Original Article

MOLECULAR METABOLISM

Transcriptional and physiological roles for STAT proteins in leptin action

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ABSTRACT

Objectives: Leptin acts via its receptor LepRb on specialized neurons in the brain to modulate food intake, energy expenditure, and body weight. LepRb activates signal transducers and activators of transcription (STATs, including STAT1, STAT3, and STAT5) to control gene expression.
Methods: Because STAT3 is crucial for physiologic leptin action, we used TRAP-seq to examine gene expression in LepRb neurons of mice ablated for *Stat3* in LepRb neurons (Stat3^{LepRb}KO mice), revealing the STAT3-dependent transcriptional targets of leptin. To understand roles for STAT proteins in leptin action, we also ablated STAT1 or STAT5 from LepRb neurons and expressed a constitutively-active STAT3 (CASTAT3) in LepRb neurons.
Results: While we also found increased *Stat1* expression and STAT1-mediated transcription of leptin-regulated genes in Stat3^{LepRb}KO mice, ablating *Stat1* in LepRb neurons failed to alter energy balance (even on the Stat3^{LepRb}KO background); ablating *Stat5* in LepRb neurons also failed to alter energy balance. Importantly, expression of a constitutively-active STAT3 (CASTAT3) in LepRb neurons decreased food intake and body weight and improved metabolic parameters in leptin-deficient (*ob/ob*) mice, as well as in wild-type animals.
Conclusions: Thus, STAT3 represents the unique STAT protein required for leptin action and STAT3 suffices to mediate important components of leptin action in the absence of other LepRb signals.

Keywords Leptin receptor; STAT1; STAT3; STAT5; Transcription; Obesity; Diabetes

1. INTRODUCTION

Obesity affects over one third of the adult population in developed countries, leading to diabetes, cardiovascular disease, and other conditions that contribute to significant morbidity and mortality [1]. Unfortunately, most current medical therapies fail to permanently reverse obesity; to design new and more effective treatments, we must first understand the mechanisms that govern food intake and energy balance.

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 suppress food intake and support the activity of neuroendocrine pathways that increase 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]. 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 [6,7]. As for other cytokine receptors, the activation of signal transducer and activator of transcription (STAT) transcription factor family members figures prominently in LepRb signaling: Tyr_{1138} recruits STAT1 and STAT3 [7,8], while Tyr_{1077} plays the dominant role in the recruitment of STAT5 (Tyr_{1138} also plays a minor role) [3]. In contrast, Tyr_{985} recruits protein tyrosine phosphatase 2 (SHP2; PTPN1) [7] and the cytokine signaling inhibitor, SOCS3 [9], and contributes to the feedback inhibition of LepRb signaling [10].

Mice containing substitution mutations of LepRb Tyr_{1138} (LepRb^{Y1138MUT} mice) display dramatic hyperphagia and obesity (although some aspects of leptin action are preserved relative to *ob/ob* and *db/db* mice) [11,12], suggesting the importance of Tyr_{1138}→STAT signaling for leptin action. STAT3 must play a role in leptin action: The activation of STAT3 by leptin *in vivo* was demonstrated early on [13], and mice in which *Stat3* is ablated from LepRb neurons (STAT3^{LepRb}KO mice) develop hyperphagic obesity, albeit with partial preservation of some physiologic parameters (as for LepRb^{Y1138MUT} mice) relative to ob/ob

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Received December 18, 2018 • Revision received January 14, 2019 • Accepted January 17, 2019 • Available online 24 January 2019

https://doi.org/10.1016/j.molmet.2019.01.007

and db/db mice [14]. Thus, Tyr_{1138} and STAT3 are crucial for leptin action, but an unidentified second LepRb signaling pathway that is independent of Tyr_{1138} and STAT3 must also play important roles in physiologic leptin action.

STAT1 and/or STAT5 could potentially contribute to leptin action. Indeed, brain-wide *Stat5* knockout mice display mild obesity [15]; potential roles for STAT1 in leptin action *in vivo* have not been directly examined, however. Furthermore, it is not known whether the crucial Tyr_{1138}- and STAT3-independent LepRb signal is required for the control of energy balance by STAT3. Thus, important parameters of STAT signaling in leptin action remain to be defined, including [1]: the transcriptional targets for STAT3 in LepRb neurons [2]; roles for alternative STAT proteins (i.e., STAT1 and STAT5); and [3] the potential ability of STAT3 to mediate leptin action in the absence of additional LepRb signals.

2. MATERIALS AND METHODS

2.1. Animals

All procedures performed on animals were approved by the University of Michigan Insitutional Committee on the Care and Use of Animals and 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 water *ad libitum* and housed in temperature-controlled rooms on a 12-hour light-dark cycle. All mice were provided food *ad libitum*, with the exception of brief fasts prior to assays of glycemic control, as noted below.

Lepr$^{cre/cre}$;*Rosa*$^{eGFP-10a/eGFP-L10a}$ (LepRb$^{eGFP-L10a}$) mice were as previously described [16]. *Stat1*$^{flox/+}$ (Jackson stock #012901) [17] and *Stat5*$^{flox/+}$ [15,18] mice were crossed twice onto the *Lepr*$^{cre/cre}$ background to generate *Lepr*$^{cre/cre}$; *Stat1*$^{flox/+}$ (and *Lepr*$^{cre/cre}$; *Stat5*$^{flox/+}$) animals that were intercrossed with the same genotype to obtain *Lepr*$^{cre/cre}$; *Stat1*$^{flox/flox}$ (STAT1^{LepRb}KO) (and *Lepr*$^{cre/cre}$; *Stat5*$^{flox/flox}$ (Stat5^{LepRb}KO)) animals plus *Lepr*$^{cre/cre}$ control littermates for study.

Stat3$^{flox/+}$ mice were crossed to LepRb-eGFP-L10a mice for two generations to obtain *Stat3*$^{flox/+}$;*Lepr*$^{cre/cre}$;*Rosa*$^{eGFP-L10a/eGFP-L10a}$ mice. These mice were subsequently intercrossed to generate *Stat3*$^{flox/flox}$;*Lepr*$^{cre/cre}$;*Rosa*$^{eGFP-L10a/eGFP-L10a}$ (STAT3^{LepRb}KO-eGFP-L10a) mice and littermate *Stat3*$^{+/+}$;*Lepr*$^{cre/cre}$;*Rosa*$^{eGFP-L10a/eGFP-L10a}$ (LepRb$^{eGFP-L10a}$) controls for TRAP-seq. To generate STAT1-STAT3^{LepRb}KO mice, *Stat1*$^{flox/+}$ mice were crossed to *Lepr*$^{cre/cre}$;*Rosa*$^{eGFP-L10a/eGFP-L10a}$;*Stat3*$^{flox/+}$ mice for two generations to generate *Lepr*$^{cre/cre}$;*Rosa*$^{eGFP-L10a/eGFP-L10a}$;*Stat*$^{flox/+}$;*Stat1*$^{flox/+}$ mice. These mice were subsequently intercrossed to generate *Lepr*$^{cre/cre}$;*Rosa*$^{eGFP-L10a/eGFP-L10a}$;*Stat3*$^{flox/flox}$ (STAT3^{LepR}KO) and *Lepr*$^{cre/cre}$;*Rosa*$^{eGFP-L10a/eGFP-L10a}$;*Stat1*$^{flox/flox}$;*Stat3*$^{flox/flox}$ (STAT1STAT3^{LepR}KO) mice for study.

