Original Article

MOLECULAR METABOLISM

Identification of the leptin receptor sequences crucial for the STAT3-Independent control of metabolism

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ABSTRACT

Background: Leptin acts via its receptor, LepRb, on specialized neurons in the brain to modulate energy balance and glucose homeostasis. LepRb→STAT3 signaling plays a crucial role in leptin action, but LepRb also mediates an additional as-yet-unidentified signal (Signal 2) that is important for leptin action. Signal 2 requires LepRb regions in addition to those required for JAK2 activation but operates independently of STAT3 and LepRb phosphorylation sites.

Methods: To identify LepRb sequences that mediate Signal 2, we used CRISPR/Cas9 to generate five novel mouse lines containing COOH-terminal truncation mutants of LepRb. We analyzed the metabolic phenotype and measures of hypothalamic function for these mouse lines.

Results: We found that deletion of LepRb sequences between residues 921 and 960 dramatically worsens metabolic control and alters hypothalamic function relative to smaller truncations. We also found that deletion of the regions including residues 1013–1053 and 960–1013 each decreased obesity compared to deletions that included additional COOH-terminal residues.

Conclusions: LepRb sequences between residues 921 and 960 mediate the STAT3 and LepRb phosphorylation-independent second signal that contributes to the control of energy balance and metabolism by leptin/LepRb. In addition to confirming the inhibitory role of the region (residues 961–1013) containing Tyr_{985}, we also identified the region containing residues 1013–1053 (which contains no Tyr residues) as a second potential mediator of LepRb inhibition. Thus, the intracellular domain of LepRb mediates multiple Tyr-independent signals.

Keywords Leptin receptor; STAT3; Truncation mutant; Obesity; Diabetes

1. INTRODUCTION

Metabolic diseases, such as obesity and type 2 diabetes, affect over one-third of the adult population in developed countries, leading to significant morbidity and mortality [1]. Unfortunately, the etiology of obesity and T2D remain poorly understood, and most current medical therapies fail to permanently reverse these disorders. It is important to understand the mechanisms that govern food intake and energy balance to enable the rational design of new and more effective treatments.

Leptin, a peptide hormone produced by white adipose tissue in proportion to energy stores, plays a central role in the control of feeding and energy balance [2,3]. Leptin acts via its receptor (LepRb) on hypothalamic neurons to decrease food intake and permit energy expenditure [4]. Thus, mice null for leptin or LepRb (*ob/ob* and *db/db* mice, respectively) are hyperphagic, obese, and prone to hyperglycemia and insulin resistance [3].

LepRb is a member of the interleukin (IL)-6 receptor family of cytokine receptors, which signal via a Janus family tyrosine kinase (JAK2 in the case of LepRb) that is associated with the receptor intracellular domain [3,5]. The first 48 intracellular amino acids of LepRb (residues 861–908) mediate the binding and activation of Jak2 [6]. Activated JAK2 phosphorylates three intracellular LepRb tyrosine residues (Tyr_{985}, Tyr_{1077}, and Tyr_{1138}), each of which recruits specific effector proteins to mediate downstream signaling [7,8]. Like other cytokine receptors, the activation of signal transducer and activator of transcription (STAT) transcription factor family members figure prominently in LepRb signaling: Tyr_{1138} recruits STAT3 [8,9], and mice containing substitution mutations of LepRb Tyr_{1138} ($LepRb^{Y1138MUT}$ mice) or lacking STAT3 in LepRb neurons display dramatic hyperphagia and obesity

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[10–12]. Leptin action remains partially preserved in LepRb^{Y1138MUT} and Stat3^{LepRb} KO mice; however, these mice are less obese and diabetic than *ob/ob* and *db/db* mice [10,13,14]. Thus, while Tyr_{1138} and STAT3 are crucial for leptin action, an unidentified second LepRb signaling pathway (Signal 2) that is independent of Tyr_{1138} and STAT3 must also play an important role in physiologic leptin action.

Previous results demonstrated that other STAT proteins, including STAT1 and STAT5, do not contribute meaningfully to leptin action *in vivo* [15]. Neither do other LepRb tyrosine phosphorylation sites mediate Signal 2: Tyr_{985} (which recruits protein tyrosine phosphatase 2 (SHP2; PTPN1) [8] and the cytokine signaling inhibitor SOCS3 [16]) contribute to the feedback inhibition of LepRb signaling, but are not otherwise involved in the control of energy balance and metabolism [17]. Furthermore, not only does Tyr_{1077} (which recruits STAT5) contribute negligibly to leptin *in vivo*, but also a LepRb mutant devoid of all tyrosine phosphorylation sites retains some ability to control body weight and metabolism *in vivo* [11]. Thus, LepRb mediates Signal 2 to control metabolism independently of STAT signaling and LepRb tyrosine phosphorylation sites. Furthermore, we previously showed that signaling by LepRb-associated JAK2 alone fails to preserve any physiologic leptin action [18], suggesting that Signal 2 must be mediated by LepRb sequences COOH-terminal to the juxtamembrane JAK2-binding region.

Because there is no *in vitro* assay to detect Signal 2, we used CRISPR/Cas9-mediated mutagenesis to generate a panel of mouse lines containing COOH-terminal truncations of LepRb. By studying these five novel mouse lines, we identified a region of the intracellular LepRb that is required to mediate Signal 2 in addition to identifying a region that mediates a previously undescribed LepRb inhibitory signal.

