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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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Abbreviated Title: Leptin receptor mutations alter bone

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

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This is the author manuscript accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jcb.27726.

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Funding: These studies were supported by funding from the National Institute of Health, grants RO1 DK101050 and RO1 AT007695-01 and R00 DE024178.

Disclosure statement: The authors have nothing to disclose and have no conflicts of interest.

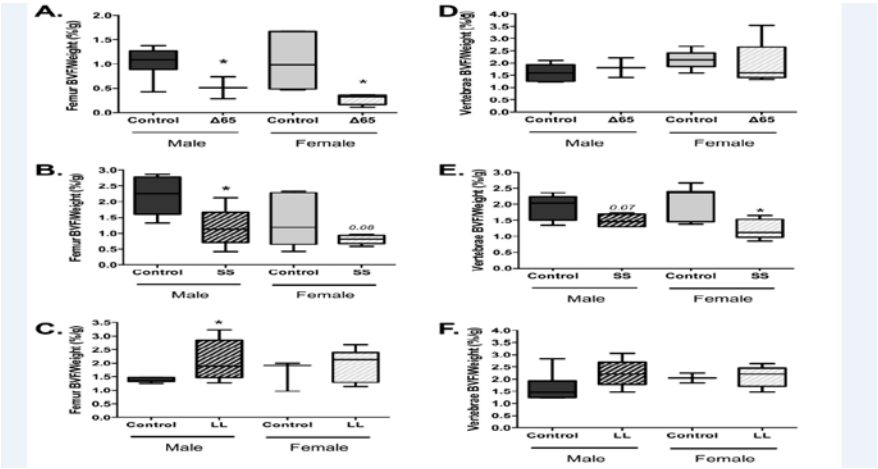
Abstract

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 STAT3, 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.

Graphial Abstract

Leptin, a hormone primarily produced by adipocytes, contributes to the regulation of bone health by modulating bone density, growth and adiposity. Our findings suggest that the phosphorylation status of specific sites of the full length leptin receptor contribute to the sex and location dependent bone responses to leptin.



Leptin is a 16 kD hormone/adipokine produced primarily by adipocytes and is involved in the regulation of body mass and bone density (Hamrick 2004, Reid 2004, Elefteriou, Ahn et al. 2005, Karsenty 2006, Iwaniec, Boghossian et al. 2007, Iwaniec, Boghossian et al. 2011, Motyl and Rosen 2012). 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 (Villanueva and Myers 2008, Abella, Scotece et al. 2017). 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 expressed on a variety of cell types including mesenchymal stem cells, which can mature into osteoblasts or adipocytes (Thomas, Gori et al. 1999, Reseland, Syversen et al. 2001, Cornish, Callon et al. 2002, Scheller, Song et al. 2010, Zhou, Yue et al. 2014).

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 mesenchymal stem/stromal cells (MSCs) and/or osteoblasts (Reseland, Syversen et al. 2001, Cornish, Callon et al. 2002, Zhou, Yue et al. 2014) or indirectly through LepRb-expressing hypothalamic neurons that can polysynaptically regulate bone (Cornish, Callon et al. 2002, Hamrick 2004, Hamrick, Della Fera et al. 2007). Mice that are completely leptin deficient (ob/ob mice) have age-dependent, site-specific phenotypes including lower femoral bone mineral content (BMC), cortical thickness, bone mineral density (BMD), trabecular bone volume, and decreased bone length (Steppan, Crawford et al. 2000, Hamrick 2004, Iwaniec, Boghossian et al. 2007, Turner, Kalra et al.

2013, Philbrick, Martin et al. 2017). Similarly, leptin receptor deficiency (db/db mice) causes decreased tibial trabecular bone volume, bone length and cortical thickness (Williams, Callon et al. 2011, Turner, Kalra et al. 2013). Absence of leptin or LepRb in mice also increases bone marrow adiposity (Steppan, Crawford et al. 2000, Hamrick 2004, Lindenmaier, Philbrick et al. 2016). The receptor-mediated mechanisms underlying these skeletal and marrow adiposity phenotypes remain unknown.