Rosa26^{CASTAT3} mice [19] were the generous gift of Sergei Koralov, PhD (New York University, New York, NY). *Rosa26*$^{CASTAT3/+}$ mice were bred to *Lepr*$^{cre/cre}$ mice for two generations to produce *Lepr*$^{cre/cre}$;*Rosa26*$^{CASTAT3/+}$ mice that were bred to *Lepr*$^{cre/cre}$;*Rosa*$^{eGFP-L10a/eGFP-L10a}$ animals to produce experimental animals. To produce *ob/ob*;LepRb-^{CASTAT3} mice and their controls, the breeders were *ob/+*;*Lepr*$^{cre/cre}$;*Rosa26*$^{CASTAT3/+}$ and *ob/+*;*Lepr*$^{cre/cre}$;*Rosa*$^{eGFP-L10a/eGFP-L10a}$ mice.

2.2. Immunohistochemistry

For the detection of pSTAT3, food was removed at the onset of the light cycle. Animals were treated four hours later with metreleptin (5 mg/kg, i.p.) and subjected to perfusion 90 min after treatment. These and all other mice for immunohistochemical analysis were anesthetized with a lethal dose of pentobarbital and transcardially perfused with phosphate buffered saline (PBS) followed by 10% buffered formalin. Brains were removed, placed in 10% buffered formalin overnight, and dehydrated in 30% sucrose for several days. Using a freezing microtome (Leica), brains were cut into 30 μm sections. Sections were treated sequentially with 1% hydrogen peroxide/0.5% sodium hydroxide, 0.3% glycine, 0.03% sodium dodecyl sulfate, and blocking solution (PBS with 0.1% triton, 3% Normal Donkey Serum). Immunostaining was performed using primary antibodies for pSTAT3 (Cell Signaling #9145, 1:1000), GFP (Aves Labs #GFP1020, 1:1000), STAT1 (Santa Cruz sc-346, 1:250). All antibodies were reacted with species-specific Alexa Fluor-488 or -568 conjugated secondary antibodies (Invitrogen, 1:200) or processed with the avidin-biotin/diaminobenzidine (DAB) method (ABC kit, Vector Labs, 1:500; DAB reagents, Sigma). Images were collected on an Olympus BX53F microscope. DAB images were pseudocolored using Photoshop software.

2.3. In situ hybridization

For *in situ* hybridization (ISH), adult STAT1^{LepR}KO, STAT3^{LepR}KO, STAT1STAT3^{LepR}KO and control mice were anesthetized with isoflurane and then euthanized by decapitation. Whole brains were dissected, flash frozen in isopentane, chilled on dry ice, and stored at −80 °C. 16 μm-thick coronal sections were cut on a cryostat (Leica), thaw-mounted to SuperFrost Plus slides, allowed to dry at −20 °C for one hour and then stored at −80 °C. Slides were then processed for ISH using RNAScope technology per the manufacturer's protocol (Advanced Cell Diagnostics) using either the 2.5 HD Assay-Brown (322310) or the multiplex fluorescent assay (320850) and Stat1 (492731) and Cre (312281-C3) probes. Images of the colorimetric assay were obtained with an Olympus BX51 and Olympus DP80 color camera under 40X objective with a 0.63X C-Mount for a total effective magnification of 25.2X. Fluorescent images were obtained with an Olympus BX53 and QImaging Retiga 6000 monochrome camera under 20X objective. Photoshop (Adobe) was used to adjust white balance (for color images) and adjust levels to remove nonspecific background (for each channel of the fluorescent images). All images from an experiment were processed identically.

2.4. Phenotyping of mouse strains

Mice were weaned into individual housing at 21 days and fed normal chow (Purina Lab Diet 5001). Weekly body weight and food intake were monitored. Blood glucose samples from *ad libitum*-fed mice were collected every other week from 4 to 12 weeks of age. Glucose tolerance test (2 g/kg body weight, i.p.) and insulin tolerance test (1 unit/kg body weight, Humulin (Eli Lilly), i.p.) were performed in 13 and 14 week old mice, respectively, after a 5 h fast beginning three hours after the start of the light-cycle. Analysis of body fat and lean mass was performed at 14–15 weeks of age using NMR-based analyzer (Minispec LF90II, Bruker Optics). Leptin and insulin were assayed by commercial ELISA (Crystal Chem).

2.5. Translating ribosome Affinity purification with deep sequencing (TRAP-seq)

At the midpoint of the light cycle, adult mice were anesthetized with isofluorane, after which their brains were removed and placed onto a mouse coronal brain matrix (1 mm sections).

For whole hypothalamic dissections, a 3x3x3mm block was dissected from the ventral diencephalon immediately caudal to the optic chiasm and immediately homogenized and processed for TRAP-seq analysis as previously described [16]. Hypothalami from 5 to 8 mice were pooled at the time of collection to produce sufficient mRNA for a single RNA-seq replicate. Messenger RNA isolated from eGFP-tagged ribosomes and from the eGFP-depleted was assessed for quality using

TapeStation (Agilent, Santa Clara, CA) and samples with RNA Integrity Numbers (RINs) of 8 or greater were prepared using the Illumina TruSeq mRNA Sample Prep v2 kit (Catalog # RS-122-2001 and #RS-122-2002) (Illumina, San Diego, CA), where 0.1–3 μg of RNA was converted to mRNA using a polyA purification. The mRNA was chemically fragmented and copied into first strand cDNA using reverse transcriptase and random primers. The 3′ ends of the cDNA were adenylated and the 6-nucleotide-barcoded adapters ligated. These products were then purified and enriched by PCR to create the cDNA library, which were checked for quality and quantity by TapeStation (Agilent) and qPCR using Kapa's library quantification kit for Illumina Sequencing platforms (catalog #KK4835) (Kapa Biosystems, Wilmington MA). They were clustered on cBot (Illumina) and sequenced 4 samples per lane on a 50 cycle single end run on a HiSeq 2000 (Illumina) using version 2 reagents according to manufacturer's protocols.

2.6. RNA-seq analysis

50 base pair single end reads underwent QC analysis (FastQC 0.11.7) and quality filtering (fastq_quality_filter with q = 20 from FASTX Toolkit 0.0.14) prior to alignment to mouse genome build GRCm38 using STAR software [20]. Differential expression was determined using DESeq2, with thresholds for differential expression set to fold change >1.5 or <0.66 and a false discovery rate of <0.05 [21]. Differential expression for enriched genes was set at an absolute fold-change of ≥ 0.5 (bead/sup). No differential expression threshold was set for regulated genes (fold-change between genotypes). Genesets were filtered using Ensembl annotation (version 94) and all analysis was performed only on protein-coding genes.

2.7. Statistics for mouse phenotyping

Data are reported as mean ± SEM. Statistical analysis of physiological data was perfomed with Prism software (version 7). Unpaired t-tests were used to compare results between two groups. Body weight gain, cumulative food intake, body length, GTT and ITT were analyzed by two-way ANOVA. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Defining STAT3-regulated genes in LepRb neurons

We previously used anti-GFP translating ribosome affinity purification (TRAP) coupled with RNA-seq (TRAP-seq) on material derived from *Lepr*$^{cre/cre}$;*Rosa26*$^{eGFP\text{-}L10a/eGFP\text{-}L10a}$ (LepRb$^{eGFP-L10a}$) mice to define the regulation of hypothalamic LepRb neuron gene expression by leptin [16,22]. To determine how the absence of STAT3 alters gene expression in LepRb neurons, we crossed LepRb$^{eGFP-L10a}$ mice onto the *Stat3*^{flox} background to generate STAT3^{LepRb}KO-eGFP-L10a mice for TRAP-seq analysis of hypothalamic LepRb neurons that lack *Stat3* (Figure 1A). As expected [14], STAT3^{LepRb}KO-eGFP-L10a mice were obese (body weight: 23.8 ± 1.8 g control vs 38.8 ± 5.1 g STAT3-^{LepRb}KO; $p < 0.0001$) and devoid of leptin-stimulated pSTAT3 (Supplemental Figure 1A).