2. MATERIALS AND METHODS

Animals. All of the procedures conducted on the animals were approved by the University of Michigan Institutional Committee on the Care and Use of Animals and were in accordance with AAALAC and NIH guidelines. All mice were bred in our colony in the Unit for Laboratory Animal Management at the University of Michigan. All mice were provided with food and water *ad libitum* and housed in temperature-controlled rooms on a 12-hour light–dark cycle.

CRISPR/Cas9 technology was utilized to generate all *Lepr* truncation mutant mouse lines. *Lepr*$^{1083\Delta}$, *Lepr*$^{1053\Delta}$, and *Lepr*$^{1013\Delta}$ were all generated by template-free random insertion/deletion by Cas9-mediated cleavage followed by non-homologous end-joining. *Lepr*$^{921\Delta}$ and *Lepr*$^{960\Delta}$ were generated using a single-stranded DNA (ssDNA) editing template to direct homologous recombination for insertion of a premature stop codon followed immediately by an EcoRI restriction motif (for screening purposes) immediately following Ser_{921} or Ser_{960}, respectively. The guide RNA (gRNA) design was performed using crispr.mit.edu and https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design. Both ssDNA editing templates and oligonucleotides containing the guide sequence and appropriate sticky ends for cloning were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Oligos corresponding to the gRNA sequences were phosphorylated, annealed, and subcloned into the linearized pX330 vector (which contained the sgRNA scaffold component as well as encoding Cas9) as described [19]. The gRNA sequences inserted into pX330 were as follows: *Lepr*$^{921\Delta}$-aaatcagtgtcgatacagct, *Lepr*$^{960\Delta}$-agaagttagcactgttacac, *Lepr*$^{1013\Delta}$-gtaatcattccccactgagg, *Lepr*$^{1053\Delta}$-acaactttcattctcggggt, and *Lepr*$^{1083\Delta}$-ccgtcaacagaagagagagt.

The finished pX330-derived plasmids were sent to the University of Michigan Transgenic Animal Model Core to be separately microinjected alone (or with a ssDNA oligonucleotide editing template in the case of *Lepr*$^{960\Delta}$ and *Lepr*$^{921\Delta}$) into fertilized mouse embryos, which were then implanted into pseudopregnant female donor mice. The editing template sequences were:

960Δ: 5′gagatggtcccagcagctatggtctccctttcttttgaccacaccagaccctgaaagcagttctatttgtattagtgaccagtgtaacagtTAGGAATTCGctaacttctctgggtctcagagcacccaggtaacctgtgaggatgagtgtcagagacaaccctcagttaaatatgcaactctggtcagcaacgataaact3′

and

921Δ: 5′ctgaaacatttgagcatctttttaccaagcatgcagaatcagtgatatttggtcctcttcttctggagcctgaacccatttcagaag*aaatTagC*TGAgaattcTGA*gtAgaCacGgct*tggaaaaataaagatgagatggtcccagcagctatggtctcccttctttgaccacaccagaccctgaaagcagttctatt3′ (note that the capitalized residues represent changes from the native sequence).

Genomic DNA sequences surrounding and including the expected Cas9/sgRNA-mediated cut sites were PCR-amplified for each potential founder and were screened for gene editing; potentially useful mutations were sequenced. Following screening, founder mice were bred to C57/Bl6 mice (Jackson Laboratories, Bar Harbor, ME, USA) to obtain germline transmission of the mutations; the sequence of each germline mutation was reconfirmed by sequencing. Heterozygous animals were intercrossed to generate homozygous and wild-type mice for study.

Longitudinal analyses. The mice were singly housed from the time of weaning and their body weight and food intake were measured weekly; their blood was collected biweekly by submandibular bleed, and their blood glucose was measured using a One Touch Ultra glucometer. At week 13, body composition analysis was performed using a Bruker MiniSpec LF90II at the Michigan Mouse Metabolic Phenotyping Center. Their serum was collected at weeks 12–13 for leptin and insulin assessment, and their snout-anus length and femur length were measured using calipers at week 13 prior to either perfusion for immunohistochemistry or hypothalamic dissection for gene expression analysis.

Perfusion and immunohistochemistry. Following week 13 assessment of their body composition, the *ad libitum*-fed mice were anesthetized with sodium pentobarbital and perfused transcardially with formalin. Their brains were removed and processed as described previously [20]. Hypothalamic sections were sectioned coronally at 30 μM on a freezing microtome and distributed into four series for analysis. The sections were pretreated with 1% hydrogen peroxide, blocked with 3% donkey serum, and subsequent washes were performed with PBS with 0.3% glycine and 0.03% SDS. The sections were incubated overnight at room temperature with rabbit anti-cFos (Santa Cruz, sc-52; 1:1000) and exposed the subsequent day with biotinylated (1:200 followed by ABC amplification and DAB reaction) donkey anti-rabbit biotin secondary antibody. The stained sections were mounted on glass slides and coverslipped with Vectashield mounting media (Vector Labs). An Olympus BX-51 microscope was used for image capture of FOS-IR. FOS-IR cells were counted from both sides of matched sections using ImageJ software, and the data were expressed as raw counts from each 1:4 series.

