A novel compound heterozygous leptin receptor mutation causes more severe obesity than in Lepr<sup>db/db</sup> mice

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Abstract The leptin receptor (Lepr) pathway is important for food intake regulation, energy expenditure, and body weight. Mutations in leptin and the Lepr have been shown to cause early-onset severe obesity in mice and humans. In studies with C57BL/6NCrl mice, we found a mouse with extreme obesity. To identify a putative spontaneous new form of monogenic obesity, we performed backcross studies with this mouse followed by a quantitative trait locus (QTL) analysis and sequencing of the selected chromosomal QTL region. We thereby identified a novel Lepr mutation (C57BL/6N-Lepr<sup>L536Hfs*6-INKB</sup>), which is located at chromosome 4, exon II within the CRH2-leptin-binding site. Compared with C57BL/6N mice, Lepr<sup>L536Hfs*6</sup> develop early onset obesity and their body weight exceeds that of Lepr<sup>db/db</sup> mice at an age of 30 weeks. Similar to Lepr<sup>db/db</sup> mice, the Lepr<sup>L536Hfs*6</sup> model is characterized by hyperphagia, obesity, lower energy expenditure and activity, hyperglycemia, and hyperinsulinemia compared with C57BL/6N mice. Crossing Lepr<sup>L536Hfs*6/</sup> with Lepr<sup>L536Hfs*6/wt</sup> mice results in compound heterozygous Lepr<sup>L536Hfs*6/</sup> mice, which develop even higher body weight and fat mass than both homozygous Lepr<sup>L536Hfs*6</sup> and Lepr<sup>L536Hfs*6</sup> mice. Compound heterozygous Lepr deficiency affecting functionally different regions of the Lepr causes more severe obesity than the parental homozygous mutations.

Supplementary key words Lepr • leptin receptor mutation • obesity • compound heterozygous • genetic background

The discovery of leptin has changed our understanding of how circuits in the central nervous system that modulate energy intake and expenditure are regulated in response to changes in body energy stores (1, 2). Secreted from adipose tissue (AT), leptin binds to receptor (Lepr), a type I cytokine receptor, expressed in various tissues including neurons of the hypothalamus. Leptin pathway not only plays a key role in regulating appetite and food intake, energy expenditure, but also in fertility and glucose homeostasis. Leptin or the obese (ob) gene was discovered in 1994 as a molecule that caused severe obesity in ob/ob mice (3). Leptin plasma concentrations correlate with AT mass and decrease after weight loss (3, 4). Loss-of-function mutations in the lepin gene cause obesity in ob/ob mice (5, 6) and also, rare cases of human obesity with severe early onset and hyperphagia (7, 8).

Comparable to leptin deficient ob/ob mice, mutations in the Lepr cause obesity in Lepr<sup>db/db</sup> mice (9, 10) and other models (11). Moreover, mutations in the human LEPR cause obesity and pituitary dysfunction (12). In addition, Lepr<sup>L536Hfs*6</sup> mice develop hyperglycemia, hyperphagia, fatty liver, insulin resistance, and are infertile (13, 14).

Lepr exists in five different isoforms due to alternative splicing and enzymatic shedding in mice (10, 15, 16). The Lepr-Ra, -Rb, -Rc, and -Rd isoforms share a common extracellular and transmembrane domain while the intracellular part differs (7). The long Lepr-Rb represents the active isoform for signal transduction and is highly expressed in nuclei of hypothalamus (9, 10, 17,19). Lepr-Ra, -Rc, and -Rd vary in intracellular length and are expressed in peripheral tissues such as the liver, lung, heart, testis, and AT (17, 18). The shortest isoform, Lepr-Re, only consists of the extracellular domain and is secreted into the blood as soluble Lepr (20).

Lepr signaling is activated by leptin and results in Janus kinase (Jak)/signal transducers and activators of transcription (Stat) signaling (1, 10). While the
stochiometry of the leptin-Lepr-complex is still under discussion, Jak2 autophosphorylation is initiated by conformational changes within the leptin-Lepr-complex, followed by phosphorylation of Lepr tyrosine residues Y985, Y1077, and Y1138 (21–24). Phosphorylated Y985 recruits two proteins, which activate the mitogen-activated protein kinase (Mapk) pathway (1). Stat3 is recruited by phosphorylated Y1138. Mice lacking Stat3 in Lepr-expressing neurons develop hyperphagic obesity supporting the important role of Stat3 signaling in mediating central leptin effects (25, 26).

Here, we describe an extremely obese mouse phenotype among offspring from backcross studies with C57BL/6N−/− mice and the discovery of a novel spontaneous single nucleotide deletion in the Lepr gene (27). To further explore the impact of this novel Lepr mutation, we compared the strain with the well-established Leprdb/db mouse and generated a compound heterozygous mouse model of LeprL536Hfs*6/wt mice.