We performed anti-GFP TRAP-seq on hypothalamic tissue from STAT3^{LepRb}KO-eGFP-L10a mice and their LepRb$^{eGFP-L10a}$ controls. The transcriptome of LepRb neurons from STAT3^{LepRb}KO mice was similar to that of wild-type and *ob/ob* mice [16,22]. The absence of STAT3 in LepRb neurons did increase the expression of some genes, which thus became enriched in LepRb neurons in the STAT3^{LepRb}KO mice (Supplemental Table 1).

Because the TRAP fraction contains low levels of contaminating mRNA derived from non-LepRb neurons, we examined transcripts enriched in hypothalamic LepRb neurons to understand alterations in LepRb neurons specifically (as previously described [16]) (Supplemental Table 2). We compared these results to controls and to our previous results from leptin-deficient *ob/ob*;LepRb$^{eGFP-L10a}$ mice [22] to define the gene expression changes in STAT3^{LepRb}KO mice that mimic leptin deficiency (as opposed to those that may result from *Stat3* deletion independently of decreased leptin signaling) (Figure 1B–D). Of the 2463 mRNA species enriched in hypothalamic LepRb neurons, 243 were different in at least one comparison among conditions (Supplemental Tables 1, 2); of these, 128 were differentially expressed between *ob/ob* and STAT3^{LepRb}KO mice.

Comparing gene expression in *ob/ob* and STAT3^{LepRb}KO mice to their controls revealed several classes of differentially-regulated genes (Figure 1D). Genes that were coordinately regulated in *ob/ob* and STAT3^{LepRb}KO mice (in regions I and I′ of Figure 1D) likely contain the crucial targets of STAT3-dependent leptin action. These include several neuropeptides important for leptin action (e.g., *Pomc*, *Cartpt*, *Agrp*, and *Npy*) [3] and the serine protease inhibitor *Serpina3n* (which is coordinately regulated by leptin across many conditions [22]), among others. Gene expression changes discordant between *ob/ob* and STAT3^{LepRb}KO mice might account for the more modest perturbation of body weight and glucose homeostasis in STAT3^{LepRb}KO mice compared to *ob/ob* animals. The genes in regions II and II′ of Figure 1D represent those dysregulated in *ob/ob*, but not in STAT3^{LepRb}KO, mice and thus could include those controlled by the physiologically-relevant STAT3-independent LepRb signal. Transcription factor protein-protein interaction (TF-PPI) analysis revealed that (with the exception of RAD21) these genes were not significantly enriched in known targets for any transcriptional regulator (Supplemental Table 3). Furthermore, because RAD21 is a component of the cohesion complex, rather than a transcription factor, this finding likely indicates that the STAT3-independent LepRb signal acts mainly by non-transcriptional mechanisms.

Many genes in regions III and III′ in Figure 1D (changed in STAT3-^{LepRb}KO but not *ob/ob* mice) and in regions IV and IV′ (changed in opposite directions in *ob/ob* and STAT3^{LepRb}KO mice) also displayed differential regulation, however. Genes in these regions include *Irf9 and Irgm2*, among others (Supplemental Table 2), which are regulated by leptin under multiple conditions [22]. Thus, the lack of STAT3 signaling *per se* provokes changes in gene expression that might contribute to the STAT3-independent control of energy balance in STAT3^{LepRb}KO mice.

Interestingly, TF-PPI analysis and ENCODE ChIP data revealed that many of the genes different between *ob/ob* and STAT3^{LepRb}KO mice represent the transcriptional targets of STAT1 (Supplemental Table 3) (Figure 1D). Furthermore, our TRAP-seq analysis revealed >3-fold increased *Stat1* expression in STAT3^{LepRb}KO hypothalamic LepRb neurons compared to controls (~6-fold compared to *ob/ob* animals) (Supplemental Table 2). Indeed, immunohistochemical (IHC) analysis and *in situ* hybridization (ISH) demonstrated that while STAT1 protein and *Stat1* mRNA, respectively, were virtually undetectable in the LepRb neurons of control mice, the detection of both dramatically increased in ARC LepRb neurons of STAT3^{LepRb}KO animals (Figure 1E; Supplemental Figure 1B). Since LepRb can activate STAT1 in cultured cells [8], this finding suggests the possibility that STAT1 plays a role in leptin action, including to partially compensate for the loss of STAT3 signaling in STAT3^{LepRb}KO animals.

3.2. STAT1 fails to compensate for the lack of STAT3 in LepRb neurons

To determine whether STAT1 might play a role in LepRb signaling and the control of energy balance by leptin, we bred *Stat1*^{flox} [17] onto the