The leptin receptor (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 (Gong, Ishida-Takahashi et al. 2007). Leptin initiates its action through binding to the single long form of the leptin receptor (LepRb) which leads to the auto-phosphorylation 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 approximately 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 suppressor of cytokine signaling 3 (SOCS3) which leads to an attenuation of LepRb signaling(Gong, Ishida-Takahashi et al. 2007). Phosphorylation of the second site, Tyr_{1077}, recruits the signal transducer, latent transcription factor, and activator of transcription 5 (STAT5)(Bjornholm, Munzberg et al. 2007). Phosphorylation of Tyr_{1138} results in the recruitment of STAT3 (Banks, Davis et

al. 2000, Munzberg, Huo et al. 2003). 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 LepRb(Bates, Stearns et al. 2003). 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 (Bates, Stearns et al. 2003). Finally, Δ65 animals lack LepRb signaling and are similar to db/db mice (Robertson, Ishida-Takahashi et al. 2010). 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.

Methods:

LepRb Mutant Mouse Models: This study examined LepRb Δ65, LL and SS mice on the C57/Bl6 background, as previously described (Bates, Stearns et al. 2003, Bjornholm, Munzberg et al. 2007, Robertson, Ishida-Takahashi et al. 2010). 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 2-4 weeks of age. Study mice were group housed until 8-9 weeks of age, then were anesthetized with a lethal dose of pentobarbital and transcardially perfused with 10% neutral buffered formalin. Bones were removed and post-fixed in formalin for 24-hours prior to 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.

Bone density measurement: Femurs and vertebrae (lumbar 3-4) were fixed in 10% formalin and imaged using a GE Explore Locus microcomputed 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 and was consistent with previous reports(Hamrick,

Pennington et al. 2004). 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 (~ 0.17mm) proximal to the growth plate extending 2 mm toward the diaphysis excluding the outer cortical bone. Trabecular bone volume fraction 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 (ROI) in the mid-diaphysis.

Leptin serum measurements: Leptin concentration in female mouse serum was determined by ELISA using the Mouse/Rat Leptin Quantikine Kit (R&D Systems).

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. Paraffin blocks were sectioned at 5 µm on a Reichert Jung 2030 rotary microtome and were H&E stained. Femur sections were examined by microscopy at 4x 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. Adipocytes greater than 30 µm in diameter were counted and

expressed relative to the total marrow area. Analyses were done blinded to conditions.

Statistical analyses:

All measurements are presented as the mean ± SE. All groups contained at least 5 mice, except for the Δ65 male mouse group which has 3 mice. Student's two tail 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 3 identified). Statistical analyses used student t-tests that compared littermate WT to mutant mouse values for each sex. Analyses were performed using GraphPad Prism software version 6 (GraphPad, San Diego, CA, USA). A *p*-value ≤0.05 was considered significant.

Results

To determine the role of LepRb and its key phosphorylation sites on the male and female mouse skeleton, we examined 8-9-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(Villanueva and Myers 2008) which lack LepRb signaling, both male and female Δ65 mice had significantly greater body mass compared to 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(Bjornholm, Munzberg et al. 2007). 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(Bjornholm, Munzberg et al. 2007, Villanueva and Myers 2008). 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 to 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 2 A-C). Both the male and female Δ65 mice displayed reduced femoral growth compared to 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 (L3-4) but in contrast to another report(Kishida, Hirao et al. 2005) 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 bone volume fraction (BV/TV) did not differ between conditions except for male LL mutant mice which displayed higher bone volume and greater trabecular thickness compared to 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 non-significant 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 to their corresponding control littermates, by 51% and 74% respectively, consistent with previous reports(Hamrick 2004, He, Liu et al. 2004, Ealey, Fonseca et al. 2006, Ramos-Junior, Leite et al. 2016). The SS mutant mice showed a significant 53% decrease for male BV/TV, and a non-significant 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 non-significant 20% increase for females, relative to littermate controls.

Examination of vertebral BV/TV, without correction to bodyweight, 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 to 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.

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 to 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 to 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 (Thomas, Gori et al. 1999, Reseland, Syversen et al. 2001, Cornish, Callon et al. 2002, Motyl and McCabe 2009, Scheller, Song et al. 2010) demonstrated that leptin can influence marrow adiposity. Therefore, we further examined the effect of the leptin receptor 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 per mm^2), compared to the effect of Δ65 on marrow fat. The female Δ65 mice exhibited an

average increase of 2.7-fold (to 51 adipocytes per mm^2) and the SS female mice displayed a 1.5-fold average increase (to approximately 5 adipocytes per mm^2) compared to 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.

