Insufficiency of Jak2-autonomous leptin receptor signals for most physiologic leptin actions

Running Title: Role of Jak2 in leptin action

Scott Robertson¹, Ryoko Ishida-Takahashi², Isao Tawara², Jiang Hu³, Christa M. Patterson², Justin C. Jones², Rohit N. Kulkarni³, and Martin G. Myers, Jr.¹,²,³

¹Department of Molecular and Integrative Physiology and ²Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109. ³Joslin Diabetes Center and Harvard Medical School, Boston, MA 02215

S.R. and R.I.T contributed equally.

Corresponding Author:
Martin G. Myers, Jr.,
Email: mgmyers@umich.edu

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**Objective:** Leptin acts via its receptor (LepRb) to signal the status of body energy stores. Leptin binding to LepRb initiates signaling by activating the associated Jak2 tyrosine kinase, which promotes the phosphorylation of tyrosine residues on the intracellular tail of LepRb. Two previously examined LepRb phosphorylation sites mediate several, but not all, aspects of leptin action, leading us to hypothesize that Jak2 signaling might contribute to leptin action independently of LepRb phosphorylation sites. We therefore determined the potential role in leptin action for signals that are activated by Jak2 independently of LepRb phosphorylation (Jak2-autonomous signals).

**Research Design and Methods:** We inserted sequences encoding a truncated LepRb mutant (LepRb$^{Δ65c}$, which activates Jak2 normally, but is devoid of other LepRb intracellular sequences) into the mouse Lepr locus. We examined the leptin-regulated physiology of the resulting $Δ/Δ$ mice relative to LepRb-deficient $db/db$ animals.

**Results:** The $Δ/Δ$ animals were similar to $db/db$ animals in terms of energy homeostasis, neuroendocrine and immune function, and the regulation of the hypothalamic arcuate nucleus, but demonstrated modest improvements in glucose homeostasis.

**Conclusions:** The ability of Jak2-autonomous LepRb signals to modulate glucose homeostasis in $Δ/Δ$ animals suggests a role for these signals in leptin action. Since Jak2-autonomous LepRb signals fail to mediate most leptin action, however, signals from other LepRb intracellular sequences predominate.
Adipose tissue produces the hormone, leptin, in proportion to fat stores to communicate the status of long-term energy reserves to the brain and other organ systems (1-4). In addition to moderating food intake, adequate leptin levels permit the expenditure of energy on myriad processes including reproduction, growth, and immune responses, as well as regulating nutrient partitioning (4-6). Conversely, lack of leptin signaling due to null mutations of leptin (e.g., Leptob/ob mice) or the leptin receptor (LepR) (e.g., Leprdb/db mice) results in increased food intake in combination with reduced energy expenditure (and thus obesity), neuroendocrine dysfunction (including hypothyroidism, decreased growth, infertility), decreased immune function, and hyperglycemia and insulin insensitivity (1;7-9). Many of the effects of leptin are attributable to effects in the CNS, particularly in the hypothalamus, but leptin also appears to act directly on some other tissues (2;3).

Alternative splicing generates several integral-membrane LepR isoforms that possess identical extracellular, transmembrane, and membrane-proximal intracellular domains. LepR intracellular domains diverge beyond the first 29 intracellular amino acids, however, with the so-called “short” isoforms (e.g., LepRa) containing an additional 3-10 amino acids, and the single “long” (LepRb isoform) containing a 300 amino acid intracellular tail (10). Like other type I cytokine receptors (11), LepRb (which is required for physiologic leptin action) contains no intrinsic enzymatic activity, but associates with and activates the Jak2 tyrosine kinase to mediate leptin signaling. The intracellular domain of LepRb possesses membrane-proximal Box1 and Box2 motifs, both of which are required for association with and regulation of Jak2; while LepRa and other short LepRs contain Box 1, they lack Box 2 and thus fail to bind and activate Jak2 under physiologic conditions (12).