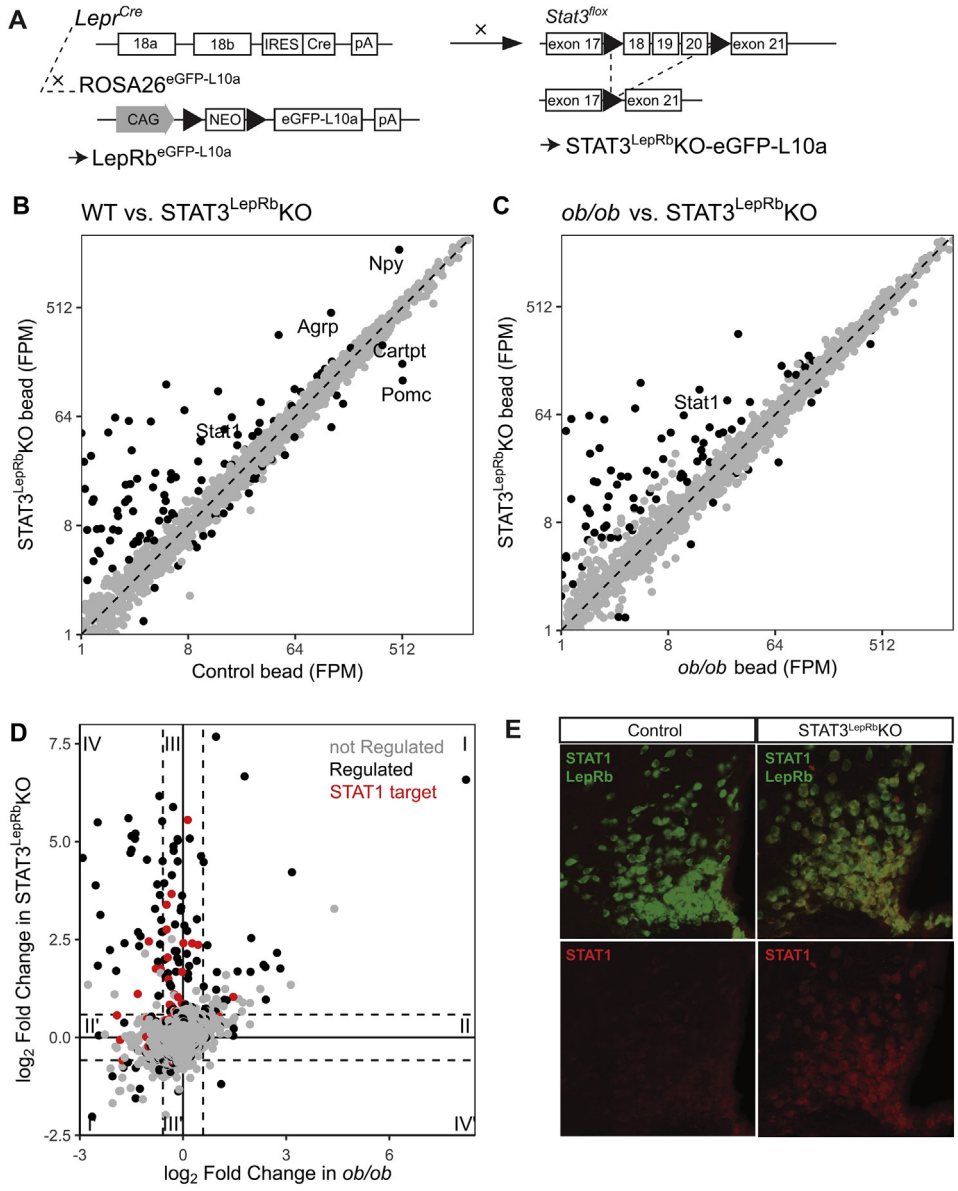

Figure 1: Transcriptional response to the deletion of *Stat3* in LepRb neurons. (A) Schematic diagram showing the *Lepr*^{cre}-mediated deletion of *Stat3*^{flox} mice on the *Rosa26*$^{eGFP-L10a}$ background to generate STAT3^{LepRb}KO-eGFP-L10a mice. **(B–C)** Expression values (fragments per million reads; FPM) for genes that were enriched in LepRb neurons under any condition, comparing STAT3^{LepRb}KO mice to control mice (**B**) or *ob/ob* mice (**C**) [16] were plotted. Black dots represent genes demonstrating statistically significant changes in gene expression for the comparison; some genes of interest are labeled. (**D**) Comparison of fold change in gene expression relative to control for STAT3^{LepRb}KO and *ob/ob* mice for all genes enriched in LepRb neurons. Dashed lines represent fold change values of 1.5 and 0.667 for both axes. Black dots represent regulated genes; red dots show genes that are regulated and known to be controlled by STAT1 [38]. Regions of the graph are denoted by Roman numerals for reference by the main text. **(E)** Representative images showing colocalization of STAT1-IR (red) and GFP-IR (green) in the ARC of Control and STAT3^{LepRb}KO mice.

Lepr^{cre} background to generate *Lepr*$^{cre/cre}$;*Stat1*$^{flox/flox}$ (STAT1^{LepRb}KO) mice null for *Stat1* in LepRb neurons. The deletion of *Stat1* from LepRb neurons failed to alter body weight, food intake, adiposity, or glucose homeostasis (Supplemental Figures 2, 3). We similarly ablated *Stat5* [18] from LepRb neurons, with no effect on these metabolic parameters (Supplemental Figures 4, 5).

To directly test the notion that STAT1-mediated transcription partially compensates for the lack of *Stat3* in STAT3^{LepRb}KO mice, we generated *Stat1*$^{flox/flox}$;*Stat3*$^{flox/flox}$;*Lepr*$^{cre/cre}$ (STAT1STAT3^{LepRb}KO, lacking both STAT1 and STAT3 in LepRb neurons) mice for comparison to STAT3^{LepRb}KO controls (Figure 2A). Note that because *Stat5* expression was not different from controls in the STAT3^{LepRb}KO mice (Supplemental Tables 1, 2), we did not ablate *Stat5* in conjunction with *Stat3*. While we readily detected *Stat1* mRNA and STAT1 protein in LepRb neurons from STAT3^{LepRb}KO mice, we detected neither in the LepRb neurons of STAT1STAT3^{LepRb}KO mice (Figure 2B, Supplemental Figure 6A), confirming the expected ablation of *Stat1* from LepRb neurons in these animals. Importantly, our analysis revealed no difference in body weight, adiposity, food intake, or glycemic control between STAT3^{LepRb}KO and STAT1STAT3^{LepRb}KO mice, demonstrating

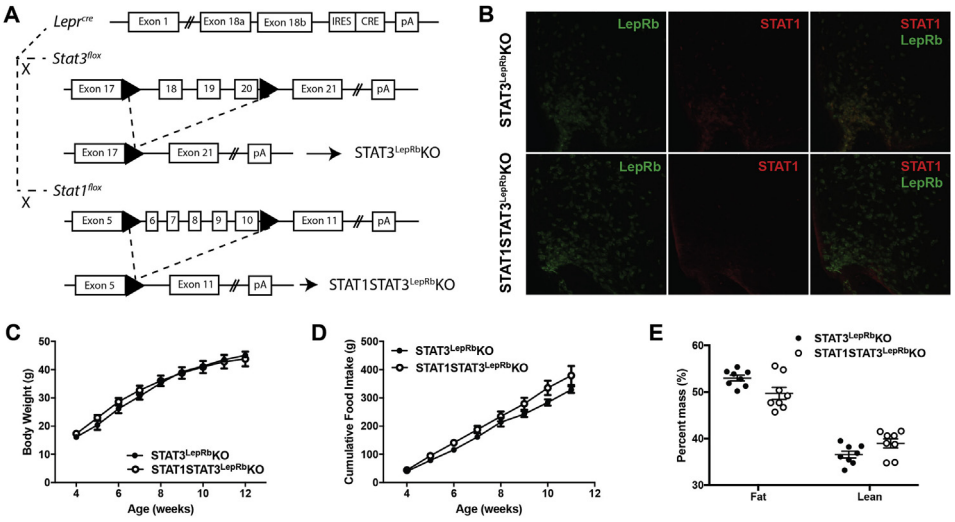

Figure 2: Deletion of STAT1 and STAT3 in LepRb expressing neurons. (A) Schematic diagram showing the cross of *Lepr*^{cre} with *Stat1*^{flox} and *Stat3*^{flox} mice to generate STAT3^{LepRb}KO and STAT1STAT3^{LepRb}KO mice. pA: polyadenylation signal. **(B)** Representative images showing colocalization of STAT1-IR (red) with GFP-IR (green) in the arcuate nucleus of STAT3^{LepR}KO and STAT1STAT3^{LepR}KO (both of which are on the *R26*$^{eGFP\text{-}L10a}$ background) mice. (**C**–**E**) Male STAT3^{LepRb}KO and STAT1STAT3^{LepRb}KO mice were placed on chow and body weight **(C)** and cumulative food intake **(D)** were measured weekly. **(E)** At 14–15 weeks of age, animals underwent body composition analysis by NMR spectroscopy. Mean, quartiles, and individual plots are shown; n = 8–14 per genotype. ANOVA analysis was performed for **(C, D)**; unpaired t-test was performed for **(E)**. All comparisons p = not significant unless indicated.

that increased *Stat1* expression did not compensate for the lack of STAT3 signaling in LepRb neurons (Figure 2C–E, Supplemental Figures 6, 7).