MATERIALS AND METHODS

Mice and breeding

All mice were housed in pathogen-free facilities in groups of three to four at 22 ± 2°C on a 12 h light/dark cycle. Animals were kept in individually ventilated cages under specific pathogen-free conditions at the University of Leipzig. All animals had free access to water and were fed with standard chow diet (Sniff GmbH, Soest, Germany). The C57BL/6N−/−LeprL536Hfs*6-1NKB (LeprL536Hfs*6) and LeprL536Hfs*6/db (Janvier Labs, France) mice were backcrossed onto C57BL/6NTac background for at least six generations by heterozygous breeding. C57BL/6NTac littermates served as wildtype (wt) controls. Compound heterozygous generations by heterozygous breeding. C57BL/6NTac litters were backcrossed onto C57BL/6NTac for at least six weeks up to an age of 12 or 30 weeks of life. Male data are shown, male data are available in the supplemental material part. Body weight was recorded weekly, body composition (fat and lean mass) analysis was measured at week 6, 10, 20, and 28 using EchoMRI700™ (Echo Medical Systems, Houston, TX). Intraperitoneal glucose tolerance tests (ITTs) and insulin tolerance tests (ITTs) were performed at the age of 12 and 24 weeks. GTT was performed after an overnight fast for 16 h by injecting 2 g/kg body weight glucose and measuring the blood glucose levels after tail vein incision at 0 (baseline), 10, 30, and 60 min after injection. ITT was performed in random-fed animals by injecting 0.75 unit/kg body weight human regular insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark). Glucose levels were determined in blood collected from the tail tip immediately before and 15, 30, and 60 min after the intraperitoneal injection (35). Indirect calorimetry was conducted by a Calorimetry Module (PhenoMaster V5.9.9, TSE Systems, Bad Homburg, Germany) at an age of 11 weeks as described before (36). Daily food intake was performed within the Calorimetry Module or for one week at the age of 21 weeks and was calculated as the average intake of chow within the time stated. Serum lipid concentrations were measured after an overnight fast at an age of 12 and 30 weeks.

Mice were sacrificed at 12 or 30 weeks of age by an overdose of anesthetic (Isofluran, Baxter, Unterschleißheim, Germany) after fasting for 14–16 h. Body- (naso-anal-length) and tail length, body weight, blood sugar (FreeStyle Freedom light, Abbott Diabetes Care, Wiesbaden, Germany), rectal body temperature (TH-5, Thermalert Monitoring Thermometer, Clifton, NJ), and glycosylated hemoglobin (HbA1c) (COBAS 7000, Roche, Basel, Switzerland) were measured. Liver,

inheritance of the cross hybrids (BC1) identified monogenic, recessive Mendelian intervals corresponding to region 4:70 Mb – 110 Mb with a 95%-confidence interval.

Whole genome sequencing

Two wt mice, two mice with the LeprL536Hfs*6 phenotype, and one presumably heterozygous mouse (judged by offspring with LeprL536Hfs*6 phenotype) were sequenced to identify the causative mutation of the LeprL536Hfs*6 phenotype.

Whole genome sequencing of the five mice was performed using paired-end sequencing (250 or 200 bp) on the Illumina HiSeq platform. Adaptor sequences were trimmed using cutadapt 1.9.1 (29) and the sequencing reads were aligned to the mouse reference genome mm38 with bwa 0.7.12 (30). Reads that mapped equally well to more than one genomic position were discarded. Reads were sorted using samtools L31 (31) and PCR duplicates removed with picard-tools:2.5.0 (http://broadinstitute.github.io/picard). Variants were called with the GATK pipeline version 3.6 (32) and annotated with the Variant Effect Predictor by Ensembl (33). Filtering the resulting VCF file in the QTL region at chromosome 4 to coding variants where LeprL536Hfs*6 mice were homozygous, the presumably heterozygous mouse was heterozygous and wt mice carried the wt allele identified the LeprL536Hfs*6 mutation (average sequencing depth of 88 reads across samples) as the only variant fulfilling those criteria.

Genotyping

LeprL536Hfs*6 genotyping was conducted with FastStart PCR Master (Roche, Mannheim, Germany), Primer (fw: CATGGCTTTTTCGCAAGATA, rev: TGAGCTTTCTCCAAAGATC, Biomers, Ulm, Germany), and the program 95°C 15 min, 95°C 30 s, 60°C 30 s, 72°C 1 min (35 cycles), 72°C 10 min, 4°C forever. The PCR-products were sequenced with the same primers by Sanger sequencing and analyzed for the mutation with FinchTV (Geospiza, Inc., Version 1.4.0). Genotyping of LeprL536Hfs*6 mice was conducted according to Peng et al. (34).

Phenotyping

In two setups, seven to eight mice of each mouse strain, LeprL536Hfs*6, and LeprL536Hfs*6/db of both sexes were studied from age of six weeks up to an age of 12 or 30 weeks of life. Female data are shown, male data are available in the supplemental material part. Body weight was recorded weekly, body composition (fat and lean mass) analysis was measured at week 6, 10, 20, and 28 using EchoMR700™ (Echo Medical Systems, Houston, TX). Intraperitoneal glucose tolerance tests (GTts) and insulin tolerance tests (ITTs) were performed at the age of 12 and 24 weeks. GTT was performed after an overnight fast for 16 h by injecting 2 g/kg body weight glucose and measuring the blood glucose levels after tail vein incision at 0 (baseline), 10, 30, and 60 min after injection. ITT was performed in random-fed animals by injecting 0.75 unit/kg body weight human regular insulin (40 units Actrapid; Novo Nordisk, Copenhagen, Denmark). Glucose levels were determined in blood collected from the tail tip immediately before and 15, 30, and 60 min after the intraperitoneal injection (35). Indirect calorimetry was conducted by a Calorimetry Module (PhenoMaster V5.9.9, TSE Systems, Bad Homburg, Germany) at an age of 11 weeks as described before (36). Daily food intake was performed within the Calorimetry Module or for one week at the age of 21 weeks and was calculated as the average intake of chow within the time stated. Serum lipid concentrations were measured after an overnight fast at an age of 12 and 30 weeks.

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subcutaneous (sc), and epidymal (epi) adipose tissue (AT) were immediately removed, weighed, and related to the whole-body mass to obtain relative organ weights. Organs were immediately frozen by liquid nitrogen. Serum was collected at 12 and 30 weeks and frozen.

Analytical Procedures

Adiponectin (Adiponectin mouse ELISA, Adipogen Life Sciences, Liestal, Switzerland), C-peptide (Mouse c-peptide ELISA, Alpeco, Salem, NH), insulin (mouse insulin ELISA, Merckodia AB, Uppsala, Sweden), and leptin (Mouse/Rat Leptin, R&D systems Europe Ltd., Abingdon, UK) were measured according to manufacturer’s protocol from mouse serum for both groups.

