Functional Consequences of the Human Leptin Receptor (*LEPR*) Q223R Transversion

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Perturbations in the functional integrity of the leptin axis are obvious candidates for mediation of altered adiposity. In a large number of genetic association studies in humans, the nonconservative *LEPR* Q223R allele has been inconsistently associated with adiposity. Subtle, long-term effects of such genetic variants can be obscured by effects of the environment and other confounders that render definitive inferences difficult to reach. We directly assessed the biological effects of this variant in 129P3/J mice segregating for the humanized *Lepr* allele at codon 223. No effects of this allele were detected on body weight, composition, or energy expenditure in animals fed diets of varying fat content over periods as long as 235 days. *In vitro*, Q223R did not affect leptin signaling as reflected by activation of STAT3. We conclude that Q223R is unlikely to play a significant role in regulation of human adiposity. This approach to vetting of human allelic variation might be more widely used.

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INTRODUCTION

Leptin plays a central role in the control of human body weight as evidenced by the profound effects on adiposity of null alleles for leptin (*LEP*) (1–4) or leptin receptor (*LEPR)* (5). A more difficult question is to what degree alleles of these genes—with more subtle effects on function or expression—contribute to human adiposity. This question is part of an even larger one: to what extent does allelic variation in genes in the known molecular pathways regulating body weight contribute in additive or epistatic ways to human adiposity?

In mice, haploinsufficiency for *Lep* or *Lepr* increases adiposity; and these effects are additive (6). In humans, comparable effects have been described for *LEP* (7). Genes in the signaling pathways engaged by leptin and other peripheral (insulin, ghrelin, peptide YY) and central (melanocortin 4 receptor, neuropeptide Y, proopiomelanocortin, carboxypeptidase E) molecules have been examined by linkage and association studies for contributions to human adiposity. For example, the neuropeptide Y Leu7Pro polymorphism has been associated with higher BMI in premenopausal women (8) and young Dutch males (9). The ghrelin Leu72Met variant is associated with the age of onset of obesity (10). Positive associations with BMI have also been found with common variants of proopiomelanocortin and the melanocortin 4 receptor (11–13). For *LEP,* associations of the G19A and Gln25Gln (CAA to CAG) polymorphisms with increased body weight have been reported (14,15). Three missense variants Q223R, K109R, K656N in *LEPR* with allele frequencies >5% have been described, and their associations with adiposity examined (16,17) (see below).

LEPR is a member of the class I cytokine receptor family with six alternative transcripts. In the mouse, the longest isoform (*Lepr*b) is predominately expressed in hypothalamic and other CNS neurons that control food intake, energy balance, and neuroendocrine function, while the shorter isoforms are predominately expressed in several peripheral tissues where their physiological roles are not entirely clear (18–21). Leptin binding to mouse LEPRb (mLEPRb) stimulates the activity of the associated Janus kinase 2, which initiates intracellular signaling by phosphorylating three sites on the intracellular domain of mLEPRb leading to transcriptional regulation of neuropeptides and other molecules that exert effects on energy homeostasis (22–33). All six LEPR isoforms share the same extracellular domain consisting of two cytokine receptor homology (CRH) domains, CRH1 and CRH2, separated by an immunoglobulin (Ig)-like domain and followed by three fibronectin type III (F3) domains (**Figure 1**). CRH2 is sufficient for leptin binding and activation of LEPR (34,35). The CRH1

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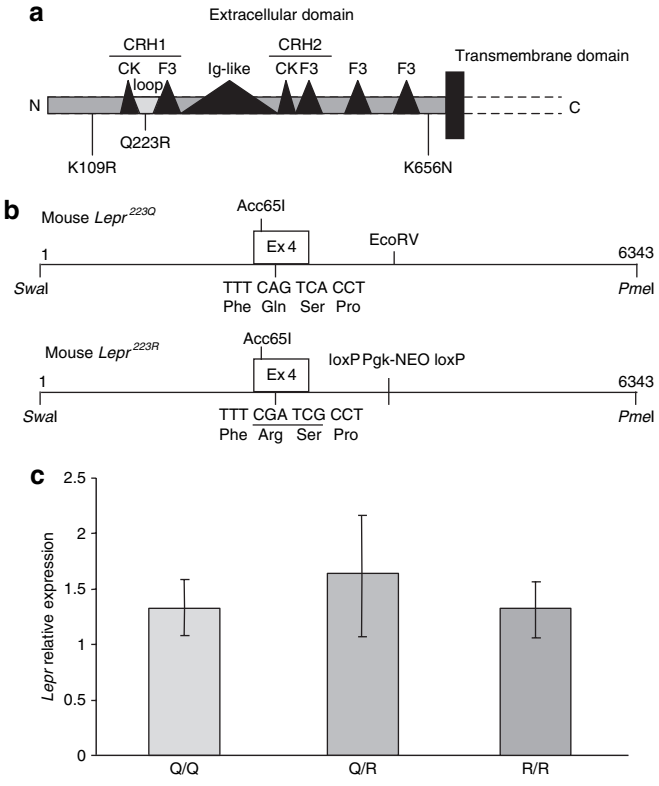

Figure 1 Extracellular domain of the leptin receptor. (**a**) Subdomains and locations of common polymorphisms of LEPR. (**b**) Generation of mice with the "humanized" leptin receptor. 223Q to 223R substitution (Q223R) in mouse exon 4. Restriction sites used for genotyping are indicated. (**c**) Expression levels in the hypothalamus of the three genotypes at amino acid 223 of *Lepr*b. Expression data were normalized to *β-actin* ($N = 3$). CK, cytokine receptor subdomain; CRH, cytokine receptor homology subdomain; F3, fibronectin type III subdomain; Ig, immunoglobulin.

domain is less conserved, and does not seem to participate in leptin binding or receptor activation (36).