3.3. Expression of CASTAT3 in LepRb neurons decreases feeding and body weight in lean mice and ob/ob mice

Since these findings reveal that STAT3 plays a unique and indispensable role in physiologic leptin action, we surmised that increased STAT3 signaling in LepRb neurons might augment leptin action and/or substitute for absent leptin action. To examine this possibility, we crossed LepRb$^{eGFP-L10a}$ mice onto *Rosa26*^{CASTAT3} [19] to generate *Lepr*$^{cre/cre}$;*Rosa26*$^{CASTAT3/eGFP\text{-}L10a}$ (LepRb^{CASTAT3}) mice expressing a constitutively-active STAT3 mutant (CASTAT3) in LepRb neurons, along with littermate *Lepr*$^{cre/cre}$;*Rosa26*$^{eGFP\text{-}L10a/+}$ controls (Figure 3A). Using TRAP-seq to examine mRNA from hypothalamic LepRb neurons in these mice, we found that CASTAT3-encoding mRNA represented approximately 6–7% of the total *Stat3* mRNA in LepRb neurons from LepRb^{CASTAT3} mice (Figure 3B). While we detected no alteration in body weight or food intake in female LepRb^{CASTAT3} mice, these mice exhibited decreased adipose tissue mass (Supplemental Figure 8). Furthermore, male LepRb^{CASTAT3} mice displayed decreased body weight due to lower adiposity (Figure 3C–E, Supplemental Figure 9). Despite decreased adipose mass, leptin concentrations were unchanged in LepRb^{CASTAT3} mice compared to controls (Figure 3F), suggesting that STAT3 in LepRb neurons may participate in the recently-described control of adipose leptin expression via CNS leptin action [23]. Insulin concentrations were unchanged, as well (Figure 3G). The male LepRb^{CASTAT3} mice also exhibited decreased food intake but unchanged energy expenditure (Figure 3D,H), suggesting that increased STAT3 signaling in LepRb neurons decreases body weight primarily by suppressing food intake.

Despite their decreased body mass and adiposity, male LepRb^{CASTAT3} mice remained sensitive to the anorectic effects of leptin (Figure 4), revealing that augmented STAT3-mediated transcription at baseline does not prohibit further increases in leptin action. In contrast, LepRb^{CASTAT3} males gained weight and became similarly obese as control mice when fed a high-fat diet (HFD) (Figure 5). Thus, while decreased LepRb→STAT3 signaling has been proposed to underlie diet-induced obesity (DIO) [24], these data suggest instead that constitutive STAT3-mediated transcription in LepRb neurons is insufficient to block DIO, despite its ability to augment leptin action and promote leanness in normal animals.

To determine whether increased STAT3-mediated transcription in LepRb neurons might suffice to mediate all or part of leptin action in the absence of other LepRb signals, we also bred LepRb^{CASTAT3} mice onto the *ob* background to produce *ob/ob*;*Lepr*$^{cre/cre}$;*Rosa26*^{CASTAT3} (*ob/ob*;LepRb^{CASTAT3}) and *ob/ob*;*Lepr*$^{cre/cre}$ controls (Figure 6; Supplemental Figure 10). We found that *ob/ob*;LepRb^{CASTAT3} mice displayed lower body weight and adiposity, consistent with their trend toward decreased food intake, compared to obese *ob/ob*;*Lepr*$^{cre/cre}$ mice. Furthermore, the *ob/ob*;LepRb^{CASTAT3} animals displayed lower blood glucose concentrations under *ad libitum*-fed conditions. Thus, even in the absence of other leptin→LepRb signals, CASTAT3-mediated transcriptional activity in LepRb neurons suffices to ameliorate the phenotype of leptin deficiency, suggesting that STAT3-mediated transcription can mediate leptin-like effects even in the absence of other LepRb signals. Since the expression of CASTAT3 in LepRb neurons does not completely normalize the body weight phenotype of LepRb^{CASTAT3}*ob/ob* mice, non-STAT3 dependent signals may also contribute to (and be required for) leptin action, even in the presence of STAT3-mediated transcription.

4. DISCUSSION

Although LepRb activates STAT1, STAT3, and STAT5 in cultured cells [6–8,25], our present findings reveal no role for STAT1 or STAT5 in leptin action *in vivo*. Furthermore, the lack of *Stat1* fails to alter the phenotype of STAT3^{LepRb}KO mice despite the increased STAT1-dependent expression of several leptin target genes in these animals. Additionally, expressing CASTAT3 in LepRb neurons not only