Liver samples were analyzed for glycogen content (Glycogen colorimetric Assay Kit II, Biovision, Inc., Milpitas, CA) and triglycerides (LabAssay® Lipid Tissue Mini Kit and QIAzol in QIACube) was used. Histology, adipocyte size, and its distribution were analyzed with Keyence BZ-X800 and Legend. Data are shown as mean ± SD or SEM, P values were considered as statistically significant. To develop QTL mapping and whole genome sequencing pipelines, bash and R programming languages were used (28).

Brain tissue preparation and immunohistochemical labeling

Mouse brain preparation was performed as previously described by Heiland et al. (37) at a postnatal age of 12 weeks. Two hours prior to brain preparation, mice were injected i.p. with 5 μg/g body weight of sterile leptin (Merck KGaA, Darmstadt, Germany) or vehicle (sterile 20 mM Tris/HCl, pH 8.0). The brains were cut at hypothalamus within the area of ventromedial hypothalamic nucleus (VMN) and paraventricular hypothalamic nucleus (PVN) as described previously (37). Primary antibody raised against pStat3 (cell signaling #9145, 1:500, overnight) was used and visualized with secondary peroxidase-conjugated goat antibodies and Ni-DAB as substrate for 3–5 min.

Histology, adipocyte size, and its distribution

ScAT, epiAT, and liver biopsies were fixed in 4% buffered formalin and imbedded in paraffin. Five micrometer sections were stained with hematoxylin and eosin, pictures were taken and analyzed with Keyence BZ-X800 and Keyence BZ-X800 Analyzer 1.1.8 (Neu-Isenburg, Germany).

Western blot

Proteins were isolated from tissue (after leptin i.p. injection (5 μg/g body weight) or 20 mM Tris/HCl injection for 2 h) with RIPA buffer and 5x Halt Protease and Phosphatase Inhibitor (Thermo Scientific, Rockford, IL) and stained with hematoxylin and eosin, pictures were taken and analyzed with Keyence BZ-X800 and Keyence BZ-X800 Analyzer 1.1.8 (Neu-Isenburg, Germany).

Statistics

Statistical analyses were performed with the Prism 6.0 software (GraphPad Software, San Diego, CA). The following statistical tests were performed: two-tailed unpaired Student’s t test, two-way ANOVA with Tukey correction or multiple t test with correction by Holm-Sidak method with alpha = 5.00%. The used statistical test is indicated in each figure legend. Data are shown as mean ± SD or SEM, P values < 0.05 were considered as statistically significant. To develop QTL mapping and whole genome sequencing pipelines, bash and R programming languages were used (28).

Study approval

All animal experiments were approved by the local ethics review committees and were performed according to local government guidelines of the Saxony animal protection law (Landesdirektion Sachsen, Approval No: TVV66/15, T02/19, TVV27/14, TVV10/20, Leipzig, Germany)

RESULTS

A novel spontaneous Lepr gene mutation is associated with an obese phenotype

We discovered a novel spontaneous single nucleotide deletion in the Lepr, which leads to a frame shift mutation and an early stop codon that causes the phenotype of our Lep-Rb L536Hfs*6 mice. These mice exhibit differences in body weight and proportion (Fig. 1A,
In a backcross with C57BL/6NCrl mice, our colleagues at the Max Planck Institute of Experimental Medicine, Göttingen, noticed a spontaneous development of obesity in one offspring. Three animals, one obese female and two lean brothers, were transferred to our facilities. The obese female mouse was used to

Fig. 1. QTL analysis and Lepr<sup>L536Hfs*6</sup> mutation. A: Pictures of 12-week-old female mice on mm scale. B: Quantitative trait locus (QTL) identification on chromosome 4 with R/qtl using Haley Knott regression (37). C: Nucleotide and amino acid sequences of the QTL region on chromosome 4 resulted in the identification of the point mutation at position 536 causing the novel Lepr<sup>L536Hfs*6</sup> spontaneous Lepr mutant mice. The mutation results in a deletion in exon 11 which leads to a frame shift and stop codon within the next 5 aa. D: Exemplary sequence of a wt, a heterozygous Lepr<sup>L536Hfs*6/wt</sup>, and a homozygous Lepr<sup>L536Hfs*6</sup> mouse. F: Schematic representation of the Lepr protein with highlighted positions for Lepr<sup>L536Hfs*6</sup> and Lepr<sup>db/db</sup> mutations as well as cytokine receptor homology (CRH1/2), Immunoglobulin domain (Ig), fibronectin III domain (FNIII).

age- and gender-matched to wt, Lepr<sup>db/db</sup>, and compound heterozygous Lepr<sup>L536Hfs*6/db</sup> mice.)

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screen for known obese gene mutations. Since the obese female did not produce pups, according to the infertility of homozygous Lepr<sup>L536Hfs*6</sup> mice, the lean brothers were crossed with C57BL/6N female mice. One of the two lean brothers was the founder and produced two litters, which were inbred with each other. Half of the inbred offspring were crossed with I29S6/EnNTac to generate F1 offspring. F1 were then backcrossed (BC) to males, which generated obese and lean pups ([Lepr<sup>L536Hfs*6</sup> × I29S6Ev]<sub>F1</sub> × Lepr<sup>L536Hfs*6</sup>) BC1. Genomic DNA samples from all 57 BC1 animals were analyzed using an 822 single nucleotide (SNP) panel. We mapped the body weight phenotype to a QTL locus on chromosome 4, 69 Mb flanked by SNPs rs27880559 and rs28253902, Fig. 1B).