The K109R polymorphism of LEPR causes a conservative change in the membrane-distal part of the LEPR extracellular domain (Figure 1). No apparent effect of K109R on BMI has been reported (16,37–42). K656N, a nonconservative change present in the membrane-proximal part of the LEPR extracellular domain, shows no association with adiposity (16,37–43). The Q223R nonconservative change is located in the CRH1 domain (Figure 1). A number of studies have reported associations of this variant with increased body weight and fat mass, whereas others failed to demonstrate the association (summarized in Table 1).

The aim of this study was to examine the functional consequences of Q223R by (i) assessing the effects of a humanized allele on body composition in the mouse; and (ii) measuring the functional activity of these *Lepr* alleles in cultured cells. Mice segregating for *Lepr* Q223R were generated, and gene dosage effects on adiposity and energy homeostasis were quantified. No effects of this allele were apparent either *in vivo* or *in vitro*; relevant caveats are discussed below. We propose that this approach will be useful in vetting the biological relevance of nonsynonymous variants in genes-mediating putative effects on quantitative traits such as adiposity.

METHODS AND PROCEDURES

Humanized *Lepr* variant Q223R

A targeting construct was designed to use homologous recombination in embryonic stem (ES) cells to replace coding exon 4 of the mouse *Lepr* gene with a segment that was identical except for codon 223. The targeting construct contained a ~6 kb fragment (extending from a *Swa*I site to a *Pme*I site) that contains coding exon 4. The codon substitution was accomplished by oligonucleotide-directed mutagenesis coupled with PCR. Two overlapping fragments were generated by PCR that encompassed coding exon 4 and flanking sequences. The codon sequence alteration was designed to introduce a novel *Pvu*I site and a novel *Taq*I site for diagnostic restriction digestions to identify the wild-type and novel alleles. The two overlapping fragments were digested with *Pvu*I, ligated, and reamplified to generate one contiguous fragment containing the codon sequence alteration. This fragment was digested with *Acc*65I and *Eco*RV and used to replace a similar restriction fragment in the targeting construct. A floxed Pgk-neo cassette was inserted into the *Eco*RV site that is downstream of coding exon 4. All amplified segments were sequenced to eliminate clones with PCR-related sequence alterations (Figure 1b). The construct was used for targeting 129P3/J ES cells. Three G418 resistant clones were identified to have homologous recombination at the PGK-neo cassette by PCR. However, only two independent clones were verified to contain the desired codon alteration. Mice carrying the humanized *Lepr* 223R (equivalent to 222R in the mouse; for simplicity, it will be referred to as 223R) allele were generated by injecting the ES clone into C57BL/6J blastocysts. The floxed *Pgk-neo* cassette was excised by mating to deleter protamine *Cre* 129 mice to prevent inadvertent effects due to interference from the *Pgk-neo* cassette. Mice carrying the *neo*-less allele were identified by PCR using primers flanking the *Pgk-neo* cassette insertion point.

The founder progeny (potentially capable of producing either 129 or B6 gametes) were crossed to 129 mice and the DNA of F1 progeny interrogated for the targeted *Lepr* allele. F1 animals segregating for the targeted *Lepr* allele (therefore 129 throughout) were intercrossed to generate the animals whose phenotypes are described below.

Husbandry

All mice were housed in groups of three to four per cage under a 12:12 hour light–dark cycle in a barrier facility at 21 °C. A total of 13 223R/R, 35 223Q/R, 18 223Q/Q male and 23 223R/R, 28 223Q/R, 16 223Q/Q female mice were fed a low-fat (LF) diet (9% of calories as fat; Purina Picolab no. 5058 chow; Granville Milling, Creedmoor, NC). In addition, 12 223R/R, 23 223Q/R, 16 223Q/Q male and 12 223R/R, 16 223Q/R, 11 223Q/Q female mice were fed a LF (Purina Picolab no. 5058 chow; Granville Milling) or a high-fat (HF) (65% of calories as fat; cat. no. D12492; Open Source Diets) diet starting at 4 weeks of age. Mice fed the LF diet from 4 weeks of age were switched to the HF diet at 121 days of age. All mice had *ad libitum* access to food and water throughout these studies.

Body mass and composition measurement

Mice were weighed weekly on an electronic scale starting on postnatal day 14. Immediately after weighing, body composition was determined by TD-NMR using a Minispec Analyst AD lean fat analyzer (Bruker Optics, Silberstreifen Germany). The TD-NMR was calibrated using mouse carcasses that ranged from 5 to 70 g in mass.