Leptin stimulation promotes the autophosphorylation and activation of LepRb-associated Jak2, which phosphorylates three LepRb tyrosine residues (Tyr985, Tyr1077 and Tyr1138). Each LepRb tyrosine phosphorylation site recruits specific SH2 domain-containing effector proteins: Tyr985 recruits SHP2 and SOCS3 and attenuates LepRb signaling, but does not appear to play other roles in leptin action in vivo (13-15). Tyr1077 recruits the latent transcription factor, STAT5, and Tyr1138 recruits STAT3 (16-18). Mice in which LepRbS1138 (mutant for Tyr1138 and thus specifically unable to recruit STAT3) replaces endogenous LepRb exhibit hyperphagic obesity, with decreased energy expenditure, but increased growth, protection from diabetes, and preservation of several aspects of hypothalamic physiology (19-21). These results thus suggest roles for Tyr1077 and/or Jak2-dependent signals that are independent of LepRb tyrosine phosphorylation (“Jak2-autonomous signals”) in mediating Tyr985/Tyr1138-independent leptin actions. While others have examined the effect of mutating all three LepRb tyrosine phosphorylation sites in mice (22), revealing potential tyrosine phosphorylation-independent roles for LepRb in leptin action, this previous study did not examine several aspects of leptin action, and could not distinguish potential effects of non-phosphorylated LepRb motifs from effects due to LepRb/Jak2 interactions specifically.

RESEARCH DESIGN AND METHODS:

Cell Culture Studies: The plasmids pcDNA3LepRb∆65 and pcDNA3LepRa were generated by mutagenesis of pcDNA3LepRb (23) using the Quickchange kit (Stratagene). The absence of adventitious mutations was confirmed by DNA sequencing for all
plasmids. Cell culture, transfection, lysis and immunoblotting were conducted as reported previously using αJak2(pY1007/8) from Cell Signaling and αJak2 from our own laboratory (24). Leptin was the generous gift of Amylin Pharmaceuticals (La Jolla, CA).

**Mouse Model Generation:** The targeting vector encoding LepRbΔ65 was generated by inserting a STOP codon (Quickchange kit) following the 65th intracellular amino acid of LepRb in the 5’ targeting arm in the pBluescript plasmid; this modified 5’-arm was subsequently subcloned into the previously-described pPNT-derived targeting vector that contained the 3’-arm (19;25;26). The resulting construct was linearized and transfected into murine ES cells with selection of clones by the University of Michigan Transgenic Animal Model Facility. Correctly targeted clones were identified and confirmed with real-time PCR and Southern Blotting as performed previously (15;21;26), and were injected into embryos for the generation of chimeras and the establishment of germline LeprΔ65/+ (∆/+), LeprΔ/∆ (∆/∆) animals. ∆/+ animals were intercrossed to generate +/+ and ∆/∆ mice for the determination of hypothalamic Lepr expression, or were backcrossed onto the C57BL/6J background (Jackson Laboratories) for six generations prior to intercrossing to generate +/+ and ∆/∆ animals for the collection of other data.

**Experimental Animals:** C57BL/6J wild-type, Lepob/+ and Lepobob/+ breeders were purchased from Jackson Laboratories. All other animals and progeny from these purchased breeders were housed and bred in our colony, and were cared for and used according to guidelines approved by the University of Michigan Committee on the Care and Use of Animals. Following weaning, all mice were maintained on 5011 Labdiet chow. Mice were given ad libitum access to food and water unless otherwise noted. For body weight, food intake, glucose monitoring and estrous monitoring, animals were weaned at four weeks and housed individually. Body weight and food intake were recorded weekly from four to eight weeks. Whole venous blood from the tail vein was used to determine blood glucose (Ascensia Elite glucometer) and to obtain serum, which was frozen for later hormone measurement. A terminal bleed was also collected at the time of sacrifice. Commercial ELISA kits were used to determine insulin (Crystal Chem), leptin (Crystal Chem) and C-peptide concentrations (Millipore).

In females, vaginal lavage was used to assess estrous cycling daily from four to eight weeks. Animals intended for glucose tolerance tests, insulin tolerance tests and body composition were weaned at four weeks and group housed. Body composition was determined using a NMR Minispec LF90II scanner (Bruker Optics) in the University of Michigan Animal Phenotyping Core.

**Analysis of hypothalamic RNA.** Hypothalami were isolated from ad libitum-fed mice and snap-frozen. Total RNA was isolated using Trizol RNA reagent (Invitrogen) and converted to cDNA using SuperScript reverse transcriptase (Invitrogen). For comparison of relative LepRb expression, total hypothalamic cDNA was subjected to PCR with LepRb-specific primers and subjected to gel electrophoresis (21). For determination of relative neuropeptide expression, total hypothalamic cDNA was subjected to automated fluorescent RT-PCR on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) (21).

**Immunonological Cell Analysis:** For counting total splenocytes and splenic T cells, splenic T cells were magnetically separated from the spleen by AutoMACS as previously described and counted by flow cytometry (27). For proliferation assays, T cells were separated using CD90 microbeads and 2 × 10^5 of these were incubated with 2 × 10^4 of B6 bone marrow–derived dendritic cells for 48,
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Cells were stimulated with soluble anti-CD3e (1 mg/mL). Incorporation of \(^{3}\)H-thymidine (1 µCi/well) by proliferating cells during the last 12 hours of culture was measured.