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 leptin receptor 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 leptin receptor 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 (Hamrick 2004, He, Liu et al. 2004, Ealey, Fonseca et al. 2006, Ramos-Junior, Leite et al. 2016). The SS mutation of LepRb Tyr_{1138}, which prevents LepRb-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 to 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 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 (Ealey, Fonseca et al. 2006, Williams, Callon et al. 2011). This is consistent with leptin having site-specific effects on bone(Hamrick, Pennington et al. 2004). 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, *i.e.*, 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 a 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 (Thomas, Gori et al. 1999, Reseland, Syversen et al. 2001, Cornish, Callon et al. 2002, Hamrick, Della-Fera et al. 2005, Scheller, Song et al. 2010), whereas reduced leptin signaling promotes bone marrow adiposity (Steppan, Crawford et al. 2000, Hamrick 2004). The latter is most evident in ob/ob and db/db (Devlin, Cloutier et al. 2010, Ecklund, Vajapeyam et al. 2010). In our study, both the Δ65 mutant and SS mutants had increased femoral bone marrow 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 (Zhou, Yue et al. 2014). *In vitro* studies demonstrate that leptin promotes bone marrow stromal cell osteogenesis rather than adipogenesis (Thomas, Gori et al. 1999, Reseland, Syversen et al. 2001, Cornish, Callon et al. 2002, Scheller, Song et al. 2010). 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 (Hamrick, Della-Fera et al. 2005, Iwaniec, Boghossian et al. 2007). Leptin treatment also prevents T1-diabetic induced bone marrow adiposity, but in this model, leptin treatment is unable to prevent T1-diabetic bone loss (Motyl and McCabe 2009).

Leptin has previously been shown to regulate bone growth, in part by its ability to affect chondrocytes (Steppan, Crawford et al. 2000, Kishida, Hirao et al. 2005, Gat-Yablonski and Phillip 2008). Leptin deficient ob/ob mice have fragile growth plates with disturbed columnar structures and increased apoptosis, a phenotype abolished by treatment with leptin (Kishida, Hirao et al. 2005). 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 (Bjornholm, Munzberg et al. 2007). 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 (Hamrick, Pennington et al. 2004).

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 (Scheller, Song et al. 2010, Yue, Zhou et al. 2016). These studies point out the complexity of LepRb signaling in the body which encompasses effects on the brain, immune system, metabolism, and eating behavior (Villanueva and Myers 2008, Turner, Kalra et al. 2013, Lindenmaier, Philbrick et al. 2016, Abella, Scotece et al. 2017, Philbrick, Martin et al. 2017), all of which impact bone. Our study suggests that the modulation of whole body LepRb signaling may outweigh the contribution of mesenchymal stem cell 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 insight into potential systemic effects of leptin which contribute bone density regulation.

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.

Acknowledgements

The authors thank the Investigative Histology Laboratory in the Department of Physiology, Division of Human Pathology for their assistance with bone histology as well as the Biomedical Imaging Center for use of the microcomputed tomography imaging. Thank you also to Daniel Barnett for his technical expertise and Drs. Fraser Collins and Natalie Wee for their critical review of the manuscript. The authors have no conflicts of interest. These studies were supported by funding from the National Institute of Health, grants RO1 DK101050, RO1 AT007695, R01 DK056731, and R00 DE024178.

Figure

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 8-9-week-old mice

expressing LepRb mutants: Δ65, SS (Tyr_{1138}) or LL (Tyr_{985}). Mutant mouse data is graphed with data obtained from corresponding littermate control mice. Values represent averages ± SE. n = 3-7 per group. * $p < 0.05$.