To determine the physiological consequences of Lepr<sup>L536Hfs*6</sup> on glucose metabolism, we performed i.p. glucose tolerance test (GTT) at the age of 24 weeks, and monitored glycosylated hemoglobin (HbA1c), insulin and C-peptide levels at the end of the observation period. Fasting blood glucose levels as well as HbA1c were increased in Lepr<sup>L536Hfs*6</sup> compared with wt mice (Fig. 3A, B). Fasting serum insulin concentrations were significantly higher in both obese mouse models compared with wt (Fig. 3C). Moreover, Lepr<sup>L536Hfs*6</sup> mice had significantly elevated C-peptide levels compared with Lepr<sup>db/db</sup> and wt mice (Fig. 3D). GTTs revealed an impaired glucose tolerance in both obese models compared with wt mice. Higher glucose levels were found in Lepr<sup>L536Hfs*6</sup> compared with Lepr<sup>db/db</sup> mice at 30 and 60 min after glucose injection (Fig. 3E). Only wt animals reached the initial blood glucose level while Lepr<sup>L536Hfs*6</sup> mice stayed at 13.8 mmol/l and Lepr<sup>db/db</sup> at 9.1 mmol/l after 120 min.

Consequences of Lepr<sup>L536Hfs*6</sup> mutation on lipid parameters and liver fat metabolism

As shown in Table 1, circulating serum levels of triglycerides, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol were significantly elevated in Lepr<sup>L536Hfs*6</sup> compared with wt mice. Total cholesterol as well as HDL-cholesterol was significantly higher in Lepr<sup>L536Hfs*6</sup> as in Lepr<sup>db/db</sup> mice. Adiponectin serum concentrations of Lepr<sup>L536Hfs*6</sup> and Lepr<sup>db/db</sup> were not significantly different.

Hepatic triglyceride content was significantly increased in both Lepr<sup>L536Hfs*6</sup> (637 μg/mg tissue) and Lepr<sup>db/db</sup> (674 μg/mg tissue) compared with wt mice (186 μg/mg tissue), while hepatic glycogen content was not different (Fig. 4A, B). Histological slides stained with hematoxylin and eosin (Fig. 4C) underlie the higher hepatic liver fat accumulation seen in both, Lepr<sup>db/db</sup> and Lepr<sup>L536Hfs*6</sup> mice compared with wt. Histological analysis of scAT and epiAT illustrated an increased mean adipocyte size of Lepr<sup>L536Hfs*6</sup> compared with Lepr<sup>db/db</sup> in both fat depots (Fig. 4D, E).

Energy expenditure and spontaneous activity are decreased in Lepr<sup>L536Hfs*6</sup> mice

Metabolic chamber analysis displayed differences between wt animals and both obese mouse models.
Looking at the differences in activity and volume of oxygen consumption (V(O₂)) as well as CO₂ emission (V(CO₂)) between light and dark phase, only wt animals had an active phase during the dark and a recovery phase during the light phase (Fig. 5A, B). In contrast, \textit{Lepr}^{L536Hfs*6} and \textit{Lepr}^{db/db} mice had a constantly low mean activity as well as a steady oxygen consumption and carbon dioxide emission in the light and in the dark phase indicating a disturbed circadian rhythm. Significantly higher volumes of O₂ and CO₂ were detected in wt mice compared with \textit{Lepr}^{L536Hfs*6} and \textit{Lepr}^{db/db}.

Mean activity, measured by x-y-axis counts, spontaneous activity, measured by z-axis counts, and voluntary running wheel activity were decreased during the dark in \textit{Lepr}^{L536Hfs*6} and \textit{Lepr}^{db/db} mice compared with wt mice. Spontaneous \textit{Lepr}^{L536Hfs*6} mutation leads to obesity and increased food intake.
wt animals (Fig. 5C–E). In contrast to wt animals, both obese models represented lower energy expenditure during light and dark phase (Fig. 5F).

**Leptin signaling pathway**

The metabolic activity of leptin is mainly determined in the hypothalamus by binding to the Lepr and triggering Stat3 activation in neurons of the arcuate nucleus and ventromedial nucleus of the hypothalamus (39, 40). To determine leptin-Lepr signaling in the brain, Lepr<sup>L536Hfs*6</sup>, Lepr<sup>db/db</sup>, and wt control mice were fasted overnight for ∼16 h prior to injection of leptin and sacrificed 2 h later for immunohistochemistry as previously published (38) or prepared for western blot. As shown in Fig. 6A, pStat3 positive cells were detected in the area of VMH (ventro-medial hypothalamus), DM (dorsomedial hypothalamic nucleus), and LH (lateral hypothalamic area) of wt, whereas Lepr<sup>db/db</sup> and Lepr<sup>L536Hfs*6</sup> mice did not exhibit pStat3 positive nuclei, indicating a complete unresponsiveness to leptin in Lepr<sup>L536Hfs*6</sup> mice.

Western blot data of Jak2, Stat3, and Socs3 showed no genotype-depended differences in protein concentration. After activation of the signal cascade via leptin stimulation, a 14.5-fold increase in the ratio of pAkt/

| TABLE 1. Serum lipid concentrations. |
|--------------------------------------|
| Serum parameters | wt | Lepr<sup>L536Hfs*6</sup> | Lepr<sup>db/db</sup> |
|--------------------|----|----------------|------------------|
| Triglycerides (mmol/l) | 1.018 ± 0.335 | 2.064 ± 0.438<sup>a</sup> | 1.392 ± 0.138<sup>b</sup> |
| Total cholesterol (mmol/l) | 2.288 ± 0.458 | 7.526 ± 0.847<sup>b</sup> | 5.798 ± 0.836<sup>c</sup> |
| HDL-cholesterol (mmol/l) | 1.790 ± 0.351 | 5.644 ± 0.708<sup>b</sup> | 4.432 ± 0.651<sup>c</sup> |
| LDL-cholesterol (mmol/l) | 0.384 ± 0.111 | 1.326 ± 0.164<sup>b</sup> | 0.814 ± 0.228<sup>c</sup> |
| Free fatty acids (mmol/l) | 1.142 ± 0.336 | 1.854 ± 0.147 | 1.358 ± 0.384<sup>c</sup> |
| Adiponectin (μg/ml) | 149.592 ± 61.928 | 26.704 ± 16.206<sup>b</sup> | 31.457 ± 4.730<sup>c</sup> |

Data shown as mean ± SD, at an age of 30 weeks, n = 5, P < 0.05 are shown in bold, tested by unpaired t test (Adiponectin), two-way ANOVA (all others).