**Immunohistochemical Analysis of Hypothalamic Brain Sections:** 

+/+, db/+ and \(\Delta/\Delta\) were intercrossed with heterozygous Agrp\(^{LacZ}\) mice expressing LacZ from the Agrp locus (28;29) before recrossing to the parent Lepr strain to generate experimental animals. For immunohistochemical analysis, ad libitum-fed animals remained with food in the cage until the time of death. Perfusion and immunohistochemistry (IHC) were performed as described (30).

**RESULTS**

Lepr\(^{\Delta 65}\) and gene targeting to generate Lepr\(^{\Delta 65}\). To examine physiologic signals generated by LepRb-associated Jak2 in the absence of LepRb tyrosine phosphorylation sites and other LepRb motifs (Jak2-autonomous signals), we utilized a COOH-terminal truncation mutant of LepRb (LepRb\(^{\Delta 65}\)) that contains all motifs required for Jak2 association and regulation, but which is devoid of other intracellular LepRb sequences (12). Since we previously demonstrated the function of this mutant intracellular domain in the context of an erythropoietin receptor (extracellular domain)/LepRb (intracellular domain) chimera (12), we initially examined signaling by the truncated intracellular domain within the context of LepRb\(^{\Delta 65}\) in transfected 293 cells (Figure 1A). Leptin stimulation promoted the phosphorylation of Jak2 on the activating Tyr\(^{1007/1008}\) sites in LepRb\(^{\Delta 65}\) - and LepRb-expressing cells but not in LepRa-expressing or control cells (Fig 1A), confirming that LepRb and LepRb\(^{\Delta 65}\), but not LepRa, contain the necessary sequences to mediate Jak2 activation in response to leptin. To understand the potential roles for Jak2-autonomous LepRb signals in leptin action, we utilized homologous recombination to replace the genomic Lepr with Lepr\(^{\Delta 65}\) (henceforth referred to as the \(\Delta\) allele, encoding LepRb\(^{\Delta 65}\)) in mouse ES cells (Fig 1B). Correctly targeted ES cell clones were confirmed by Southern blotting (Fig 1C). This strategy mediates LepRb\(^{\Delta 65}\) expression from the native Lepr locus, ensuring correct patterns and levels of LepRb\(^{\Delta 65}\) expression, as previously for other homologously-targeted LepRb alleles (15;21;26). Indeed, RT-PCR analysis of hypothalamic mRNA confirmed similar Lepr mRNA expression in homozygous \(\Delta/\Delta\) animals and wild-type animals (Fig 1D). Prior to subsequent study, we backcrossed heterozygous \(\Delta/+\) animals to C57BL/6J mice for 6 generations to facilitate direct comparison to LepRb\(^{db/db}\) (db/db) animals on this background.

**Energy Homeostasis in \(\Delta/\Delta\) mice.**

Since our previous analysis suggested some role for Tyr\(^{985}\)/Tyr\(^{1138}\)-independent LepRb signals in regulating energy balance, we initially examined parameters of energy homeostasis in \(\Delta/\Delta\) compared to db/db animals (15;19;21). We weaned and singly-housed \(\Delta/\Delta\), db/db and control mice from 4-8 weeks of age for the longitudinal determination of body weight and food intake (Fig 2A-D). Compared to age- and sex-matched db/db animals, \(\Delta/\Delta\) mice displayed similar body weights and food intake over the study period. Furthermore, age- and sex-matched \(\Delta/\Delta\) and db/db mice displayed similar proportions of fat and lean mass (Table 1); revealing that \(\Delta/\Delta\) and db/db mice are similarly obese. Core body temperature in \(\Delta/\Delta\) and db/db animals was also similarly reduced compared to control animals (Table 1). Thus, LepRb\(^{\Delta 65}\) fails to alter major parameters of energy homeostasis compared to entirely LepRb-deficient db/db mice, suggesting that Jak2-autonomous LepRb signals are not sufficient to modulate energy balance in mice.
Linear growth and reproductive function in Δ/Δ mice. In addition to modulating metabolic energy expenditure, leptin action permits the utilization of resources on energy-intensive neuroendocrine processes, such as growth and reproduction. While db/db and ob/ob animals that are devoid of leptin action thus display decreased linear growth and infertility, animals lacking Tyr985 or Tyr1138 of LepRb display normal or enhanced linear growth and preserved reproductive function, suggesting a role for other LepRb signals in mediating these leptin actions (15;19;21). Similar to db/db mice, Δ/Δ males displayed decreased snout-anus length and femur length relative to control animals (Table 1), however, suggesting the inability of Jak2-autonomous LepRb signals to mediate linear growth in the absence of other LepRb signals.