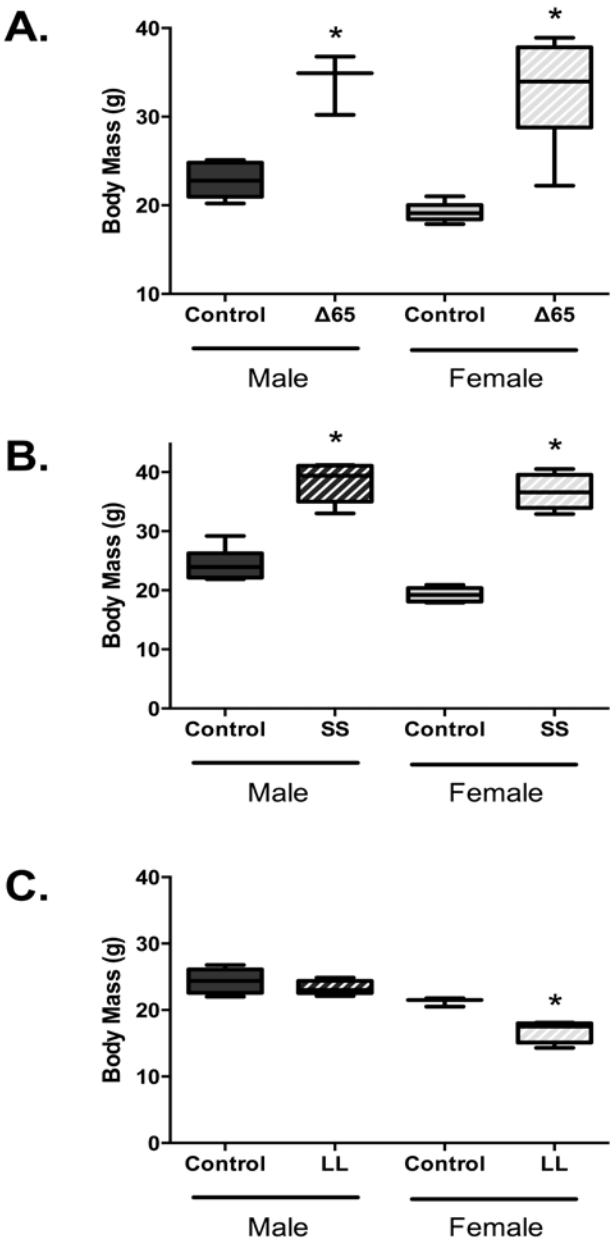


Figure 2. **Only LepRb mutation Δ65 effected femur length/growth.** Femur lengths were measured for male and female 8-9-week-old mice expressing LepRb containing mutations Δ65, SS or LL. Mutant mouse data is graphed and analyzed relative to data obtained from corresponding littermate control mice. Values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

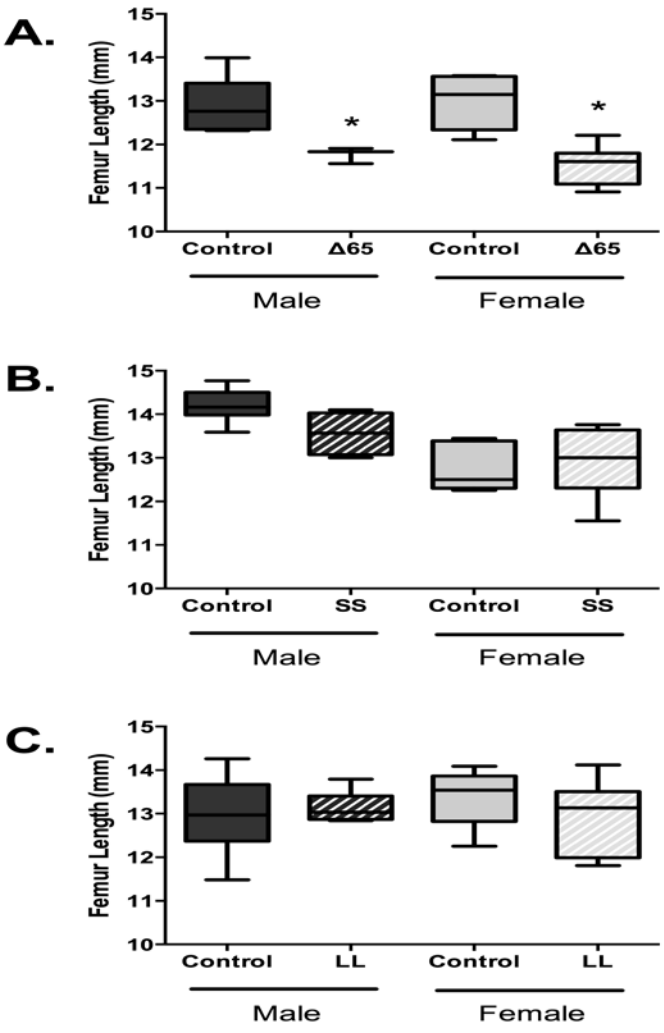


Figure 3. **LepRb mutations have sex and location dependent effects on bone volume fraction.** Femur (A-C) and vertebral (D-E) bone volume fraction (BVF) was determined, in 8-9-week-old mice, by microcomputed tomography and expressed relative to mouse body weight. LepRb mutant mouse data (Δ65, SS or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