<sup>a</sup>Lepr<sup>L536Hfs*6</sup> versus Lepr<sup>db/db</sup>
<sup>b</sup>Lepr<sup>L536Hfs*6</sup> versus wt.
<sup>c</sup>Lepr<sup>db/db</sup> versus wt.
Akt was measured in wt, while no change of the ratio was measured in Lepr\textsuperscript{L536Hfs*6} and Lepr\textsuperscript{db/db} mice. The ratios of pErk1/Erk1 and pErk2/Erk2 were also increased in wt and for Lepr\textsuperscript{db/db} mice of pErk2/Erk2 after leptin injection, while protein concentrations in Lepr\textsuperscript{L536Hfs*6} were not affected significantly (Fig. 6B, C).

**Compound heterozygosity of Lepr\textsuperscript{L536Hfs*6/db} results in higher body weight whole body fat mass and HbA1c levels**

The presence of two different mutant alleles at a particular gene locus, one on each chromosome of a pair is called compound heterozygous. Starting at 5 weeks until the end of the observation period, compound heterozygous Lepr\textsuperscript{L536Hfs*6/db} gained more weight compared with the three comparator genotypes (Fig. 7A). Lepr\textsuperscript{L536Hfs*6/db} had a higher scAT weight than Lepr\textsuperscript{L536Hfs*6}, whereas relative epiAT and liver weights were indistinguishable between the obesity mouse models (Fig. 7B). Focusing on body composition, Lepr\textsuperscript{L536Hfs*6/db} animals had a higher whole-body fat mass at 6 and 10 weeks of age than both homozygous animals (Fig. 7C). Interestingly, both body weight and naso-to-anal length were higher in compound

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Fig. 4. Consequences of the Lepr\textsuperscript{L536Hfs*6} mutation on liver and adipose tissue. Comparison of Lepr\textsuperscript{L536Hfs*6} to C57BL/6N wildtype (wt) and Lepr\textsuperscript{db/db} mice with regard to liver (A) triglyceride content (n = 4–5, unpaired t test, mean ± SD) and (B) glycogen content (n = 5, unpaired t test, mean ± SD). C: Histological differences as displayed in representative slides of liver stained with hematoxylin and eosin. Scale bars represent 100 μm. D–E: Comparison of adipose tissue between Lepr\textsuperscript{L536Hfs*6} and Lepr\textsuperscript{db/db} mice. D: Representative histological slides of subcutaneous adipose tissue (scAT) stained with hematoxylin and eosin. Scale bars represent 100 μm. E: Representative histological slides of epigonadal adipose tissue (epiAT) stained with hematoxylin and eosin. Scale bars represent 100 μm. EpiAT fat cell size distribution and mean fat cell size in epiAT from Lepr\textsuperscript{L536Hfs*6} compared with Lepr\textsuperscript{db/db} mice (n = 581–600, unpaired t test, median ± min/max).
heterozygous mice than in Lepr<sup>L536Hfs*6</sup> mice (Fig. 7D). While body temperature (Fig. 7E) and fasting blood glucose (Fig. 7F) were not affected by the Lepr compound heterozygosity, HbA1c (Fig. 7G) was higher in Lepr<sup>L536Hfs*6</sup>/<sup>db</sup> compared to wildtype (wt) mice independently of the light or dark phase (Lepr<sup>L536Hfs*6</sup> and Lepr<sup>db/db</sup> vs. wt). C: Mean activity level (n = 5–6, two-way ANOVA), D: Spontaneous activity (n = 6, two-way ANOVA). E: Voluntary running wheel activity (n = 5–6, two-way ANOVA), and F: energy expenditure (EE, n = 6, two-way ANOVA) during the light and dark phase in Lepr<sup>L536Hfs*6</sup>, wt and Lepr<sup>db/db</sup> mice. Data shown as mean ± SEM (A, B) or mean ± SD (C–F), *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Lepr expression and Jak/Stat signaling in Lepr<sup>L536Hfs*6</sup>/<sup>db</sup>

Protein expression of full-length Lepr in hypothalamus was reduced in Lepr<sup>L536Hfs*6</sup> and Lepr<sup>db/db</sup> compared to wt levels (Fig. 8A), while gene expression of Lep-Ra, Lep-Rb and total Lepr was not different between the four different genotypes (Fig. 8B).

Immunohistochemical analysis of Stat3 phosphorylation in hypothalamus upon leptin stimulation displayed a lack of Stat3 activation like in the homozygous mouse models (Fig. 8C). Western blot data for proteins of the STAT signaling in the hypothalamus of
Fig. 6. Characterization of hypothalamic signaling in Lepr<sup>L536Hfs*6</sup>, wildtype, and Lepr<sup>db/db</sup> mice. A: Immunohistochemistry of pStat3 on brain slides. Wildtype (wt) +Leptin with pStat3 positive cells within VMH (ventromedial hypothalamus), DM (dorsomedial hypothalamic nucleus), and LH (lateral hypothalamic area), wt -Leptin, Lepr<sup>L536Hfs*6/+</sup>Leptin, and Lepr<sup>db/db</sup> +/−Leptin without signal. Mice were fasted for 14–16 h prior to leptin injection (i.p. 5 μg/g body weight, +Leptin) or vehicle administration (−Leptin), 2 h later mice were sacrificed, brains were fixed by 4% PFA and removed, scale 200 μm. B: Induction of leptin signaling measured by western blot quantification of Jak2, Stat3, pAkt, Akt, Erk1/2, pErk1/2, and Socs3 in total hypothalamus protein. β-Actin served as loading control. Mice were fasted for 14–16 h prior to leptin injection (i.p. 5 μg/g body weight, +Leptin) or vehicle administration (−Leptin), and brains were removed 2 h later and hypothalamus was dissected out of the brain. Data shown as mean ± SEM, two-way ANOVA, n = 3/group, *P < 0.05.
Lepr<sup>L536Hfs*6</sup>/db did not reveal a response to leptin treatment for all measured proteins of the Jak/Stat signaling cascade in comparison to Lepr<sup>L536Hfs*6</sup>/db mice without leptin treatment (Fig. 8D, E).