To determine the potential role for Jak2-autonomous LepRb signals in the regulation of reproductive function, we monitored estrous cycling from 4-8 weeks of age in female mice, along with their ability to deliver pups following housing with wild-type males. In addition to examining Δ/Δ, db/db, and wild-type females in these assays, we included mice homozygous for LepRbmt1mgmj (a.k.a. LepRbs1138 or s/s mice; mutant for Tyr1138/BarB right STAT3 signaling) (21) as a positive control for our ability to detect residual reproductive function in obese mice with altered leptin action (Table 2). While essentially all wild-type females and approximately half of the s/s females displayed vaginal estrus when housed in the absence of males and delivered pups following cohabitation with male mice, Δ/Δ females, like db/db animals, failed to undergo estrus or deliver pups. Gross examination also revealed the reproductive organs of Δ/Δ females were atrophic and similar to those of db/db females (data not shown). Thus, Jak2-autonomous LepRb signals are not sufficient to mediate leptin action on the reproductive axis.

Regulation of the hypothalamic arcuate nucleus (ARC) in Δ/Δ animals. A number of aspects of leptin action in the hypothalamus are beginning to be unraveled, including the role of leptin in regulating ARC LepRb/pro-opiomelanocortin (POMC)-expressing neurons and their opposing LepRb/agouti-related protein/neuropeptide Y (AgRP/NPY)-expressing neurons (2;3;31;32). Leptin promotes anorectic POMC expression, while inhibiting the expression of orexigenic AgRP and NPY and attenuating the activity of AgRP/NPY neurons. While LepRb Tyr1138→STAT3 signaling is required to promote Pomc mRNA expression; signals independent of Tyr985 and Tyr1138 contribute to the suppression of Agrp and Npy mRNA expression, and to the inhibition of AgRP neuron activity (15;21;29). To determine the potential role for Jak2-autonomous LepRb signals in the regulation of these ARC neurons, we examined the mRNA expression of Pomc, Npy and Agrp in the hypothalamus, and examined c-fos-immunoreactivity (-IR) as a surrogate for activity in AgRP neurons (29) of male Δ/Δ, db/db and wild-type mice (Figure 3). As expected based upon the known role for Tyr1138 in regulating Pomc, Δ/Δ and db/db mice exhibited significantly diminished Pomc mRNA expression compared to wild-type controls (Figure 3A); Pomc levels in Δ/Δ mice were even lower than those in db/db mice. We found similarly elevated Npy and Agrp mRNA expression in Δ/Δ and db/db mice compared to wild-type mice (Fig 3B-C).

To analyze c-fos-IR in AgRP neurons, we generated +/+, Δ/Δ and db/db animals heterozygous for AgrpLacZ, in which β-galactosidase (β-gal) is expressed from the Agrp locus, facilitating the detection of AgRP-expressing neurons by β-gal immunofluorescence (29). While fed wild-type animals displayed c-fos-IR in few AgRP
neurons, a similarly large percentage of AgRP neurons in ∆/∆ and db/db animals contained c-fos-IR, suggesting their activity (Fig 3D-E). Thus, Jak2-autonomous LepRb signals are neither sufficient to mediate the normal regulation of ARC neuropeptide gene expression nor the suppression of c-fos-IR/activity in AgRP-neurons.