Calorimetry and energy intake

150-day-old male mice (four 223Q/Q and four 223R/R) were individually caged in a LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany) and trained to use the water dispenser. Mice were weighed before being placed in their cages. Indirect calorimetry was performed for ~96 h while the mice had free access to the LF diet. O_2 and CO_2 measurements were taken every ~14 min during the entire

Table 1 Association studies of *LEPR* Q223R with human adiposity

Authors	Ethnicity/subject type	Sample	Control	Association	Allele frequency	Predisposing allele	Dosage
Positive association studies							
Thompson *et al*. (42)	Pima Indians	10 obese (body fat = 40 ± 5%)	10 lean (body fat = 23 ± 5%)	Obesity	R-0.75	R	Hom
Chagnon *et al*. (58)	White 99 families: 522 subjects			4U increase of BMI, 5% increase in %fat	R-0.43	R	Het/Hom
Yiannakouris *et al*. (40)	White, 120 17 years male and female			2.7U increase of BMI, 4.6% increase in %fat (overweight/ obese)	R-0.32	R	Hom
Quinton *et al*. (59)	White, 220 postmenopausal women			2U increase of BMI, 11% increase in fat mass	R-0.6	R	Het/Hom
Mattevi *et al*. (8)	Brazilian men and women of European descent	123 overweight and 30 obese (BMI > 30)	153 lean (BMI < 25)	2.8U increase of BMI	R-0.45	R	Het/Hom
Guizar-Mendoza *et al*. (60)	Mexican male and female adolescent	55 obese (BMI = 31.1 ± 3.6)	48 lean (BMI = 20.7 ± 3.1)	11% increase in % fat	R-0.6	R	Het/Hom
Fairbrother *et al*. (61)	White postmenopausal women			4% increase in fat mass	R-0.45	R	Hom
Negative association studies							
Echwald *et al*. (39)	Danish adolescent males	156 obese (BMI ≥ 31)	205 lean (BMI = 21.5 ± 2.2)	None with BMI			
Gotoda *et al*. (43)	British white men	190 obese males (BMI > 28)	132 lean males (BMI< 22)	None with BMI	R-0.43		
Matsuoka *et al*. (41)	Japanese male and female	47 obese (BMI = 35 ± 6.5)	68 lean (BMI = 21.6 ± 2.2)	None with BMI	R-0.85		
Silver *et al*. (37)	White	175 obese (BMI = 6.75 ± 9.6)	107 lean (BMI = 21 ± 1.4)	None with BMI			
Chagnon *et al*. (62)	QFS/169 families/314-325 sib. pairs	114 obese (BMI ≥ 27)	167 lean (BMI < 27)	None with BMI	R-0.51		
de Silva *et al*. (63)	Nauruan males	232 obese (BMI = 37)		None with BMI	R-0.89		
Chagnon *et al*. (58)	African-American 115 families: 319 subjects			None with BMI, % fat	R-0.51		
Stefan *et al*. (64)	Pima Indians	268 with low subcut. fat	184 with high sub. fat	None with abd., sub. fat	R-0.33		
Ogawa *et al*. (65)	Japanese middle-aged men and women	175 obese (BMI = 6.75 ± 9.6)	107 lean (BMI = 21 ± 1.4)	None with BMI	R-0.85		
van der Vleuten *et al*. (66)	FCH patients, diverse	158 FCH patients (BMI = 27.3)	479 relatives and spouses (BMI = 24.3, 26.4 resp.)	None with BMI, waist circumference	R-0.4		
Wang *et al*. (67)	Taiwanese male and female Aborigines	226 obese (BMI ≥ 27) 33 extreme (BMI ≥ 35)	182 lean (BMI < 25)	None with BMI	R-0.1		
Mergen *et al*. (68)	Turkish male and female	262 obese (BMI ≥ 30)	138 lean (BMI ≤ 25)	None with BMI	R-0.38		