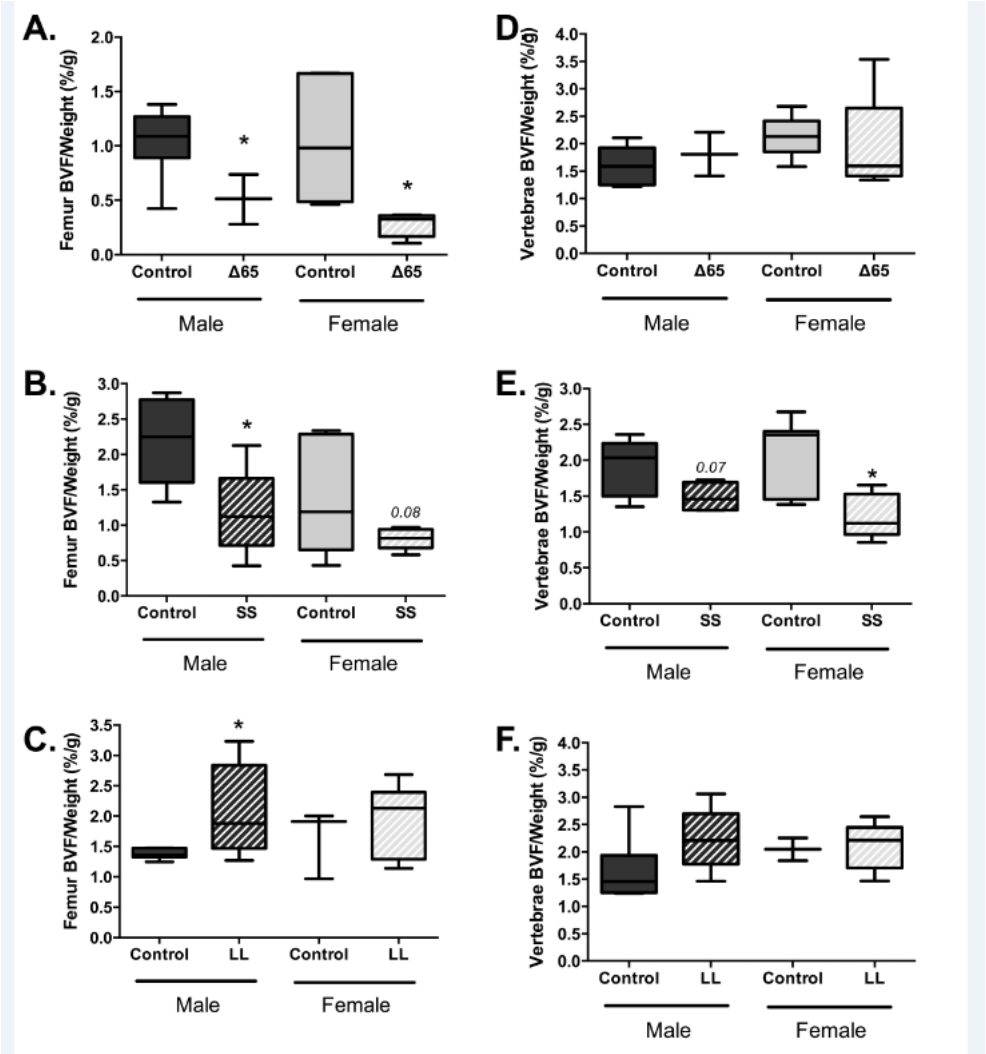


Figure 4. **LepRb mutations have sex and location dependent effects on bone marrow adipocyte density.** Distal femur bone marrow, proximal to the growth plate, was examined for marrow adipocytes. Adipocytes were counted and expressed relative to the marrow area. LepRb mutant mouse data (Δ65, SS or LL)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