Regulation of T cell function by LepRb signals. Leptin signals the status of energy stores to the immune system, as well as to the brain systems that control energy balance and neuroendocrine function. Leptin deficiency results in thymic hypoplasia, reduced T cell function, and consequent immune suppression (6;33). While we previously examined thymocyte numbers in young s/s mice deficient for LepRb Tyr1138 signaling, suggesting improved immune function in s/s compared to db/db mice (23), many other parameters of immune function in these and other models of altered LepRb signaling remain poorly understood. To better understand the signaling mechanisms by which LepRb modulates the immune system, we thus determined the numbers of total and CD4+ splenocytes, as well as the ex vivo proliferative capacity of splenic CD4+ cells from a panel of mouse models of altered LepRb signaling. In addition to examining wild-type, ob/ob, ∆/∆, and s/s animals, we also studied mice homozygous for Lepr^tm2mgmj (a.k.a. Lepr^l985 or l/l) mutant for LepRb Tyr985 (15) (Figure 4). Note that ob/ob animals were utilized as the control for the absence of leptin action in this study, as sufficient numbers of age-matched db/db animals were not available at the time of assay. In addition to revealing the expected decrease in total and CD4+ splenocytes and their proliferation in ob/ob animals relative to wild-type controls, we found decreases in these parameters in ∆/∆ animals, and normal parameters of immune function in s/s animals. Interestingly, while l/l animals exhibit increased sensitivity to the anorexic action of leptin (15), these parameters of immune function actually trended down (albeit not significantly) compared to wild-type animals. Thus, these data suggest that although Tyr985 and Tyr1138 are not required for the promotion of splenic T cell function by leptin, Jak2-autonomous LepRb signals are not sufficient to mediate these aspects of leptin action.

Glucose homeostasis in ∆/∆ mice. Given the role for leptin in modulating long-term glucose homeostasis (2;20;34), we examined glycemic control in ∆/∆ mice. Interestingly, we found that at 4 weeks of age, the blood glucose levels in male and female ∆/∆ mice were normal, while db/db mice were already significantly diabetic (Fig 5A-B). At later time-points, however, ∆/∆ animals exhibited elevated blood glucose levels similar to those of db/db mice. Similarly, male ∆/∆ mice displayed significantly lower fasting glucose levels than db/db mice at 6 weeks of age (Fig 5C), and fasted females, while not significantly different than db/db animals, also tended to have decreased blood glucose at this early age. Fasted blood glucose in ∆/∆ animals of both sexes were elevated and not significantly different from db/db levels at older ages, however (Fig 5D). These data suggest that Jak2-autonomous LepRb signals in ∆/∆ mice suffice to delay the onset of diabetes compared to db/db mice, but cannot reverse the later progression to diabetes. For female mice, there was no difference in serum insulin levels between db/db and ∆/∆ mice at any age. In male mice, insulin was also similar between db/db and ∆/∆ mice at four weeks of age, but older ∆/∆ males displayed increased circulating insulin levels compared to age-matched db/db males. In order to discriminate potential alterations in insulin clearance, we also examined serum C-peptide levels, which mirrored insulin concentrations (Supplemental Figure 1 which can be found in an online appendix at http://diabetes.diabetesjournals.org). These
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data suggest that the relative euglycemia of Δ/Δ compared to db/db animals at four weeks of age is not due to differences in insulin secretion (since insulin and C-peptide levels are similar between genotypes, but Δ/Δ animals have decreased blood glucose at this age), but must be secondary to modest improvements in hepatic glucose output and/or insulin sensitivity. This difference is transient, however, as the increased insulin levels in older male Δ/Δ animals fail to decrease blood glucose levels relative to those observed in db/db animals. No difference in β-cell mass was detected between 12 week-old Δ/Δ and db/db mice (Supplemental Figure 2).

We additionally performed glucose-and insulin-tolerance tests (GTT and ITT, respectively) in the Δ/Δ and db/db animals (Supplemental Figure 3). While 6 week-old female Δ/Δ animals displayed a diminished hyperglycemic response to GTT relative to db/db controls, no other differences between Δ/Δ and db/db mice were observed. This suggests that the difference in glucose homeostasis between Δ/Δ and db/db animals is small and insufficient to reveal differences in the face of a substantial glucose load and/or the increased insulin resistance of advancing age.

DISCUSSION

In order to determine the potential roles for Jak2-autonomous LepRb signals in leptin action in vivo, we generated a mouse model in which LepRb is replaced by a truncation mutant (LepRb^Δ65) that contains within its intracellular domain only the sequences required to associate with and activate Jak2. We found that the hyperphagia, obesity, linear growth, ARC physiology and immune function of these Δ/Δ mice closely resembled that of entirely LepRb-deficient db/db mice. Δ/Δ and db/db animals did demonstrate some modest differences in glucose homeostasis, however: both male and female Δ/Δ mice exhibited a delayed progression to frank hyperglycemia compared to db/db mice. Taken together, these findings demonstrate that Jak2-autonomous LepRb signals may contribute modestly to the modulation of glucose homeostasis by leptin, but emphasize the necessity of signals emanating from the COOH-terminus of LepRb (beyond the Jak2-associating Box1 and Box 2 motifs) for most leptin action.