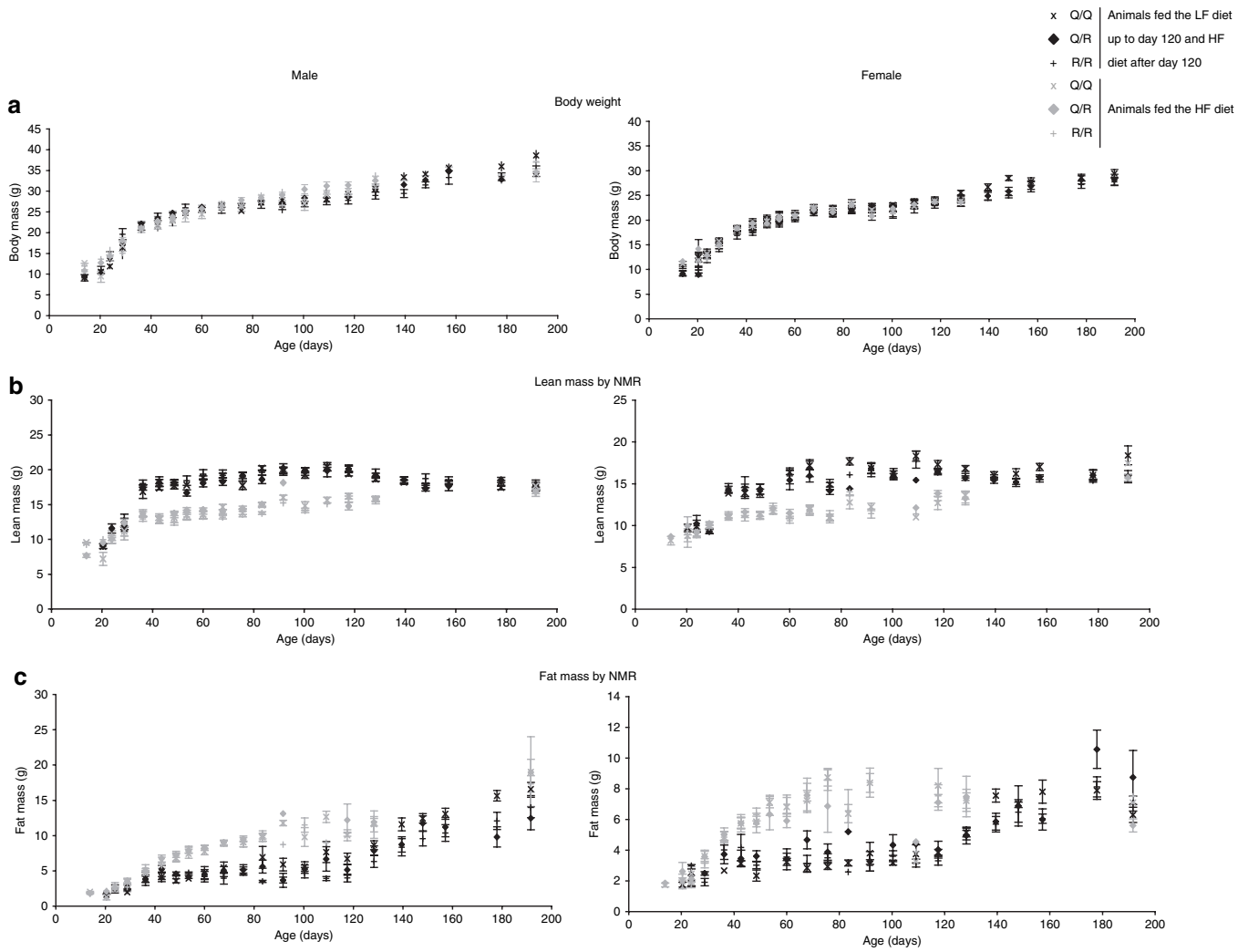

Figure 2 Body composition of mice fed the high-fat (HF) or low-fat (LF) diet by 223 genotype. Time course of (**a**) body weight, (**b**) lean mass, and (**c**) fat mass of male and female mice by genotype (at *Lepr* codon 223) fed the LF or HF diet. Body weight, lean and fat mass measurements of mice fed the HF diet were made every week from 14 to 130 days of age; an additional measurement was made at 190 days of age. For mice fed the LF diet, these measurements were made every week from 14 to 120 days of age. At day 121, their diet was switched to HF. Each data point is the arithmetic mean of all animals. Error bars represent s.e.m. For numbers of animals in each study, see Husbandry in Methods and Procedures section.

period, and VO_2 and VCO_2 values were expressed in ml/kg/h. Mice were taken out of the calorimeter and fed the HF diet *ad libitum* for 72 h. They were then returned to the calorimeter cages with *ad lib* access to the HF diet and studied for ~96 h as before. Data from only the last 72 h on the HF and LF diets were analyzed, as the mice were allowed to acclimate during the first ~24 h. Energy intake was calculated by multiplying cumulative food intake for a 24-h period by the metabolizable energy present in the HF (5.24 kcal/g) and LF (3.56 kcal/g) diets.

Real time PCR

Total RNA was extracted from hypothalami of 15 mice of each of the three 223 genotypes and reverse transcribed using random hexamers (Invitrogen SuperScript III; Carlsbab, CA). Real-time PCR was performed in a LightCycler II (Roche, Pleasanton, CA) using the LightCycler FastStart DNA Master SYBR Green kit (Roche) according to the manufacturer's specifications. The following *Lepr* exonic primers were used: 5′-CCTCTGCCCCCACTGAAAGACA; 5′-GGGTCACTGTCACTCTGAAGTGCAA.

Lepr expression levels were normalized with *β-actin* using the following exonic primers: 5′-CTTTGCAGCTCCTTCGTTGC; 5′-TCTGACCCATTCCCACCATC.

STAT3 activation assay

Materials. Generation of anti-LEPRb antibody has been described previously (44). Antibody against Tyr_{705} phosphorylated STAT3 was purchased from Cell Signaling Technology (Boston, MA). Leptin was purchased from NHPP (Los Angeles, CA). mLEPRb/pCDNA3 has been described previously (45). The function of the Δ65 truncation in the intracellular domain of mLEPRb has been described and LEPRbΔ65/pCDNA3 is the nonchimeric variant which was used previously (46). mLEPRb/pCDNA3 was used as a template for mutagenesis. $mLEPRb^{R223}$/pCDNA3 was constructed using a two-stage PCR method.

First, the 5′ flanking primer (5′-CGACTCACTATAGGGAGACCC AAGCTTG), and the antisense mutagenesis primer (5′-GACATCAGA GGTGACCGAAAACTCACACC), were used to amplify the upstream fragment.