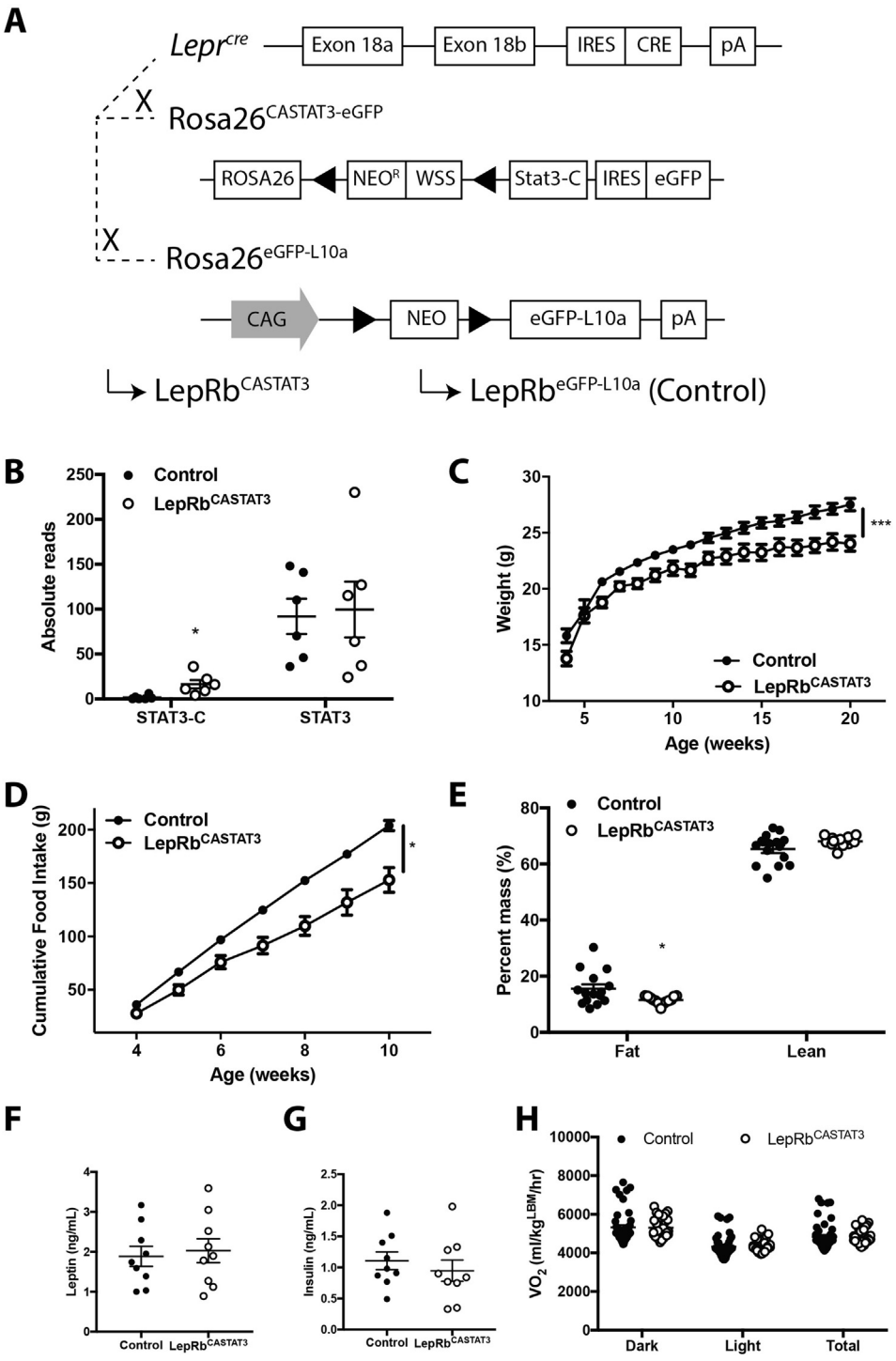

Figure 3: CASTAT3 activity in LepRb neurons decreases body weight and adiposity. (A) Schematic diagram showing the cross of *Lepr*^{cre} with *Rosa26*$^{CASTAT3\text{-}eGFP}$ *and* *Rosa26*$^{eGFP\text{-}L10a}$ mice to generate LepRb^{CASTAT3} and LepRb$^{eGFP-L10a}$ (Control) mice. pA: polyadenylation signal. **(B)** Number of RNA-seq reads for the CASTAT3-specific and normal *Stat3* exon for male mice of each genotype. **(C–H)** Male LepRb^{CASTAT3} and LepRb$^{eGFP-L10a}$ mice were placed on chow and body weight **(C)** and food intake **(D)** were measured weekly. **(E)** At 21–22 weeks of age, animals underwent body composition analysis by NMR spectroscopy. Serum from 16-week-old mice were assayed for leptin **(F)** and insulin **(G)**. 10-12 week-old mice were subjected to CLAMS analysis to determine VO_2 normalized to total body mass **(H)** over a 72-hour period. n = 15–20 for **(C,D)**, n = 8–14 for **(E–H)**. Mean, quartiles, and individual plots are shown; *p < 0.05, ***p < 0.001, by ANOVA **(C, D)** or unpaired t-test **(B, E, F–H)**.

decreases food intake and body weight in normal mice, but also ameliorates the obese and diabetic phenotype of *ob/ob* mice. Thus, STAT3 represents the only STAT that contributes meaningfully to the control of energy balance by leptin/LepRb, and STAT3-mediated transcription suffices to mediate leptin-like effects independently of other LepRb signals.

Our previous analysis of STAT proteins in leptin action focused upon LepRb Tyr_{1138}, which is required for the leptin-simulated activation of

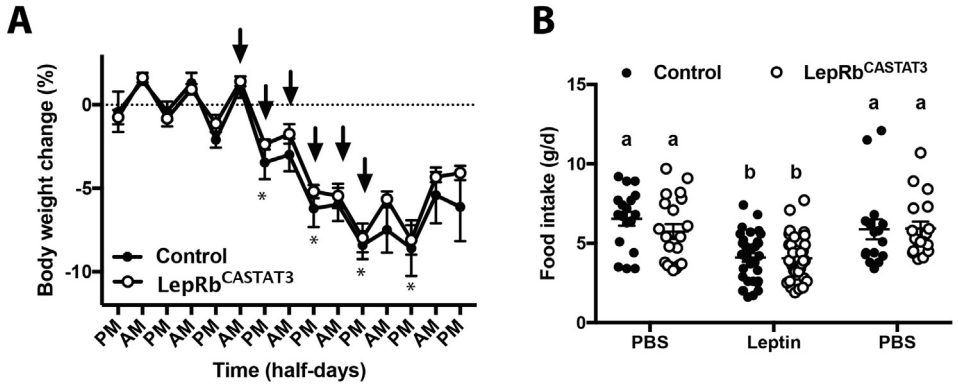

Figure 4: Effect of exogenous leptin in LepRb^{CASTAT3} mice. Eight-week-old male LepRb^{CASTAT3} and LepRb$^{eGFP-L10a}$ (Control) mice fed normal chow were injected twice daily (at 8:30AM and 4:30PM) with PBS for 3 days, with leptin for 3 days (5 mg/kg; i.p.), and then with PBS for 2 days. **(A)** Body weight was measured twice per day during the injection period; arrows indicate times of leptin injections. *$p < 0.05$ compared to baseline. No significant differences by genotype. **(B)** Food intake was measured daily and compiled for the initial PBS period, the leptin period, and the second PBS period. Mean ± SEM is shown. Plots with different letters were significantly different at $p < 0.01$; there was no significance between plots with same letters. Both panels: $n = 9–12$ per genotype.

STAT1 and STAT3 in cultured cells and also plays a minor role in the activation of STAT5 by leptin [6,7]. Mutation of LepRb Tyr$_{1138}$ in mice causes hyperphagic obesity [11], suggesting a role for STAT signaling by this residue in leptin action. The deletion of STAT3 from the brain, or from LepRb neurons specifically, promotes a similar phenotype [26,27], suggesting a unique and required role for STAT3 in the control of energy balance by LepRb.

The brain-wide deletion of *Stat5* promotes a mild obesity phenotype [15], suggesting a potential role for STAT5 in leptin action. Another study attempting to delete *Stat5* and/or *Stat3* in LepRb neurons utilized a single copy of *Lepr^{cre}* and yielded little or no phenotype for any of these manipulations [28]. The lack of a substantial phenotype for mice presumed to be deleted for *Stat3* in LepRb neurons in that study suggests incomplete deletion of the floxed alleles by the single copy of *Lepr^{cre}* employed [28]. Indeed, our present findings (using two copies of *Lepr^{cre}*) recapitulate the finding of dramatic obesity from Tyr$_{1138}$ mutation in mice lacking *Stat3* in LepRb neurons. But, using two copies of *Lepr^{cre}* to mediate the deletion of *Stat5* from LepRb neurons failed to alter energy balance, suggesting that STAT5 plays little role in the control of energy balance by leptin. Indeed, mutation of LepRb Tyr$_{1077}$ (the major site controlling STAT5 activation by leptin) minimally alters parameters of energy balance [29]. Moreover, mice mutated for all three LepRb phosphorylation sites (LepRb^{3F} mice) exhibit a phenotype similar to LepRb^{Y1138MUT} animals [12]. These findings are consistent with a minimal role for Tyr$_{1077}$ and/or STAT5 in physiologic leptin action. Thus, the obesity phenotype of mice lacking *Stat5* throughout the brain [15] likely indicates a role for STAT5 in energy balance in non-LepRb neurons, in response to a non-leptin stimulus (such as GM-CSF) [30].

We found that deleting *Stat3* from LepRb neurons dramatically increases *Stat1* expression in LepRb neurons, as well as increasing the expression of canonical STAT1-regulated genes (including *Irf9*, *Irf1* and *Psmb8*) in LepRb neurons. Furthermore, *Irf9* and other previously-identified leptin target genes are oppositely regulated in *ob/ob* and STAT3^{LepRb}KO mice, suggesting that increased STAT1-dependent signaling might partially compensate for the disruption of LepRb→STAT3 signaling in STAT3^{LepRb}KO mice, moderating their phenotype. The disruption of *Stat1* in LepRb neurons fails to alter energy balance in normal mice, or in STAT3^{LepRb}KO mice, however. Thus, STAT1 is unable to compensate, even in part, for the lack of STAT3 in leptin action. Similarly, Tyr$_{1138}$ recruits both STAT1 and STAT3, and the phenotypes of STAT3^{LepRb}KO and LepRb^{Y1138MUT} mice are similar and milder than *ob/ob* or *db/db* mice [11,12,14], consistent with our data arguing against a physiologically important role for STAT1 in LepRb signaling *in vivo*. In the future, it could be interesting to examine the potential role for these STAT proteins in LepRb cells for the response to IL-6 or other immune signals.