The finding that four week-old Δ/Δ mice display similar insulin and C-peptide levels as db/db animals, but exhibit improved blood glucose levels, suggests improved glucose disposal or decreased glucose production in the Δ/Δ mice independent of insulin production. This is consistent with data suggesting that CNS leptin action suppresses hepatic glucose production, and with our previous finding that some portion of this is mediated independently of Tyr^1138→STAT3 signaling (2;20;34). The Δ/Δ mice progress rapidly (by 6 weeks of age) to dramatic hyperglycemia and parameters of glucose homeostasis indistinguishable from db/db animals, however. Indeed, even at 5 weeks of age, the response to a glucose bolus is comparably poor in male Δ/Δ and db/db animals, and barely improved in female Δ/Δ compared to db/db animals. Furthermore, the increased insulin production of Δ/Δ relative to db/db males at 6 weeks of age and beyond fails to ameliorate their hyperglycemia. Thus, the improvement in glucose homeostasis mediated by Jak2-autonomous LepRb signals in Δ/Δ mice compared to db/db animals is very modest, as it is easily overwhelmed by a large glucose load and/or the increasing insulin resistance and diabetes of advancing age. No difference in β-cell mass was detected between Δ/Δ and db/db males. The mechanism(s) mediating the increased insulin production of the Δ/Δ relative to db/db males is unclear, but could represent an improvement in β cell function due either to the later time at which Δ/Δ animals become
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diabetic or due to some residual leptin action in the Δ/Δβ cell (35).

While the overall similarity of ARC gene expression and physiology between Δ/Δ and db/db mice indicates little role for Jak2-autonomous LepRb signals in ARC leptin action, the finding of decreased (worsened) Pomp mRNA expression in Δ/Δ compared to db/db mice was surprising. One possible explanation for this observation is that some residual signal mediated by LepRbΔ65 modestly attenuates Pomp expression, and that this attenuating signal is overwhelmed under normal circumstances by the LepRb signals that enhance Pomp expression. Unfortunately, the low Pomp content of db/db and Δ/Δ animals and low activity of these neurons at baseline rendered the examination of POMC c-fos uninformative.

While the molecular mechanisms underlying Jak2-autonomous LepRb action remain unclear, several pathways could contribute. In cultured cells, the activation of Jak2 by LepRbΔ65 and similar receptor mutants mediates some activation of the ERK pathway (13;36). Indeed, chemical inhibitor studies have suggested a role for ERK signaling in the regulation of autonomic nervous system function by leptin, and the autonomic nervous system underlies a major component of the leptin effect on glucose homeostasis (37). Phosphatidylinositol-3 kinase (PI3-K) also plays a role in the regulation of glucose homeostasis by leptin (38-41). As we have been unable to observe the regulation of PI3-K by leptin in cultured cells and the analysis of this pathway in the hypothalami of obese, hyperleptinemic mice remains problematic, the molecular mechanism by which LepRb engages this pathway remains unclear, however. Although difficult to test directly, it is thus possible that Jak2-autonomous LepRb signals might modulate this pathway in vivo. We have previously demonstrated that the major regulation of hypothalamic mTOR, including in response to nutritional alteration, occurs indirectly, via neuronal activation (42). We examined this pathway (along with the phosphorylation of STAT3) in the hypothalamus of Δ/Δ animals (Supplemental Figure 4), revealing the expected absence of STAT3 signaling and increased mTOR activity (secondary to the activation of orexigenic neurons) in the mediobasal hypothalamus of db/db and Δ/Δ animals. Thus, the regulation of mTOR is similar in these mouse models.

A potential intermediate linking Jak2 activation to PI3-K activation is SH2B1, a SH2-domain containing protein that binds phosphorylated Tyr813 on Jak2 (43). Cell culture studies show that SH2B1 binds directly to Jak2, augments its kinase activity and couples leptin stimulation to insulin receptor substrate (IRS) activation, a well-known activator of PI3-K (44). Indeed, SH2B1 null mice display hyperphagia, obesity and diabetes (45). SH2B1 could also mediate other, unknown, Jak2-autonomous LepRb signals.

Importantly, the phenotype of mice expressing LepRbΔ65 differs significantly from a mouse model in which LeprY123F (mutated for the three tyrosine phosphorylation sites, but with an otherwise intact intracellular domain) replaces LepRb (22). Unlike Δ/Δ animals, these LeprY123F mice display improved energy homeostasis and more dramatic improvements in glucose homeostasis (both of which are sustained into adulthood), compared to db/db animals. Unfortunately, the C57 genetic background strain utilized to study the LeprY123F mice differs not only compared to the C57Bl/6J background that we employed to study our Δ/Δ and db/db animals, but also diverges from that of the db/db animals used as comparators for the LeprY123F mice (22). Similarly, it is also possible that minor differences between the incipient C57Bl/6J backgrounds of db/db and Δ/Δ mice could contribute to the modest
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distinctions observed between these two models.