The 3′ flanking primer (5′-GACATCGATCACGTATAATTCAGCAT AGCGGT), and the 5′ mutagenesis primer (5′-GGTGTGAGTTTTCGG TCACCTCTGATGTC) were used to amplify the downstream fragment. These PCR products combined and used as template for a second round of PCR using the flanking primers listed above. This PCR product was then inserted into mLEPRb/pCDNA3 using *Hind*III and *Xho*I restriction

Table 2 Statistical analyses of body mass and composition measurements

		Male	Difference	Male	Difference	Female	Difference	Female	Difference
A	Days 21–190								
	Dependent variable	HF: Q/Q,Q/R,R/R		LF: Q/Q,Q/R,R/R		HF: Q/Q,Q/R,R/R		LF: Q/Q,Q/R,R/R	
	Body weight	$F = 0.69$, df = 2,48, $P = 0.52$	—	$F = 0.42$, df = 2,63, $P = 0.66$	—	$F = 1.61$, df = 2,36, $P = 0.24$	—	$F = 0.94$, df = 2,64, $P = 0.41$	—
	Fat mass	$F = 1.92$, df = 2,48, $P = 0.19$	—	$F = 0.33$, df = 2,63, $P = 0.71$	—	$F = 2.49$, df = 2,36, $P = 0.13$	—	$F = 0.31$, df = 2,64, $P = 0.73$	—
	Lean mass	$F = 1.81$, df = 2,48, $P = 0.21$	—	$F = 0.09$, df = 2,63, $P = 0.90$	—	$F = 0.03$, df = 2,36, $P = 0.97$	—	$F = 2.66$, df = 2,64, $P = 0.10$	—
	% fat	$F = 2.47$, df = 2,48, $P = 0.13$	—	$F = 0.37$, df = 2,63, $P = 0.69$	—	$F = 2.06$, df = 2,36, $P = 0.17$	—	$F = 0.24$, df = 2,64, $P = 0.78$	—
B	Days 21–120								
	Dependent variable	HF,LF	HF vs. LF			HF,LF	HF vs. LF		
	Body weight	$F = 6.40$, df = 1,115, $P = 0.07$	—			$F = 8.10$, df = 1,104, $P = 0.06$	—		
	Fat mass	$F = 17.61$, df = 1,115, $P = 0.0002$	+50%			$F = 49.27$, df = 1,104, $P < 0.0001$	+54%		
	Lean mass	$F = 185.51$, df = 1,115, $P < 0.0001$	–27%			$F = 116.56$, df = 1,104, $P < 0.0001$	–27%		
	% fat	$F = 17.35$, df = 1,115, $P = 0.0003$	+30%			$F = 55.65$, df = 1,104, $P < 0.001$	+50%		

ANOVA for body mass and body composition by genotype at *Lepr* 223 in mice fed high-fat (HF) or low-fat (LF) diets up to day 190. Area under the curve from data points taken every week was calculated for each mouse and grouped by (A) sex, diet, and genotype (up to day 190), or (B) sex and diet (up to day 120).

sites. The presence of the desired mutation and the absence of adventitious mutations was confirmed by DNA sequencing.

Cell lines and transfection. HEK 293 cells were maintained in a humidified incubator at 37 °C with 5% CO_2. Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin was used as growth medium. The appropriate LEPRb and luciferase reporter plasmids were transiently transfected into 293 cells in 12-well plates using Lipofectamine (Invitrogen). 500 ng LEPRb plasmid, 50 ng GAS-Luc (encodes STAT3-responsive γ interferon-activated sequence driven luciferase), and 50 ng pRL-TK (encodes Renilla luciferase) was transfected per well.

Immunoblotting. Following transfection, cells were switched to serum-free medium and stimulated for 6 h with various doses of leptin. Cells were harvested in lysis buffer (20 mmol/l Tris pH 7.4, 150 mmol/l NaCl, 1% Nonidet P-40) and insoluble material was cleared by centrifugation. Lysate was denatured in 4× Laemmli buffer and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE gels were transferred to nitrocellulose membranes in Towbin buffer containing 0.02% SDS and 20% methanol. Membranes were blocked for 1 h in buffer containing 20 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, and 0.01% Tween 20 (wash buffer) supplemented with 3% bovine serum albumin (block buffer). Membranes were incubated with primary antibody for 2 h at room temperature. Membranes were washed three times and incubated with a horseradish peroxidase conjugated secondary antibody for 1 h. Membranes were then washed three times, treated with luminescence reagent (Lumi-Light, Roche) and exposed to film.

Luciferase assays. Following transfection, cells were switched to serum-free medium and stimulated for 6 h with various doses of leptin. Cells were lysed and assayed with the Stop-n-Glo dual luciferase reporter kit (Promega, Madison, WI) according to the kit's instructions; GAS-Luc firefly luciferase activity was normalized for transfection efficiency with Renilla luciferase from the constitutive pRL-TK plasmid.

Statistical analysis

Changes in body composition over time of mice fed LF or HF diet up to days 120 or 190 were assessed using areas under the curves of body mass, lean mass, and fat mass over time for each mouse. One way ANOVA (StatView 5.0; SAS Institute) was used, grouping by sex, genotype, and diet. Effects of the LF and HF diet on energy intake and expenditure, RQ and VO_2 were assessed by *t*-testing the arithmetic mean of the calorimetry readings over 72 h. Levels of statistical significance were set at $P_\alpha < 0.05$.