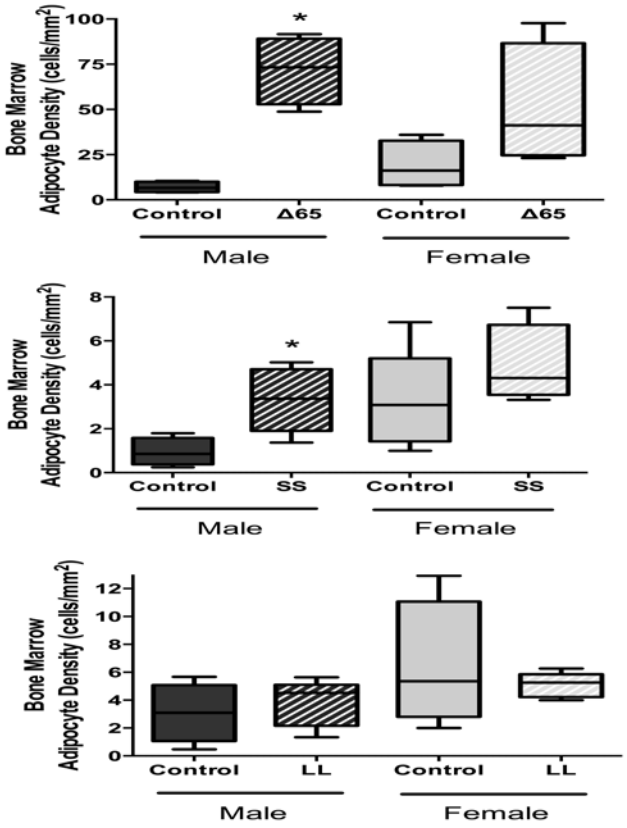


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Table 1. Femoral Trabecular Bone Parameters

	MALE		FEMALE	
	Control	**Mutant**	**Control**	**Mutant**
Δ*65*				
BV/TV	23.9 ± 3.6	17.7 ± 3.8	19.6 ± 4.1	9.8 ± 1.7
Tb. Th. (mm)	0.032 ± 0.003	0.029 ± 0.002	0.030 ± 0.002	0.026 ± 0.001
Tb. N. (1/mm)	7.288 ± 0.847	5.957 ± 0.964	6.150 ± 0.873	3.651 ± 0.547
Tb. Sp. (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
Tb. Th. (mm)	0.064 ± 0.007	0.056 ± 0.011	0.029 ± 0.003	0.037 ± 0.001
Tb. N. (1/mm)	8.308 ± 0.342	7.973 ± 0.839	5.805 ± 0.874	7.874 ± 0.199
Tb. Sp. (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
Tb. Th. (mm)	0.038 ± 0.001	0.054 ± 0.009 *****	0.039 ± 0.003	0.039 ± 0.003
Tb. N. (1/mm)	8.891 ± 0.509	8.862 ± 0.459	8.095 ± 0.576	8.157 ± 0.687
Tb. Sp. (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). * $p<0.05$

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
Tb. Th. (mm)	0.039 ± 0.002	0.058 ± 0.016	0.044 ± 0.002	0.059 ± 0.008
Tb. N. (1/mm)	8.923 ± 0.360	10.145 ± 1.272	9.069 ± 0.215	9.319 ± 0.312
Tb. Sp. (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
Tb. Th. (mm)	0.048 ± 0.004	0.055 ± 0.002	0.044 ± 0.003	0.044 ± 0.003
Tb. N. (1/mm)	9.570 ± 0.414	9.944 ± 0.089	8,834 ± 0.290	9.706 ± 0.341
Tb. Sp. (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*
Tb. Th. (mm)	0.040 ± 0.003	0.051 ± 0.002*	0.048 ± 0.001	0.036 ± 0.002*
Tb. N. (1/mm)	9.780 ± 0.433	10.287 ± 0.522	9.190 ± 0.257	9.658 ± 0.400
Tb. Sp. (mm)	0.064 ± 0.007	0.048 ± 0.007	0.061 ± 0.003	0.069 ± 0.005

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

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

Values represent the mean ± standard error ($n \geq 3$ per group); *$p<0.05$; **$p<0.01$ compared to corresponding WT control.

Table 4. **Summary of Findings**