Hypothalamic gene expression. Following week 13 assessment of their body composition, a separate *ad libitum*-fed cohort of mice was anesthetized using isoflurane, decapitated, and the whole hypothalamus was rapidly dissected on ice using a matrix and ice-cold razor blades. Hypothalamic tissue was frozen on dry ice and stored at −80°C until the entire cohort of mice was collected. TRIzol (Invitrogen) extraction was used for RNA isolation, and the RNA was subsequently stored at −80°C. cDNA was prepared from 1 μg RNA using an iScript cDNA Synthesis Kit (Bio-Rad) and stored at −20°C. *Gapdh* and target genes were analyzed via TaqMan assay (Applied

Biosystems) on an Applied Biosystems 7500 Real-Time PCR system. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Hormone analysis. The Chemistry Laboratory of the Michigan Diabetes Research Center Clinical Core employed ELISA kits from Crystal Chem for the assessment of insulin (Ultra-Sensitive Mouse Insulin ELISA) or leptin (Mouse Leptin ELISA). Samples from obese mice were diluted 1:10 prior to assay to ensure readings within the appropriate spectroscopic range.

Statistics. The body weight and blood glucose data over multiple time points was analyzed by two-way repeated measures ANOVA with Fisher's LSD *post hoc* test. One-way ANOVA was used to analyze differences among the genotypes. The threshold for determining significance was $p \leq 0.05$. All statistical analyses were conducted using GraphPad Prism.

3. RESULTS

3.1. Generation of mice carrying LepRb truncation mutants

To generate COOH-terminal LepRb truncation mutations, we initially employed CRISPR/Cas9-mediated cleavage of the *Lepr* gene and relied on non-homologous end-joining to generate mutations that would frame-shift the *Lepr* coding sequences and truncate LepRb following Val_{1083}, Ser_{1053}, or Ser_{1013} (Figure 1). The resultant mutant alleles ($Lepr^{1083\Delta}$, $Lepr^{1053\Delta}$, and $Lepr^{1013\Delta}$, respectively) thus each contained 2–6 non-native residues at their COOH-termini. We subsequently generated $Lepr^{960\Delta}$ and $Lepr^{921\Delta}$ utilizing CRISPR/Cas9-mediated cleavage in the presence of single-stranded DNA homologous repair templates to introduce STOP codons immediately downstream of Ser_{960} and Ser_{921}, respectively (Figure 1). All of these mutants contained the sequences required for JAK2 activation [6,18] but lacked Tyr_{1138} (the STAT3 recruitment site [8,21,22]). Thus, each mutant could have affected physiology only via non-STAT3 signals, such that the more severe metabolic phenotype of a mutant strain relative to those retaining more COOH-terminal sequences identified the region required for the action of Signal 2.

3.2. Energy balance phenotype of LepRb mutant mice

We began by examining the energy balance phenotypes of the mice of both sexes homozygous for mutant or wild-type *Lepr* alleles for each line (Figures 2-3). Because the wild–type (WT) mice of each strain demonstrated identical phenotypes (Supplemental Figs. 1–2), we present data combined from the WT mice of all of the strains to simplify the presentation of the data in each manuscript figure.

The mice of both sexes homozygous for $Lepr^{1083\Delta}$ (1083Δ mice) displayed increased body weight and food intake relative to the WT animals (Figure 2A–B and 3A-B). Most of the increased body weight represented increased fat mass, with commensurate increases in circulating leptin concentrations. This phenotype was consistent with previous findings that demonstrated increased food intake, adipose mass, and body weight in mice mutant for Tyr_{1138} or STAT3 in LepRb neurons [10,13].

None of the measured energy balance phenotypes of the mice homozygous for $Lepr^{1053\Delta}$ (1053Δ mice) were different from those of the 1083Δ mice (Figures 2, 3), revealing that the sequences contained within LepRb residues 1054–1083 were not required for Signal 2. This was consistent with previous findings suggesting that LepRb Tyr_{1077} contributes little to leptin action *in vivo* [11,23].

Interestingly, the female mice homozygous for $Lepr^{1013\Delta}$ and $Lepr^{960\Delta}$ (1013Δ and 960Δ mice, respectively) displayed decreased body mass and food intake compared to the 1053Δ and 1083Δ mice (Figure 2A–B and 3A-B). Similarly, the male 1013Δ and 960Δ mice displayed decreased body weight and a trend toward decreased adiposity and food intake relative to the 1053Δ and 1083Δ males.

LepRb Tyr_{985} mediates feedback inhibition on leptin signaling, and mutation of Tyr_{985} decreases body weight, food intake, and adiposity in mice [16,17]. Thus, it is not surprising that the 960Δ mice displayed ameliorated obesity compared to the other lines. In contrast, the unexpectedly decreased body weight and adiposity in the 1013Δ mice suggested the presence of a previously unsuspected inhibitory signal contained within LepRb residues 1014–1053. Furthermore, the finding that body weight, food intake, and fat mass tended to be decreased and that leptin concentrations were significantly lower in the