Ethical use of animals

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. All protocols were approved by the Columbia University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

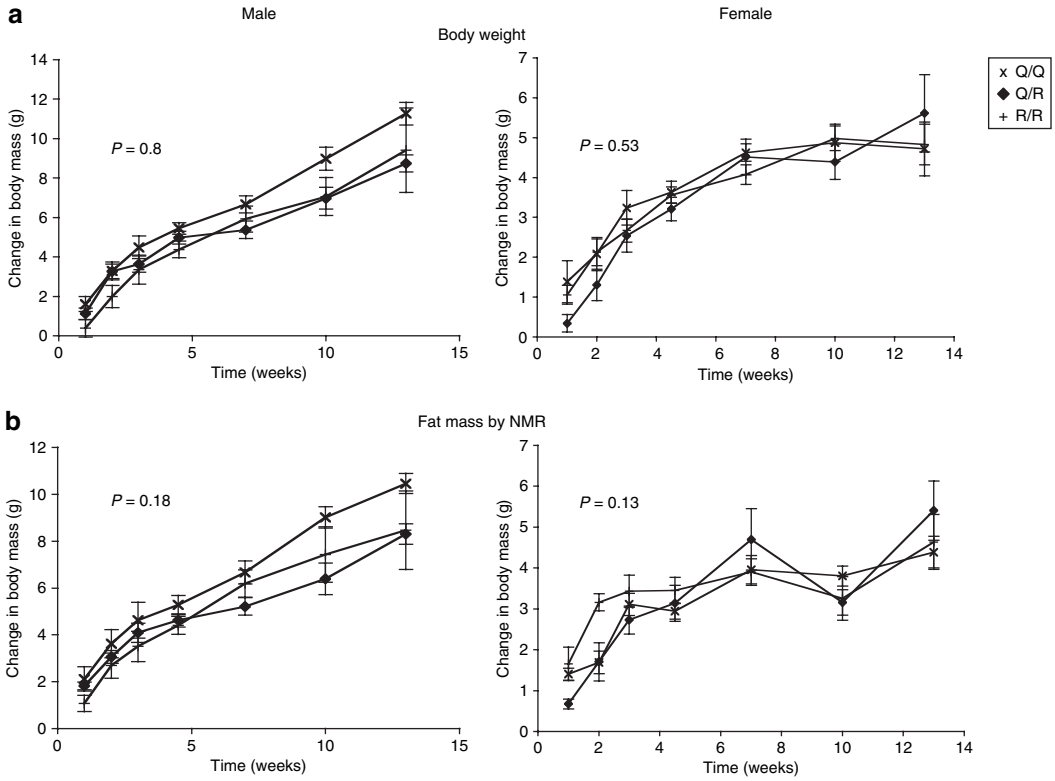

Figure 3 Body weight and composition by *Lepr* 223 genotype of mice switched to the high-fat (HF) diet at 121 days, Time course of (**a**) body weight, and (**b**) fat mass of male and female mice fed a HF diet for 13 weeks. Week 1 begins at day 121 of age, when mice were switched to the HF diet. Each data point represents a mean of all animals. Error bars represent s.e.m. Data analyzed by ANOVA. For numbers of animals in each study, see Husbandry in Methods and Procedures section.

RESULTS

Body weight and composition

Exon 4 of mouse *Lepr* is 76% identical at the DNA level and 70% identical at the amino acid level to the human *LEPR*. The substitution of the mouse wild-type glutamine residue (Q^{CAG}) with arginine (R^{CGA}) did not affect *Lepr* expression in the hypothalamus of mice heterozygous and homozygous for R^{CGA} (**Figure 1c**). Genotype did not affect body mass in homozygous (Q/Q, R/R) and heretozygous (Q/R) male or female mice from 14 to 21 days of age (**Figure 2a**). Mice of all three genotypes were then fed the HF or the LF diet until 120 days of age. From 14 to 120 days of age, there were effects of diet composition and sex—but not genotype—on changes in weight, lean and fat mass. (**Figure 2**; **Table 2**). Likewise, responses to the change to a HF diet at 120 days were unaffected by genotype at *Lepr* 223 (**Figure 3**; **Table 2**).

Calorimetry

Indirect calorimetry on Q/Q and R/R mice fed either the LF or HF diet showed, as expected, that mice on the LF diet had a higher RQ than mice on the HF diet; but this difference was unaffected by genotype at *Lepr* (**Figure 4c**). During these calorimetry studies, mice of neither genotype gained weight after 3 days on the HF or LF diet (**Figure 4a**), and had the same energy intake (**Figure 4b**) and energy expenditure (**Figure 4e**) per day while being fed the HF or LF diet. A time course of VO_2 per hour per body mass$^{2/3}$ indicated that the 24-h energy expenditure and diurnal patterns of energy expenditure were indistinguishable between Q/Q and R/R mice fed the HF or LF diets (**Figure 4d**).