Thus, STAT3 represents the unique STAT protein necessary for physiologic leptin action, and the non-Tyr$_{1138}$/STAT3-dependent LepRb signal that contributes to the difference in phenotype between STAT3^{LepRb}KO (or LepRb^{Y1138MUT} and LepRb^{3F}) mice and *ob/ob* or *db/db* animals must be mediated by something other than a STAT protein. Indeed, previous findings from others and us suggests that the STAT3-independent LepRb signal contributes to the control of energy balance via LepRb sequences that are independent of JAK2 binding and activation, and also distinct from the LepRb tyrosine phosphorylation sites [12,31]. To identify this STAT3- and phosphorylation-independent second LepRb signal, it will be important to identify the LepRb sequences that mediate the physiologic processes associated with this signal. The crucial LepRb phosphorylation-independent motif could act via phosphatidylinositol 3-kinase (PI 3-kinase), ERK, and/or WNT, which have been suggested to mediate aspects of leptin action *in vivo* [32–34].

To determine whether enhanced STAT3 signaling can augment or mimic endogenous leptin action, we expressed CASTAT3 in LepRb neurons. This manipulation decreased food intake and body weight in male mice and diminished adipose mass in male and female mice on standard chow. In contrast, expressing CASTAT3 in AgRP neurons increases locomotor activity without affecting food intake, while expressing CASTAT3 in POMC neurons reportedly increases body weight and adiposity in mice [19,35]. Thus, the crucial set of LepRb neurons in which STAT3 acts to control food intake and body weight is likely distinct from POMC and AgRP neurons but may involve unidentified populations of DMH neurons that contribute to these leptin effects [36].

Importantly, expressing CASTAT3 in LepRb neurons did not prevent DIO in males. Thus, although decreased LepRb→STAT3 signaling has been proposed to underlie DIO [24], our findings suggest instead that constitutive STAT3-mediated transcription in LepRb neurons is insufficient to combat DIO, despite its ability to augment leptin action and promote leanness in normal animals. These findings are consistent with the notion that there exists a ceiling for the efficacy of STAT3

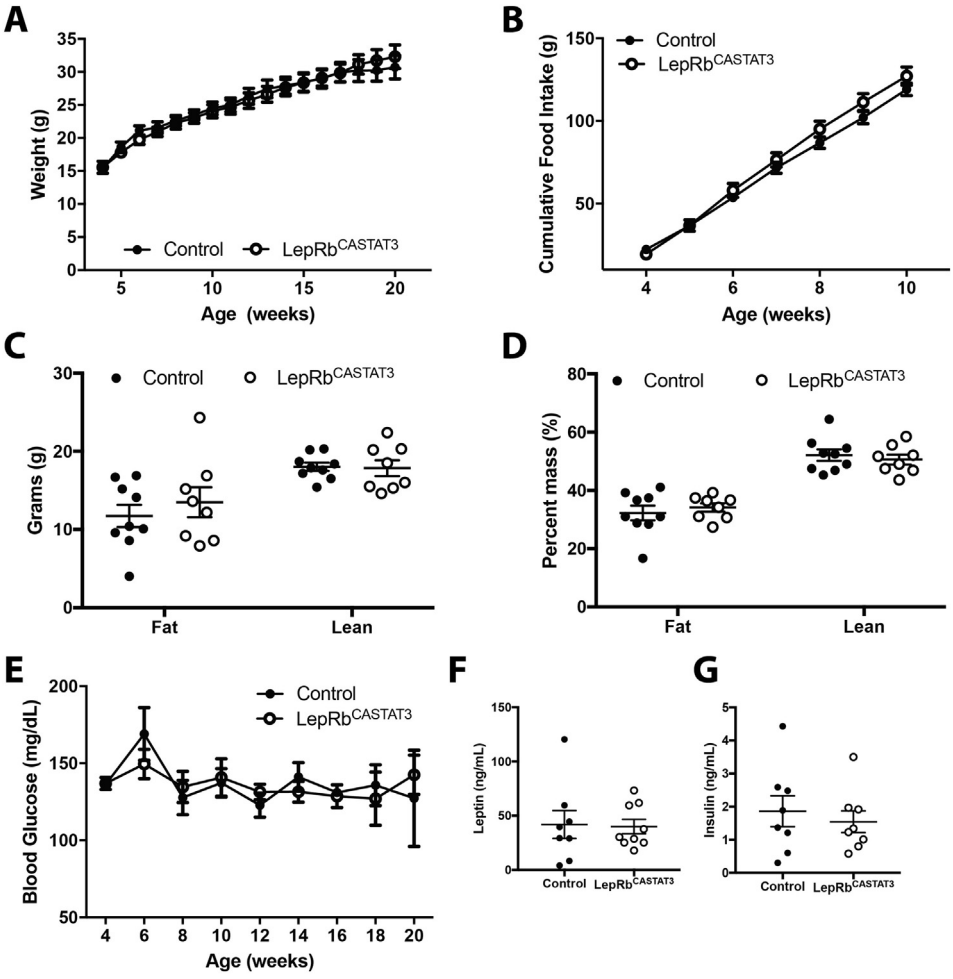

Figure 5: Enhanced STAT3 activity does not mitigate high-fat diet-induced obesity. Male LepRb^{CASTAT3} and LepRb$^{eGFP-L10a}$ (Control) mice were placed on high-fat diet and body weight **(A)** and food intake **(B)** were measured weekly. At 21–22 weeks of age, animals underwent body composition analysis **(C–D)** by NMR spectroscopy. **(E)** Unfasted blood glucose was measured biweekly. Serum from 16-week-old mice were assayed for leptin **(F)** and insulin **(G)**. Mean, quartiles and individual plots are shown; n = 8–12 per genotype. All comparisons, p = NS.

signaling in LepRb neurons beyond which increased STAT3 fails to augment the catabolic efficacy of leptin. STAT3 signaling in LepRb neurons may lie at or near its effective ceiling in DIO (but not lean) male mice.

The milder phenotypes of STAT3^{LepRb}KO, LepRb^{Y1138MUT}, and LepRb^{3F} mice relative to *ob/ob* or *db/db* mice demonstrate the existence of a second (STAT3-independent) LepRb signal that can contribute to the control of physiology by leptin [11,12,14]. While this signal can mediate a component of leptin action in the absence of Tyr_{1138}/STAT3 signaling by LepRb, the requirement for this second LepRb signal in the STAT3-dependent component of leptin action has not been clear. Since expressing CASTAT3 in LepRb neurons in *ob/ob* mice decreases their food intake, body weight, and adiposity and restores normoglycemia, STAT3 signaling can mimic substantial portions of leptin signaling in the absence of other LepRb signals.

CASTAT3 expression in LepRb neurons failed to completely normalize the phenotype of *ob/ob* mice, however, suggesting that the STAT3-independent second LepRb signal contributes to leptin action even in the presence of STAT3 signaling. Since CASTAT3 requires some phosphorylation for full activity and represents a minority of the total STAT3 in LepRb neurons, it remains possible that the failure to restore normal physiology with CASTAT3 in LepRb neurons reflects subnormal STAT3-mediated transcription in *ob/ob*;LepRb^{CASTAT3} mice.