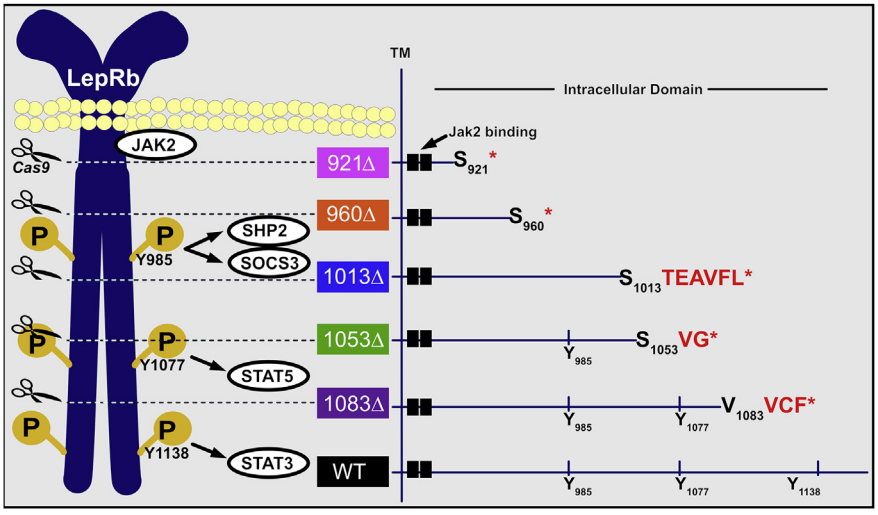

Figure 1: Schematic diagram of the generated and analyzed LepRb mutants. The left-hand portion of the diagram shows an illustration of LepRb, including the transmembrane region (covered by pale yellow circles representing the lipid bilayer), the JAK2-binding region, the tyrosine phosphorylation (PY) sites and their downstream signaling mediators, and the Cas9 cut sites at which the receptor was truncated in the indicated mutant strains. The right-hand portion of the diagram shows a schematic of the transmembrane (TM) domain and the remaining intracellular domain for each mutant, including the JAK2-binding sites (black boxes), remaining Tyr residues (Y), and terminal native residue plus any additional non-native amino acids (in red).

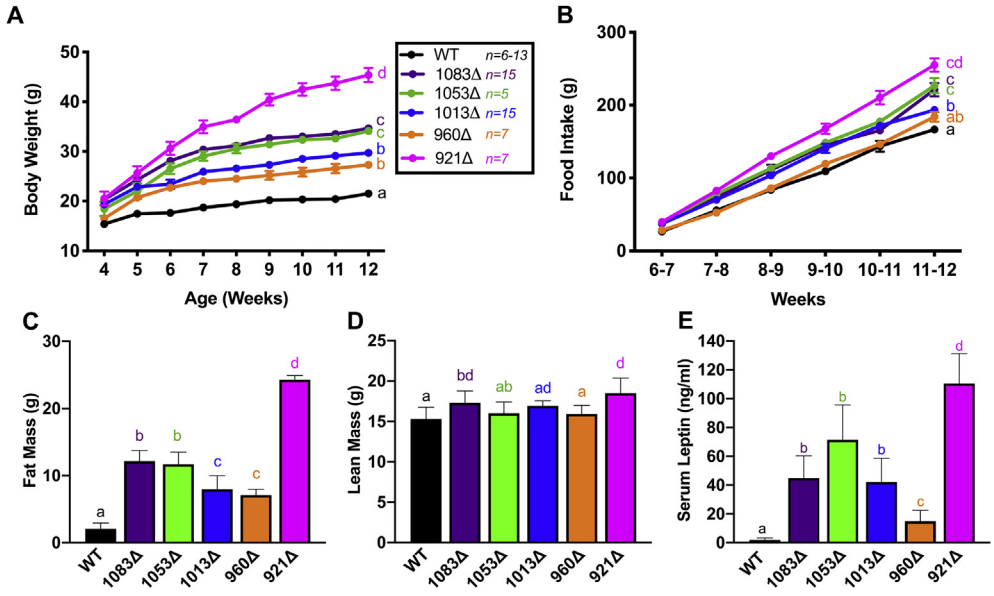

Figure 2: Adiposity phenotypes of the female mice with altered LepRb. (A) Body weight of the female mice from each strain from 4 to 12 weeks of age. **(B)** Cumulative food intake from weeks 4–12 of the mice from each strain. **(C–E)** Fat mass (C), lean mass (D), and serum leptin concentrations (E) of the mice at 12–13 weeks of age for each strain. All of the panels show mean +/− SEM; n for each strain is given in the key. Lines or bars with different letters indicate differences at $p<0.05$ by ANOVA.