In vitro signaling effects of codon 223 genotypes

To investigate a possible alteration in signaling capacity between the 223Q and 223R *Lepr* alleles, we transfected plasmids encoding, STAT3-signaling defective *Lepr*Δ65C, native *Lepr*b^{223Q} or humanized *Lepr*b^{223R} into 293 cells for the analysis of downstream signaling after treatment with various doses of leptin. The analysis of STAT3 activation by the detection of Tyr_{705} phosphorylation on endogenous STAT3 by immunoblotting revealed no differences in the extent of phosphorylation of STAT3 by *Lepr*b^{223Q} or *Lepr*b^{223R} (**Figure 5b**). Similarly, when *Lepr*b^{223Q} or *Lepr*b^{223R}-mediated STAT3 transcriptional activation was assayed in 293 cells co-transfected with the STAT3-responsive GAS-luciferase reporter plasmid, no differences in leptin-dependent luciferase stimulation were detected between the two *Lepr*b alleles over a range of leptin concentrations (Student's *t*-test, $P > 0.05$) (**Figure 5a**).

DISCUSSION

The Q allele of LEPR is conserved in species from monotremes to humans (Ensemble; Genomic Sequence Alignment). The Q allele is also present in all mouse substrain sequences available at NCBI

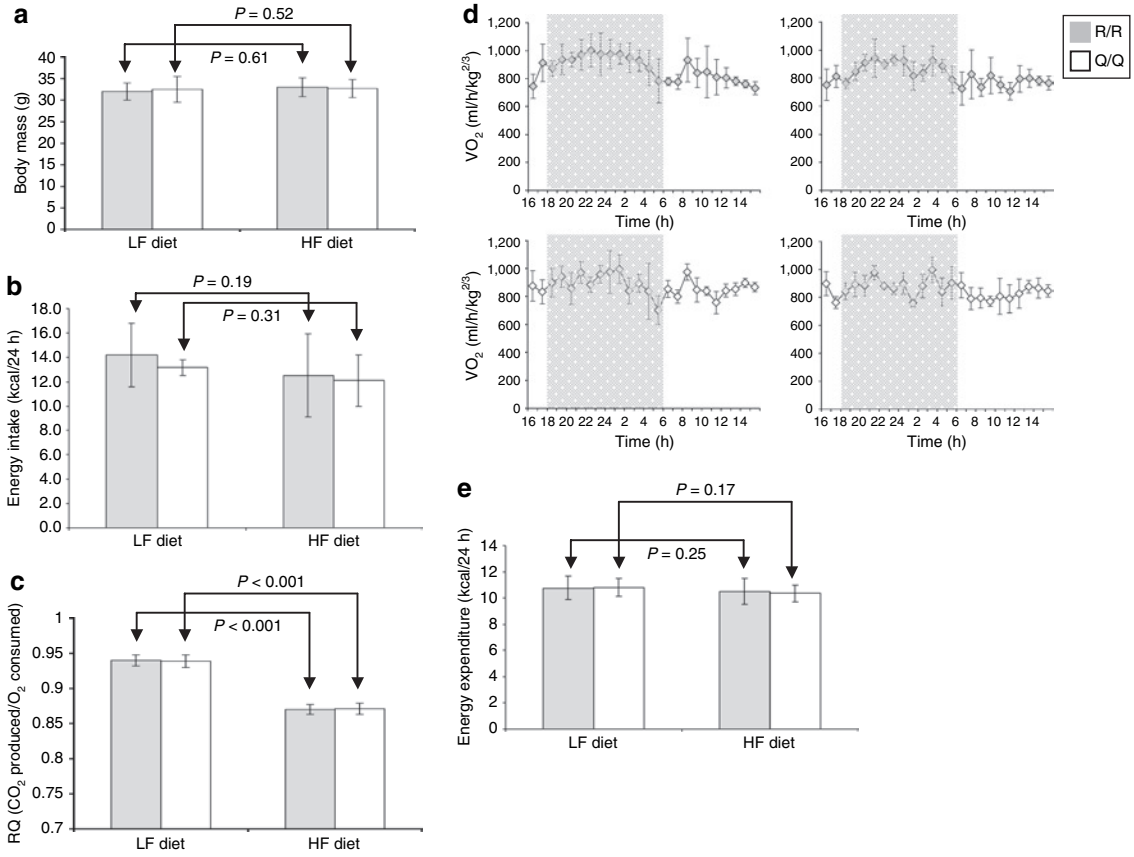

Figure 4 Metabolic studies of mice fed the high-fat (HF) or low-fat (LF) diet. Indirect calorimetry performed on mice homozygous for the 223Q or 223R *Lepr* alleles fed the LF or HF diets. Means and s.d. of (**a**) total body mass, (**b**) energy intake calculated from food intake, (**c**) respiratory quotient (RQ), (**d**) time course of oxygen utilization per hour over body mass raised to the 2/3 power (dark background represents hours with lights out) and (**e**) energy expenditure. Measurements were taken every ~14 min over a period of 72 h. Columns and error bars represent means of respective readings and s.d., respectively. For numbers in each study, see Calorimetry in Results section.

(http://www.ncbi.nlm.nih.gov/SNP/MouseSNP.cgi). A number of studies have explored the hypothesis that the common, non-conservative variant, *LEPR* Q223R, predisposes to increased adiposity in humans. Interest in *LEPR* in this regard derives from its central role in energy homeostasis and the high frequency of this allele in most populations. The results, as noted, have been mixed, and two meta-analyses (17,47) have concluded that *LEPR* Q223R is not associated with relative adiposity or obesity. We used the mouse to attempt to make a more definitive finding regarding the biological significance of these alleles of *LEPR*.