Aside from background strain, the intriguing possibility arises that the intracellular domain of LepRb may mediate heretofore unsuspected signals independently from tyrosine phosphorylation. As it is, future studies will be needed to carefully compare the phenotype of Lepr$^{Y123F}$, ∆/∆, and db/db animals within the same facility and on the same genetic background; should these differences remain, further work will be necessary to confirm the importance and determine the identity of the underlying signaling.

In summary, our findings reveal the insufficiency of Jak2-autonomous LepRb signals for the bulk of leptin action. These finding do not rule out the possibility that Jak2-autonomous signals may be required to support the action of LepRb phosphorylation, however. Indeed, our present findings suggest a modest role for Jak2-autonomous LepRb signals in the regulation of glucose homeostasis by leptin. Understanding collaborative roles for Jak2-autonomous signals in leptin action and deciphering the mechanisms underlying these signals and potential tyrosine phosphorylation independent signals mediated by the COOH-terminus of LepRb will represent important directions for future research.

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**Table 1.** Phenotypic Data for Mice Expressing Mutant LepRb. Fat and lean content were determined at 10 weeks and are expressed as a percentage of body weight. Body temperature was determined at 11 weeks. Snout-anus length, femur length and femur weight were determined at 9 weeks. Data are plotted as mean +/- SEM; *p<0.05 compared to WT by Student’s unpaired, two-tailed t test. Sample size noted in parentheses.

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<th>Genotype</th>
<th>WT</th>
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<th>∆/∆</th>
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<td>Fat Content (%)</td>
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<td>Femur Mass (mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>42.4 ± 0.6 (9)</td>
<td>34.8 ± 0.8* (8)</td>
<td>35.3 ± 0.9* (9)</td>
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**Table 2.** Fertility Data for Mice Expressing Mutant LepRb. Single-housed females were examined daily from 4 to 8 weeks of age. Cytological examination of vaginal lavage was used to monitor estrus. Females were paired with one wild-type male and monitored daily for 6 weeks for the production of offspring.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>db/db</th>
<th>∆/∆</th>
<th>s/s</th>
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<tbody>
<tr>
<td>Estrus</td>
<td>8/9</td>
<td>0/9</td>
<td>0/8</td>
<td>3/8</td>
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<td>Litters</td>
<td>9/9</td>
<td>0/7</td>
<td>0/8</td>
<td>5/8</td>
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Figure 1. Generation of mice expressing LepRb\textsuperscript{∆65}. A) HEK293 cells were transfected with plasmids encoding the indicated lepR isoforms, made quiescent overnight, incubated in the absence (-) or presence (+) of leptin (625 ng/mL) for 15 minutes before lysis, and immunoprecipitated with αJak2 (24). Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. The figures shown are typical of multiple independent experiments. B) Diagram of gene-targeting strategy to replace wild-type exon 18b with that encoding the COOH-terminally truncated LepRb\textsuperscript{∆65}. C) Southern blotting of control (WT) and correctly targeted (D/+, C1, C2) Lepr\textsuperscript{∆65} ES lines, using a Lepr-specific probe. M indicated marker lane. D) Image of gel electrophoresis of Lepr-specific RT-PCR products from hypothalamic mRNA of 5 wild-type and 5 ∆/∆ animals.

Figure 2. Similar hyperphagia and obesity in ∆/∆ and db/db mice. WT (black squares), db/db (white circles) and ∆/∆ (black triangles) mice of the indicated age (n = 8-10 per genotype), were weaned at 4 weeks and (A-B) body weight and (C-D) food intake were monitored weekly from 4 to 8 weeks of age. Food intake represents cumulative food intake over the time-course. Data are plotted as mean +/-SEM. *p<0.05 compared to WT by one-way ANOVA and Tukey’s post test.