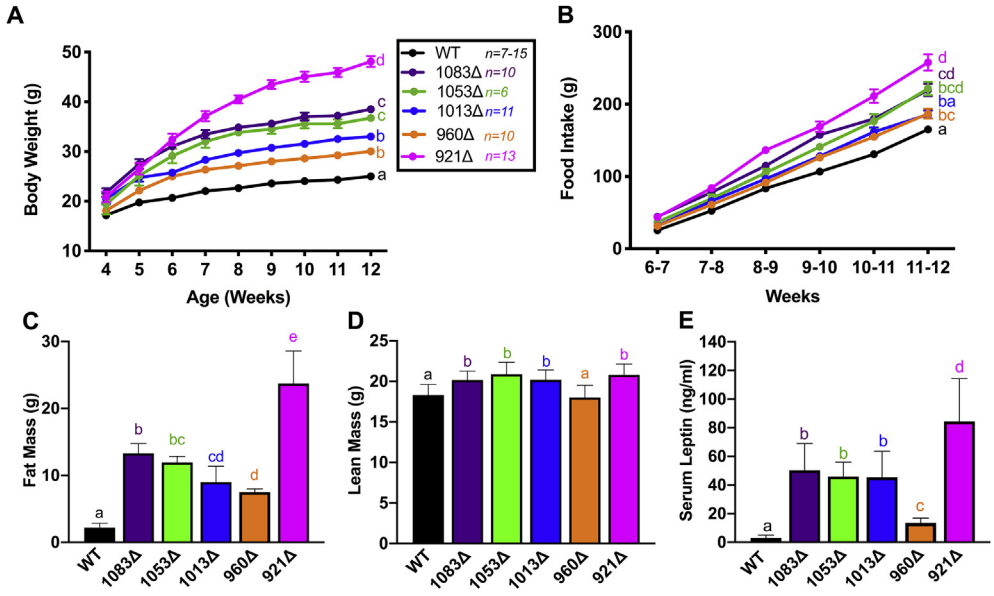

Figure 3: Adiposity phenotypes of the male mice with altered LepRb. (A) Body weight of the male mice from each strain from 4 to 12 weeks of age. **(B)** Cumulative food intake from weeks 4–12 of the mice from each strain. **(C–E)** Fat mass (C), lean mass (D), and serum leptin concentrations (E) of the mice at 12–13 weeks of age for each strain. All of the panels show mean +/− SEM; n for each strain is given in the key. Lines or bars with different letters indicate differences at $p<0.05$ by ANOVA.

960Δ mice than the 1013Δ mice is consistent with an independent inhibitory effect of Tyr_{985} relative to the 1014–1053 region. Thus, the intracellular domain of LepRb appeared to contain two inhibitory signals, Tyr_{985} and an unknown and previously unsuspected signal contained within LepRb residues 1014–1053.

Both the female and male mice homozygous for *Lepr*$^{921\Delta}$ (921Δ mice) displayed dramatically increased body weight, food intake, adiposity, and leptin concentrations relative to all of the other lines (Figures 2-3), consistent with the absence of Signal 2 on this LepRb isoform and the previously described phenotype of *Lepr*$^{924\Delta}$ mice [18]. Thus, LepRb sequences contained within residues 925–960 must have been required to mediate Signal 2. Note that because of the obesity and attendant severe hyperleptinemia in each of the deletion lines, the leptin action was likely maximal in these animals.

3.3. Glucose homeostasis in LepRb mutant mice

Because Tyr_{1138}/STAT3-independent signals are also required for the control of glucose homeostasis by leptin [10,14], we longitudinally examined the blood glucose and insulin concentrations in the *ad libitum*-fed mice of all of the strains (Figure 4). As with the body

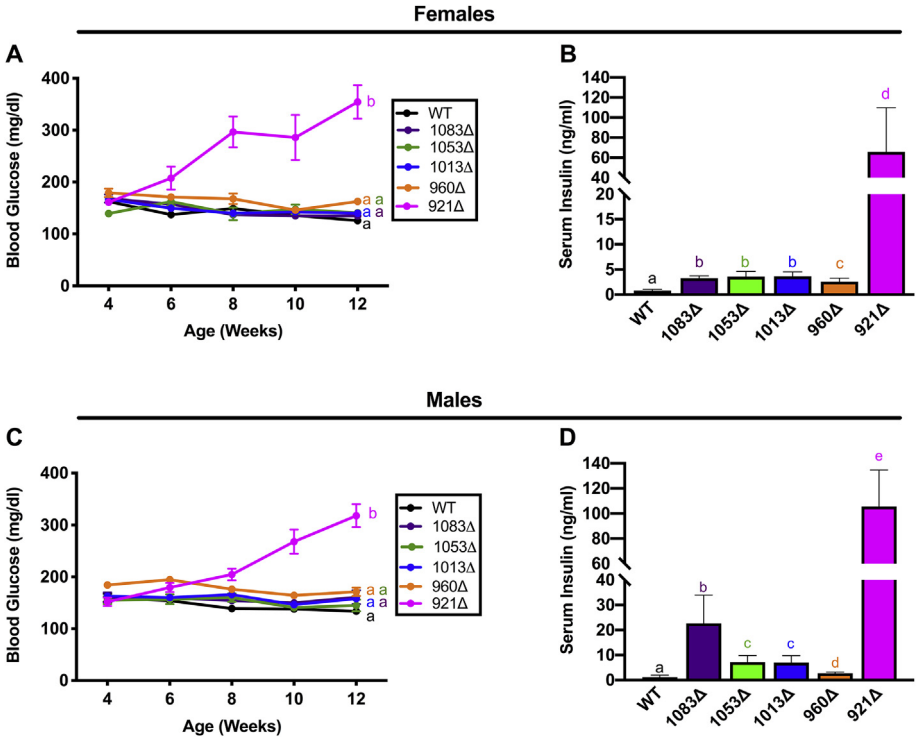

Figure 4: Glucose homeostasis phenotypes of the mice with altered LepRb. (A, C) Blood glucose of the female (A) and male (C) mice of each strain measured from 4 to 12 weeks of age. **(B, D)** Serum insulin concentrations of the female (B) and male (D) mice measured at 12–13 weeks of age for each strain. All of the panels show mean +/− SEM. Lines or bars with different letters indicate differences at $p<0.05$ by ANOVA.