Characterization of energy metabolism in Lepr<sup>L536Hfs*6</sup>, Lepr<sup>L536Hfs*6/6</sup>, wt and Lepr<sup>db/db</sup> mice

Analyzing the results of the metabolic chamber, Lepr<sup>L536Hfs*6/6</sup> mice had comparable rate of oxygen consumption and carbon dioxide production as homozygous Lepr<sup>L536Hfs*6</sup> and Lepr<sup>db/db</sup> mice (Fig. 9A, B). Mean activity, voluntary running wheel activity and energy expenditure were also not significantly between Lepr<sup>L536Hfs*6</sup>, Lepr<sup>L536Hfs*6/6</sup> and Lepr<sup>db/db</sup> mice, while spontaneous activity of Lepr<sup>L536Hfs*6/6</sup> mice was increased in light phase in comparison to all other analyzed genotypes, in dark phase it was only higher compared with Lepr<sup>L536Hfs*6</sup> and Lepr<sup>db/db</sup> mice (Fig. 9C–F). Food intake was elevated in the leptin receptor deficient mouse models compared with wt mice, but not significantly between Lepr<sup>L536Hfs*6</sup>, Lepr<sup>L536Hfs*6/6</sup> and Lepr<sup>db/db</sup> mice in dark phase. During light phase, there was no difference in food intake between the genotypes (Fig. 9G).
DISCUSSION

We discovered a novel spontaneous mutation within the Lepr gene, which causes severe obesity in homozygous mice. The 1 bp deletion on mouse chromosome 4 within exon 11 at position 1607 (counting from start codon) of the Lepr gene leads to a frameshift and a predicted early stop codon 5 aa later. Exon 11 is part of the cytokine receptor homology 2 (CRH2), which is the receptors’ main leptin-binding region (41). The early stop codon in this region leads to a decreased expression of full-length Lepr at the cell surface, leading to unfolded protein response (supplemental Fig. S2). In the hypothalamus, leptin-induced phosphorylation of Stat3 revealed that leptin signaling was differentially affected in neurons localized in the DM, VMN and LH of the mutant mice. Abrogated signaling in response to exogenous leptin was observed in the DM, VMN, and LH, suggesting that the Lepr<sup>L536Hfs*6</sup> product predominates in these neurons and prevents responsiveness to leptin in this region. With the lower leptin binding and pStat3 signal transduction, energy balance is disturbed in Lepr<sup>L536Hfs*6</sup> (4). We compared our new monogenic mouse model with the extensively characterized Lepr deficient Lepr<sup>db/db</sup> mice. Since it is known that the background significantly influences metabolic parameters (42), we decided to backcross Lepr<sup>L536Hfs*6</sup> and Lepr<sup>db/db</sup> mice on the same BL/6NTac background to make sure that the observed phenotype of
Lepr<sup>L536Hfs*6</sup> mice is the result of differences in Lepr mutations and not background driven. Lepr<sup>L536Hfs*6</sup> mice are characterized by significantly higher body weight, hyperphagia, lower energy expenditure, and spontaneous activity compared with wt littermate controls. Our model underscores previous discoveries that genes that are critical for body weight regulation are mainly expressed in the hypothalamus and that mutated genes in monogenic forms of obesity play a key role in the leptin and melanocortin pathway (43, 44). In this context our monogenic mouse model of obesity considered confirmatory. However, using Lepr<sup>L536Hfs*6</sup>/db mouse cross-breeding, we provide evidence that compound heterozygous mutation at the Lepr locus results in an even more pronounced obesity and impaired glucose metabolism phenotype than one would expect from the parental models. Our data may stimulate further human genetics research to test the hypothesis that compound heterozygous variants in the Leptin and or LEPR genes contribute to an increased obesity risk in sub-populations. Indeed, it has been recently shown that carriers of compound heterozygous mutations in the adenylate cyclase 3 (ADCY3) gene cause severe childhood obesity similar to ADCY3 homozygous mutations (45).

**Fig. 9.** Characterization of energy metabolism in Lepr<sup>L536Hfs*6</sup>, Lepr<sup>L536Hfs*6/db</sup>, wildtype, and Lepr<sup>db/db</sup> mice. All mice were housed in a metabolic chamber for 72 h. Data of two consecutive dark and light phases (in total 48 h) after a 24 h adaptation phase. A: Oxygen consumption (V(O<sub>2</sub>), n = 4–6, two-way ANOVA) and (B) CO<sub>2</sub> production (V(CO<sub>2</sub>), n = 4–6, two-way ANOVA) are significantly lower in Lepr<sup>L536Hfs*6</sup>, Lepr<sup>L536Hfs*6/db</sup>, and Lepr<sup>db/db</sup> compared with wildtype (wt) mice independently of the light or dark phase (* Lepr<sup>L536Hfs*6</sup>, Lepr<sup>L536Hfs*6/db</sup> and Lepr<sup>db/db</sup> vs. wt). C: Mean activity (n = 4–6, two-way ANOVA), (D) spontaneous activity level (n = 4–6, two-way ANOVA), (E) voluntary running wheel activity (n = 4–6, two-way ANOVA), (F) energy expenditure (EE, n = 4–6, two-way ANOVA) and (G) Food intake (n = 4–6, two-way ANOVA) during the light and dark phase in Lepr<sup>L536Hfs*6</sup>, Lepr<sup>L536Hfs*6/db</sup>, wt and Lepr<sup>db/db</sup> mice. Data shown as mean ± SEM (A, B) or mean ± SD (C–F), *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
In collaboration with Antje Körner, we recently found that heterozygosity of newly identified LEPR variants may contribute to the development of childhood morbid obesity (46). The finding that Lepr\textsuperscript{L536Hfs*6} mice have higher body weight and expandability of adipose tissue than Lepr\textsuperscript{db/db} mice further suggests that the genetic location of the mutation represents an important factor defining the phenotype. These differences between monogenic obesity models affecting the same gene at different loci are also reflected at the level of obesity-related metabolic alterations.