We performed TRAP-seq to define the changes in gene expression in LepRb neurons in animals lacking STAT3 in LepRb neurons. We were unable to perform TRAP-seq on *ob/ob*;LepRb^{CASTAT3} mice due to the inability to produce enough mice to generate sufficient mRNA for his type of analysis. Our analysis revealed 19 genes that were coordinately regulated in *ob/ob* and STAT3^{LepRb}KO mice (i.e., both significantly changed in the same direction) compared to controls, however. Although many of these genes represent neuropeptides known to be important for the control of energy balance (e.g., *Agrp*, *Cartpt*, *Npy*, *Nts*, *Pomc*) and which likely contribute to the phenotype of mice lacking LepRb→STAT3 signaling, our previous analysis suggests that these do not represent the first-order transcriptional targets of leptin action [22]. Other candidates for first-order mediators of LepRb signaling include several genes whose expression is controlled by leptin under other conditions [22], with known roles in energy balance, and/or that demonstrate expression patterns consistent with LepRb specificity (including *Serpina3n*, *Fam159a*, *Npy2r*, and *Tmem176a*), as

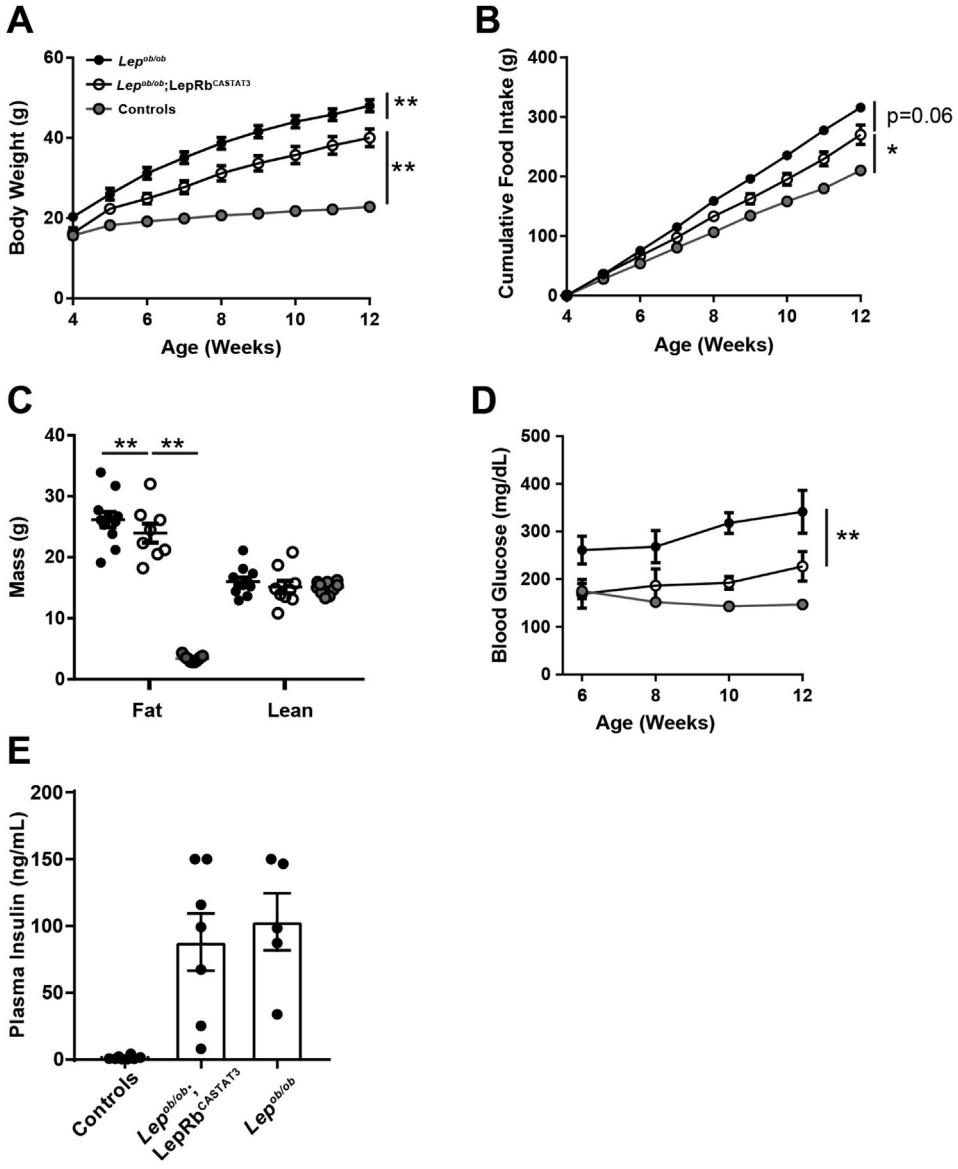

Figure 6: Improved control of energy balance and glucose homeostasis in *ob/ob*;LepRb^{CASTAT3} mice. Control (grey circles), *ob/ob*;LepRb^{CASTAT3} (open cirlces), and *ob/ob*;LepRb$^{eGFP-L10a}$ (*ob/ob*) (black circles) mice were weaned onto chow diet. Body weight **(A)** and food intake **(B)** were measured weekly. **(C)** Blood glucose was measured biweekly in *ad libitum*-fed mice. At 14–15 weeks of age, animals underwent body composition analysis **(D)** by NMR spectroscopy. Insulin concentrations are plotted in (**E**). Data are combined from male and female animals shown in Supplemental Figure 10. Mean ± SEM is shown; n = 6–12 per genotype. *p < 0.05; **p < 0.01 by ANOVA.

well as other genes whose function is not yet clear. It will be important to define the potential roles for these as we seek to understand mechanisms of leptin action. Note also that LepRb→STAT3 signaling may contribute to energy balance via the developmental control of crucial leptin-regulated circuits [37], as well as via the control of gene expression in adult animals.

A STAT3-independent second LepRb signal also contributes to leptin action; the genes that are altered in *ob/ob* compared to control mice, but that are differently expressed in STAT3^{LepRb}KO mice compared to *ob/ob* animals, may be controlled by this signal. We have identified 32 genes in this category (excluding genes that were changed in STAT3^{LepRb}KO mice but not *ob/ob* animals), but increased *Stat1* expression in the LepRb neurons of STAT3^{LepRb}KO mice may contribute to some of the observed differences (which thus may not be relevant). Unfortunately, the breeding scheme necessary to produce sufficient STAT1STAT3^{LepRb}KO animals renders impractical the notion of performing TRAP-seq on this line.

Interestingly, TF-PPI analysis of these genes revealed no transcription factors that are likely to explain their changes in expression. Indeed, while many of these genes are altered by RAD21, RAD21 is a component of the cohesion complex, rather than a transcription factor. Thus, the unidentified second (STAT3 independent) physiologically relevant LepRb signal likely mediates its effects non-transcriptionally, potentially via PI 3-kinase, ERK, and/or WNT signaling. For a full understanding of the mechanisms by which LepRb controls energy balance, it will be important to manipulate the expression of candidate genes that may contribute to leptin action in response to LepRb→STAT3 signaling, as well as to identify the cellular pathways by which the second LepRb signal controls energy balance.

AUTHOR CONTRIBUTIONS

PS, AR, JA, CP, and JCJ researched and analyzed data and proofread the manuscript. WP, MBA, DPO, and MGM designed experiments, researched and analyzed data, and wrote and edited the manuscript. MGM is the guarantor of the manuscript.

ACKNOWLEDGEMENTS:

We thank MedImmune, Inc. and Drs. James Trevaskis and Chris Rhodes for the generous gift of leptin. We thank Drs. Sergei Karalov and Jens Bruning for generously providing the CASTAT3 allele. We thank members of the Myers and Olson labs for helpful discussions. Research support was provided by the Michigan Diabetes Research Center (NIH P30 DK020572, including the Molecular Genetics, Animal Phenotyping, and Clinical Cores), the American Diabetes Association (MGM), the Marilyn H. Vincent Foundation (MGM) and the NIH (MGM: DK056731; WP: T32 GM007315; MBA: DK097861).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest relevant to this manuscript.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.01.007.

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