weight, food intake, and measures of energy balance, we observed no differences in any parameters among the WT controls of all of the strains (Supplemental Fig. 3). Although the insulin concentrations were increased in the male 1083Δ mice compared to the 1053Δ and 1013Δ animals, we found that all of the strains, with the exception of 921Δ, maintained normoglycemia through at least 12 weeks of age. The insulin concentrations were similarly elevated compared to the WT animals in the females of the 1083Δ, 1053Δ, and 1013Δ strains (commensurate with their increased adiposity). In contrast, the insulin concentrations tended to be lower in the 960Δ mice compared to the other mutant strains, consistent with the improved energy balance phenotype of these mice relative to the other strains.

In addition to hyperglycemia, the 921Δ mice of both sexes demonstrated much higher circulating insulin concentrations compared to the other strains, consistent with the more dramatically impaired glucose homeostasis in the mice lacking Signal 2 than the mice mutant only for Tyr_{1138}/STAT3 signaling [11,14]. Thus, the LepRb region containing residues 922–960 mediated the STAT3-independent signal required for the control of glucose homeostasis.

3.4. Control of ARC neurons in LepRb mutant mice

To determine how Signal 2 might impact hypothalamic physiology to modulate energy balance and glycemic control, we examined the control of neuronal activity and gene expression in the hypothalamic arcuate nucleus (ARC) of our mouse models (Figures 5-6). Prior research demonstrated that the absence of leptin or LepRb signaling increases the activity of ARC neurons that contain neuropeptide Y (Npy), agouti-related peptide (Agrp), and gamma aminobutyric acid (GABA) (NAG neurons) [3,24,25]. Increased NAG neuron activity can be monitored by examining FOS-immunoreactivity (IR) in the medial basal ARC (mbARC) [20]. Furthermore, while mutation of Tyr_{1138} increases mbARC FOS-IR and the activity of NAG neurons, the complete ablation of LepRb further augments these parameters, suggesting that Signal 2 may contribute to the suppression of NAG neuron activity [20]. We thus examined mbARC FOS-IR in our mutant lines, revealing that all of our mutant lines, with the exception of the 960Δ mice, demonstrated increased mbARC FOS-IR relative to the WT controls and that mbARC FOS-IR increased in the 921Δ mice to a greater extent than in the other lines. Furthermore, the 960Δ mice demonstrated decreased mbARC FOS-IR than the other mutant strains, suggesting that interfering with the inhibitory signal in this region enhances the ability of LepRb to STAT3-independently suppress that activity of NAG neurons. Hence, the suppression of mbARC FOS-IR by LepRb mutants correlates with the ability of the LepRb isoform to control food intake, body weight, and glucose homeostasis.

We also dissected the hypothalami from the WT, 1013Δ, 960Δ, and 921Δ mice and prepared RNA for the quantification of *Pomc*, *Cart*, *Agrp*, and *Npy* mRNA by qPCR (Figure 6). While we observed no differences in *Npy* expression by genotype, *Agrp* was similarly increased, and *Pomc* was similarly decreased in all of the examined mutants, consistent with the known role of STAT3 in the control of these genes [10,18]. Interestingly, *Cart* expression was only diminished in the 921Δ line, however, suggesting a specific role for Signal 2 in the control of this gene.

4. DISCUSSION

By generating and studying the physiologic and hypothalamic phenotypes of five novel LepRb truncation mutants, we identified two regions of the LepRb intracellular domain that are required to mediate

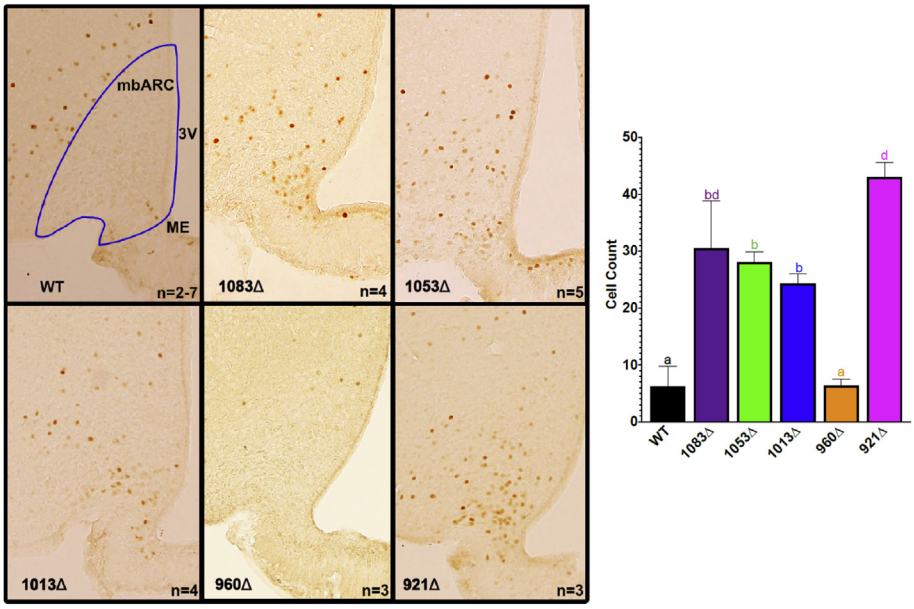

Figure 5: Quantification of mbARC FOS-IR of the mice with altered LepRb. Panels on the left show representative images of mbARC FOS-IR (brown nuclei) in the mice of the indicated genotypes. FOS-IR cells were counted from both sides of the matched sections using ImageJ software, and the raw counts are shown as mean +/-SEM; the sample size for each group is shown in the corresponding panel. Bars with different letters indicate differences at $p<0.05$ by ANOVA.