Lepr\textsuperscript{L536Hfs*6} mice have significantly higher fasting glucose, HbA1c, fasting insulin and C-peptide serum concentrations than wt mice. In comparison to other mouse models with mutations in extracellular parts of Lepr, Lepr\textsuperscript{L536Hfs*6} have worsened glucose tolerance than db\textsuperscript{3} (with a peak at app. 21 mmol/l at age of 2 months) and a similar glucose dynamic as Lepr\textsuperscript{G506S} (at the age of 20 weeks) (11, 47). Although Lepr\textsuperscript{G506S}, db\textsuperscript{3}, and our Lepr\textsuperscript{L536Hfs*6} have in common that Lepr mutations are located in the extracellular part (11, 47). Our model suggests that the exact variant may have an influence on glucose and insulin response, which seems to be distinct to each model. Interestingly, circulating C-peptide was 2-fold higher in Lepr\textsuperscript{L536Hfs*6} mice compared with Lepr\textsuperscript{db/db} mice suggesting that different Lepr mutations might affect beta cell function distinctly. However, the exact mechanisms underlying these differences are not clear. Based on the altered leptin signaling observed in Lepr\textsuperscript{L536Hfs*6} mice, we speculate that worse glucose tolerance results from diminished function of neurons in the VMN (48). Although, we cannot definitively rule out an indirect effect caused by the different body composition and higher scAT fat mass in Lepr\textsuperscript{L536Hfs*6} mice compared with Lepr\textsuperscript{db/db} mice, we hypothesize that elevated scAT fat mass may contribute to differences in glucose tolerance by differences in adipokine secretion patterns.

Interestingly, Lepr\textsuperscript{L536Hfs*6} mice did not have significantly altered leptin or adiponectin serum concentrations compared with Lepr\textsuperscript{db/db} mice. We can therefore only hypothesize that other adipokines or metabolites may contribute to the observed differences in glucose metabolism parameters between Lepr\textsuperscript{L536Hfs*6} and Lepr\textsuperscript{db/db} mice.

Importantly, spontaneous Lepr knock-down models may differ in the extent of how leptin mediated signaling, leptin binding, receptor expression on cell surfaces, receptor dimerization and activation are affected by a specific mutation. In a previous human study, it has been shown that even despite computational structure prediction suggested impaired leptin binding for all LEPR variants, biological experiments revealed important mutation-related differences in leptin binding or LEPR cell surface expression (46). Of the five Lepr isoforms, only the long Lepr isoform (Lep-Rb) has a functional intracellular domain, which mediates leptin signaling (9, 10). Conformational changes within the leptin-Lepr-complex initiate Jak2 autophosphorylation followed by phosphorylation of three tyrosine residues (Y985, Y1077, and Y1138) only present in the intracellular domain of Lep-Rb. Phosphorylation of Y1138 causes recruitment of signal transducer and activator of transcription (Stat3) to the Lep-Rb and Jak2 complex (26). Activation of Stat3 signaling pathway has been shown to mediate the effects of leptin on melanocortin production and energy homeostasis (25). In both Lepr\textsuperscript{L536Hfs*6} and Lepr\textsuperscript{db/db} mice, we could not detect pStat3 positive cells upon leptin treatment in VMH, DM, and LH, indicating a complete unresponsiveness to leptin upon this mutation. In addition, Lepr\textsuperscript{L536Hfs*6} show lower body temperature and energy expenditure, in accordance with other published Lepr mutated obese mouse models (49). Moreover, Lepr\textsuperscript{L536Hfs*6} may be considered as a model for fatty liver disease and impaired lipid metabolism due to the fact that the phenotype showed is similar to other reported fatty liver disease animal models (50).

In humans, the first family with a LEPR mutation was identified by Clement et al. (12). The prevalence of pathogenic LEPR mutations in a cohort with severe, early-onset obesity was 3% (51). In the homozygous mutation subjects, a truncation of the receptor before the transmembrane domain completely abolishes leptin signaling, leading to a form of massive obesity similar to that seen with leptin deficiency, along with significant growth retardation and central hypothyroidism (12). Patients with LEPR mutations typically develop very high circulating leptin levels. Chromatography of circulating leptin revealed that the hormone was bound to the truncated LEPR, leading to an increased plasma leptin half-life, although AT leptin expression correlates with fat mass (12). Therefore, the full knockout of the leptin pathway in humans was not responsible for the compensatory hypersecretion of leptin (32). Interestingly, the two subjects with the LEPR mutation in the study of Clement et al. neither developed type 2 diabetes nor dyslipidemia suggesting that mechanisms compensating for impaired leptin signaling are not entirely translatable between mice and men (12).

