoral trabecular bone volume, bone length, and cortical thickness, and increased bone marrow adiposity (Table 4). The SS mutant, which lacks Tyr_{1138}/STAT3 signaling, recapitulated the excess body mass phenotype of the db/db and Δ65 mice. However, its impact on bone parameters was reduced. Specifically, femoral length and cortical thickness were normal and marrow adiposity, though marginally increased, was an order of magnitude less than Δ65 animals. This suggests that signals in addition to Tyr_{1138}/STAT3 are necessary to

promote maximal bone loss, growth restriction, and marrow fat accumulation. In the absence of excess peripheral and bone marrow adiposity, as present in LL mice, bone mass was unchanged (females), or increased (males). These results are relevant to understanding how disruptions in leptin signaling, whether due to monogenetic or diet-induced obesity, may impact bone development and growth. This may be particularly relevant in the context of juvenile obesity, where loss of leptin signaling via LepRb could compromise bone development and growth. Understanding how leptin modifies bone through LepRb will impact the design of strategies to promote bone growth and integrity in such at-risk populations.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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