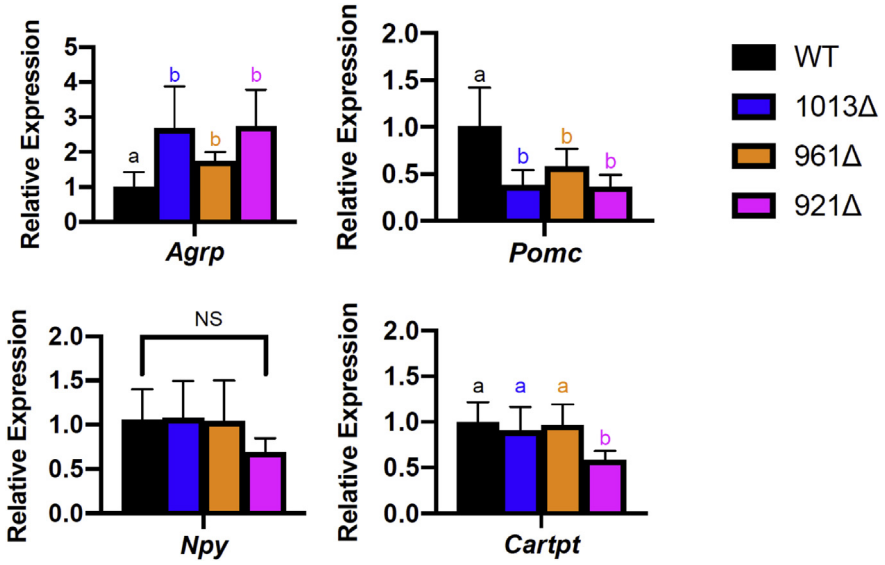

Figure 6: Hypothalamic gene expression of the male mice with altered LepRb. The hypothalami of the male mice of the indicated strains were dissected and snap frozen. RNA was isolated, converted into cDNA, and samples were subjected to qPCR to determine the expression of the indicated mRNA for the indicated mouse strains. Expression values for each strain were normalized to their own controls to permit comparisons among samples run on different plates. Controls are shown aggregated in the graphs. All panels show mean +/− SEM; n = 8 for 921Δ, 8 for 1013Δ, and 6 for 960Δ, and between 5 and 9 for the WT controls. Bars with different letters indicate differences at $p<0.05$ by ANOVA.

Tyr-independent effects on leptin action (Figure 7). In addition to identifying a region (within residues 922–960) that is required to mediate the STAT3/Tyr-independent control of energy balance, we identified a previously unsuspected region (within residues 1014–1053) that decreases LepRb action. Going forward, it will be important to study these LepRb regions to determine their cellular mechanisms of action and their direct effects on neurophysiology. Note that all mutations studied were within the LepRb-specific exon away from the splicing sequences and were unlikely to affect the expression of the "short" LepRb isoforms.

The LepRb region that we have identified as being important to mediate Signal 2 is largely conserved among mammalian species (Supplemental Fig. 4). At this stage, we cannot know whether this region recruits a specific downstream signaling molecule or participates in receptor trafficking and/or some other cellular function important for signal propagation. Furthermore, it is possible that the important motif(s) within residues 922–960 are redundant with other sequences further COOH-terminal to this region. For instance, LepRb might contain multiple sequences that could mediate receptor trafficking, with the one(s) contained within residues 922–960 simply

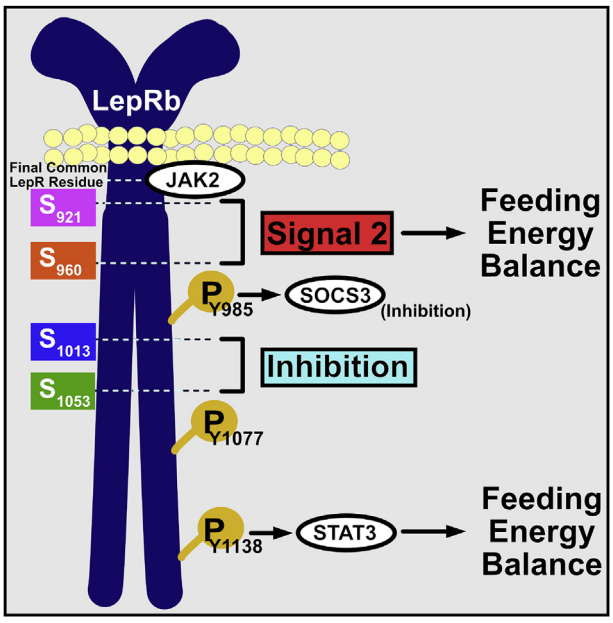

Figure 7: Schematic diagram of Tyr-dependent and -independent LepRb signals. **(A)** Diagram showing an illustration of LepRb, including the transmembrane region (covered by pale yellow circles representing the lipid bilayer), the final common amino acid (K889) shared by LepRb and the other transmembrane *Lepr* isoforms, the JAK2-binding region, the tyrosine phosphorylation (PY) sites, and physiologically relevant downstream signaling mediators. Also shown are the two PY-independent LepRb regions demonstrated by our analysis to affect physiology: the region between S921—S960, which mediates the Signal 2-dependent control of feeding and energy balance (such as $Tyr_{1138} \rightarrow$ STAT3), and S1013-1053, which blunts LepRb action (such as $Tyr_{985} \rightarrow$ SOCS3).