Compound heterozygous mutations are rarely found for LEPR and only seven different mutations in eight cases are described in humans up to now (46, 51, 53–56). All of them had early onset of extreme obesity, seven had hyperphagia, three had altered growth and elevated leptin levels, two had hypogonadotropic hypogonadism, dyslipidemia, and hyperinsulinemia (46, 51, 53–56). A study of Huvenne et al. analyzed 535 patients with severe obesity, only three of them had compound heterozygous mutations (54). These subjects probably had a truncated and/or damaged protein which results in a short LEPR only consisting of the extracellular part (54). We generated a genotype similar to this human example (36) by a crossbreeding strategy. We aimed to test the hypothesis that Lepr\textsuperscript{L536Hfs*6}\textsuperscript{db} mice will develop a phenotype distinct to that of the
parental carriers of the homozygous mutations. \( \text{Lepr}^{L536Hfs*6/\text{db}} \) results in a deletion of the transmembrane and intracellular part of Lepr. Unexpectedly, \( \text{Lepr}^{L536Hfs*6/\text{db}} \) mice develop even more pronounced body weight gain upon hyperphagia, adiposity, and impairment of glucose metabolism than homozygous \( \text{Lepr}^{L536Hfs*6} \) and \( \text{Lepr}^{\text{db/db}} \) mice.

In contrast to humans with compound heterozygous \( \text{LEPR} \) mutations (46, 54), \( \text{Lepr}^{L536Hfs*6/\text{db}} \) mice do not have high circulating leptin levels at the age of 12 weeks. The higher extent of obesity and impairment of glucose metabolism in \( \text{Lepr}^{L536Hfs*6/\text{db}} \) compared with parental homozygous \( \text{Lepr} \) mutations cannot easily be explained, because both mutations are supposed to result in a functional knockout of the Lepr. In \( \text{Lepr}^{\text{db/db}} \) mice, there is no Lep-Rb protein produced and the transcript that should encode the Lep-Rb isoform is replaced by a novel transcript encoding the Lep-Ra isoform (9). Based on this assumption, \( \text{Lepr}^{L536Hfs*6/\text{db}} \) mice should either produce no Lepr protein at all or only a soluble truncated version, because \( \text{Lepr}^{\text{db/db}} \) used as controls in our study can still produce the Lep-Ra isoform. However, although we could confirm that \( \text{Lepr}^{\text{db/db}} \) indeed produce the Lep-Ra isoform, both \( \text{Lepr}^{L536Hfs*6} \) and \( \text{Lepr}^{L536Hfs*6/\text{db}} \) displayed Lep-Ra gene expressions that were indistinguishable to those produced by \( \text{Lepr}^{\text{db/db}} \) mice. It has been proposed that the long intracellular Lepr domain determines intracellular signal transduction and that functional absence of this Lepr form leads to the severe obese phenotype found in \( \text{Lepr}^{\text{db/db}} \) mice (9). Despite the fact that we did not find significant differences in Lepr isoform gene expression between the genotypes, we hypothesize that forced AAV-vector mediated expression of the short Lepr-Ra isoform in \( \text{Lepr}^{L536Hfs*6} \) mice could recapitulate the exacerbated phenotype of \( \text{Lepr}^{L536Hfs*6/\text{db}} \) compound heterozygous mice.

We cannot exclude that both homozygous mutations result in an incomplete loss of Lepr despite the fact that soluble Lepr was not detectable by ELISA and western blot data suggest a lower concentration of long Lepr in homozygous obese mice compared with wt. Incomplete deletion of the Lepr or residual signaling activity of Lepr fragments may account for phenotype differences between \( \text{Lepr}^{L536Hfs*6} \) and \( \text{Lepr}^{\text{db/db}} \) mice that are reduced in the \( \text{Lepr}^{L536Hfs*6/\text{db}} \) model.

Taken together, we identified a novel spontaneous \( \text{Lepr} \) mutation, which causes obesity, hyperphagia, and impaired glucose metabolism similar to the well-described \( \text{Lepr}^{\text{db/db}} \) (13, 14) or other Lepr knockout mouse models (11).

CONCLUSION

Beyond the known associations between \( \text{Lepr} \) mutations and a severely obesity phenotype, our study suggests that the phenotype of monogenic Lepr deficient mice depends on the molecular localization of the \( \text{Lepr} \) mutation. This assumption is further supported by the phenotype of compound heterozygous \( \text{Lepr}^{L536Hfs*6/\text{db}} \) mice that developed more severe obesity and hyperglycemia than the parental carriers of homozygous mutations. Although our model does not reveal a new obesity mechanism, \( \text{Lepr}^{L536Hfs*6} \) and \( \text{Lepr}^{L536Hfs*6/\text{db}} \) may serve the scientific community as an additional model to study mechanisms of extreme adipose tissue expandability and mechanisms how heterozygosity in candidate genes of obesity may worsen the phenotype. The latter model might be of particular interest to better understand how allelic and locus heterogeneity affects variation in human genotype-phenotype associations.

Data availability

Data are available on request to Nora Klöting (Helmholtz Institute for Metabolic, Obesity and Vascular Research (HI-MAG), nora.kloeting@helmholtz-muenchen.de)

Supplemental data

This article contains supplemental data.

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Author contributions

N. K. conceptualization; C. B. and H. O. H. formal analysis; M. B. and N. K. funding acquisition; C. B., H. O. H., T. H., S. D., C. H., J. L., and M. D. investigation; C. B., H. O. H., M. D., and N. K. methodology; N. K. project administration; S. R., M. D., P. K., M. B., and N. K. resources; E. G.-J. and N. K. supervision; C. B., H. O. H., and C. H. visualization; C. B. writing—original draft; H. O. H., T. H., S. D., C. H., E. G.-J., S. R., M. D., P. K., M. B., and N. K. writing—review and editing.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations
ADCY3, adenylyl cyclase 3; AT, adipose tissue; BC, backcross; DM, dorsomedial hypothalamic nucleus; epi, epigonalad; fl, flank; HK, hepatic kinase; IGF-1, insulin-like growth factor-1; JH, juvenile hypothalamic area; Mapk, mitogen-activated protein kinase; PVN, paraventricular hypothalamic nucleus; QTL, quantitative trait locus; sc, subcutaneous; Stat, signal transducers and activators of transcription; VMN, ventromedial hypothalamus.

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