Figure 3. ARC neuropeptide expression and AgRP neuron c-fos-IR in WT, db/db and ∆/∆ mice. (A-C) mRNA was prepared from the hypothalami of 10-11 week old male mice. qPCR was used to determine (A) Pomc, (B) Npy and (C) Agrp mRNA levels (n = 14-19 per genotype). D) C-fos-IR in AgRP neurons of ad libitum-fed WT, db/db, and ∆/∆ animals. All mouse groups were bred onto a background expressing LacZ under the AgRP promoter, enabling the identification of AgRP neurons by staining for β-Gal. Representative images showing immunofluorescent detection of c-Fos (top), β-Gal (middle), and merged c-Fos/ β-Gal (bottom). E) Quantification of double-labeled c-Fos/AgRP-IR neurons. Double-labeled AgRP neurons are plotted as percentage of total AgRP neurons +/-SEM. (A-C, E) *P<0.05 compared to WT;†P<0.05 compared to db/db by one-way ANOVA with Tukey’s post test.

Figure 4. Reduced numbers and proliferation of splenic T cells in ∆/∆ and ob/ob, but not s/s or l/l mice. Spleens were isolated from the indicated genotypes of male mice, separated using autoMACS and counted for (A) total splenocytes and (B) CD4+ cells using a flow cytometer (n = 7-22 per genotype). Data are plotted as mean +/-SEM; *p<0.05 compared to WT by one-way ANOVA and Tukey’s post test. C) For proliferation assays, CD4+CD25- Naïve T cells were isolated by autoMACS (n = 4-7 per genotype), incubated in the presence of bone marrow–derived dendritic cells from C57BL/6J mice, and stimulated with anti-CD3e. Incorporation of H\textsuperscript{3}-thymidine (1 mCi/well) by proliferating cells was measured during the last 6 hours of culture. Proliferation is expressed as a percentage of a paired WT sample analyzed concurrently (dashed line), and are plotted as mean +/-SEM; *p<0.05 by one-way ANOVA and Tukey’s post test.

Figure 5. Delayed onset of hyperglycemia in ∆/∆ compared to db/db mice. A-D) Blood glucose was determined for ad libitum-fed (A-B) or fasted (5 hrs) (C-D) animals of the indicated genotype (WT (black squares), db/db (white circles) and ∆/∆ (black triangles)) and sex at the indicated ages (n = 8-12 per genotype). (E-F) Serum was collected from mice of the indicated genotype and sex at the indicated ages (n = 8-10 per genotype), and insulin content was determined by ELISA. All panels: Data are plotted as mean +/-SEM; db/db *p<0.05 compared to ∆/∆ at the indicated time-points by one-way ANOVA and Tukey’s post test.
Role of Jak2 in leptin action

Fig 1A

IP: αJak2(475)

Leptin: - + - + - + - +

αY1007/8

αJAK2

Fig 1B

Linearized targeting vector

LepR gene

Correctly targeted mutation

STOP

Exon 18b Δ65

Neo

TK

STOP

Exon 18b

STOP

Exon 18b Δ65

Neo

Recombination
Role of Jak2 in leptin action

Fig 1C

<table>
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<th>C2</th>
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![Image of gel electrophoresis with LepRΔ65 labeled]

Fig 1D

<table>
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<th>Δ/Δ</th>
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</thead>
</table>

![Image of gel electrophoresis]
Fig 2A

Fig 2B
Role of Jak2 in leptin action

Fig 2C

![Graph showing cumulative food intake for males](image)

Fig 2D

![Graph showing cumulative food intake for females](image)

Cumulative Food Intake (gm)

Age (weeks)
Fig 3A

![Graph showing Pomc mRNA Level comparison between WT, db/db, and Δ/Δ genotypes.]

Fig 3B

![Graph showing Agrp mRNA Level comparison between WT, db/db, and Δ/Δ genotypes.]

* indicates statistical significance compared to WT, † indicates additional significance compared to db/db.
Role of Jak2 in leptin action

**Fig 3C**

![Graph showing Npy mRNA Level](image)

**Fig 3D**

![Images of WT, db/db, Δ/Δ showing c-fos expression](image)

**Fig 3E**

![Graph showing Colocalized c-fos in AgRP Neurons (%)](image)
Role of Jak2 in leptin action

**Fig 4A**

![Bar chart showing total splenocytes](chart1.png)

**Fig 4B**

![Bar chart showing CD4+ cell number](chart2.png)

**Fig 4C**

![Bar chart showing CPM (CPM % WT)](chart3.png)
Role of Jak2 in leptin action

![Graph showing blood glucose levels in males and females over weeks.](image)

**Fig 5A**

**Fig 5B**
Role of Jak2 in leptin action

Fig 5E

![Graph showing insulin levels in males over age (weeks) for Db/Db, Δ/Δ, and WT groups.]

Fig 5F

![Graph showing insulin levels in females over age (weeks) for Db/Db, Δ/Δ, and WT groups.]

* *