being the most NH_2-terminally positioned. It is also possible that residues 922—960 do not encompass the entire sequences required to mediate Signal 2, but that additional sequences NH_2-terminal to residue 922 also participate. It will also be important to understand the role(s) of Signal 2 in STAT3-dependent LepRb signaling. Addressing these questions will require the future generation of finer deletions as well as internal (not just COOH-terminal) deletions.

Residues 922—960 do not alter the expression of hypothalamic genes known to be targets of STAT3 (for example, *Agrp* and *Pomc*) [10,15,26], consistent with the Tyr- and STAT3-independent effects on energy balance and glucose homeostasis mediated by Signal 2. Rather, Signal 2 appears to modulate the activity of at least some neuronal targets of leptin action (mbARC NAG neurons) and the expression of *Cart*, which is thought to be controlled by neuronal activity [27]. In contrast, residues 922—960 are required for the suppression of mbARC FOS-IR. Thus, the physiologic role of this region may be to control neuronal activity rather than gene expression.

In addition to confirming the inhibitory role of residues 961—1013, which contain the known SOCS3-dependent feedback inhibitor Tyr_{985} [16,17], we identified the region containing residues 1014—1053 as another site that sensitizes leptin action when deleted. Thus, within residues 1014—1053, there likely lies one or more motifs that mediate the inhibition of LepRb action. As for the region containing Signal 2, 1014—1053 contains no Tyr residues and thus may not recruit a classic tyrosine kinase second messenger protein but may affect other LepRb functions. Importantly, because the loss of these two inhibitory elements [961-1013and1014-1053] can decrease body weight, food intake, and adiposity relative to 1083Δ (or 1053Δ), these elements interfere with physiologic regulation by Signal 2 as well as STAT3.

While the analysis we present herein was facilitated by the ability of CRISPR/Cas9 to rapidly and (relatively) inexpensively generate multiple novel gene-edited mouse lines, the time and resources required to thoroughly phenotype these lines remains rate-limiting. For this reason, we focused our present analysis on robust measures of food intake, adiposity, and glucose homeostasis, since examining more detailed measures of energy expenditure, reproduction, and other neuroendocrine functions [10,14,28] for these lines would have required considerable additional time and resources for relatively little additional information. Note that while the hyperleptinemia (>40 ng/ml in most cases) of the deletion lines studied suggests that leptin action was likely maximal in these lines, it is possible that Signal 2 mediated developmental changes (rather than leptin action in adult animals). Future studies using leptin antagonists in adult mice could test this possibility. While we cannot entirely rule out potential background differences among strains (for instance, due to potential CRISPR off-target mutations), we attempted to avoid such off-target effects by utilizing gRNA design algorithms that minimized the chance of off-target cutting. We also screened by DNA sequencing for changes at the top 10 predicted off-target sites for each gRNA, revealing no alterations at these sites. Furthermore, because we found the expected phenotypes of 1083Δ (similar to $LepRb^{Y1138MUT}$ [10,11,14,28]) and 921Δ (similar to *db/db* and the previously generated 924Δ [18]) and because we observed sensitization of leptin action by deletion of the Tyr_{985}-containing region, the signaling phenotypes of our mutations dominated any theoretical background effects. Note also that the energy balance and glucose homeostasis phenotypes of the control mice from each line did not differ in any way, consistent with the lack of off-target mutations that might have impacted the energy balance. As previously mentioned, however, definitively addressing these questions will require the future generation of finer deletions as well as internal (not just COOH-terminal) LepRb deletions.

Ultimately, defining the precise LepRb sequences that mediate Signal 2 and the novel 1014—1053 region-dependent LepRb inhibition will permit us to understand the potential cellular nature of these signals and the mechanisms by which they control neuronal physiology and function. This deeper understanding of cellular leptin action may reveal novel targets for potential therapeutic intervention in metabolic disease.

AUTHOR CONTRIBUTIONS

KS conducted the experiments, analyzed the data, and proofread the manuscript. MBA and GKS generated the 1083, 1053, and 1013 mutants and proofread the manuscript. QZ and CF bred the mutants to establish germline transmission and helped with the initial genotyping of either the 921 mutants or the 1083, 1053, and 1013 mutants, respectively, and proofread the manuscript. DG, PVS, AJT, and JCJ contributed the hypothalamic dissection and/or immunohistochemical work and proofread the manuscript. TB and MGM designed the experiments, researched and analyzed the data, and wrote and edited the manuscript. MGM is the manuscript guarantor.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.12.013.

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