129P3/J mice were transgenically engineered to segregate for the human alleles of *LEPR* at codon 223. Weight and body composition were assessed in male and female mice fed LF and HF diets over 235 days. Mice were also studied by indirect calorimetry. No effect of the *Lepr* Q223R genotype was observed for any of the metabolic or body weight/composition phenotypes. Possible reasons for our failure to detect any functional consequence of amino acid substitution at this locus include: (i) use of *Lepr* DNA sequence from a mouse strain with unique characteristics rendering the molecule insensitive to the allelic variant; (ii) a corollary possibility related to interactions between the mouse *Lepr* sequence and the "background" genome of the mice examined in this study; (iii) the presence

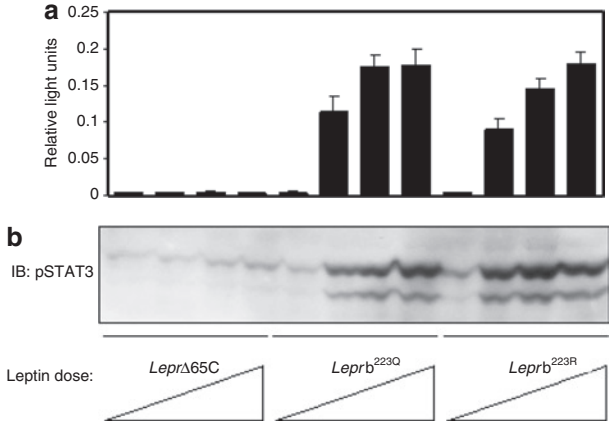

Figure 5 *In vitro* assessment of leptin receptor activity. Activation of STAT3 in response to leptin stimulation. HEK 293 cells were transfected with the *Lepr*Δ65C (truncated *Lepr*), *Leprb*^{Q223} (native mouse *Lepr*) and *Leprb*^{R223} ("humanized" *Lepr*) plasmids, as well as the *STAT3*-responsive GAS-luciferase and control pRL-TK plasmids. Following transfection, cells were made quiescent, then treated with leptin for 6 h before lysis. Leptin doses were 0, 20 ng/ml, 200 ng/ml, and 2 mg/ml. Lysates were either subjected to (**a**) dual luciferase analysis or (**b**) resolved by SDS–PAGE before transferring to nitrocellulose and immunoblot with the indicated antibody. Bars represent the means of triplicate determinations plus or minus the s.d.

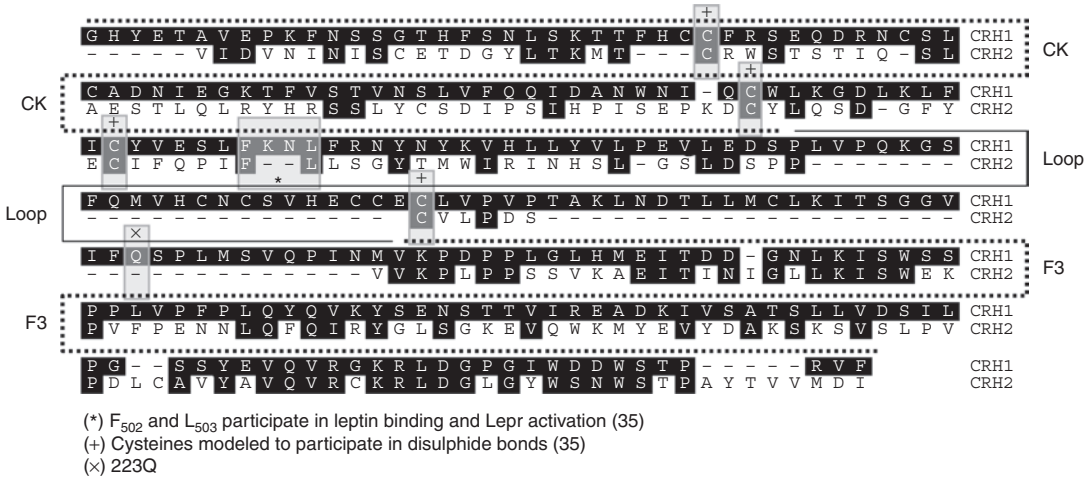

Figure 6 Comparison of CRH1 and CRH2. Alignment of human CRH1 and CRH2 using the Clustal V method. Although the two subdomains show weak identity, key residues are conserved.

of critical differences between mouse and human amino acid sequences in LEPR limiting the impact of the Q223R on a relevant signaling pathway. With regard to (i), there are no coding or intronic splice site sequence differences in *Lepr* among mouse strains examined to date (NCBI; http://www.ncbi.nlm.nih.gov/SNP/MouseSNP.cgi; database includes sequence from 125 mouse strains). Point (ii) is addressed in the penultimate paragraph of the article, just below. With regard to (iii), the mouse LEPRb is ~75% identical at the amino acid level to the human protein (mLEPRb NCBI accession no. CAA71342; Human LEPRb NCBI accession no. NP_002294). In particular, mLEPRb residues associated with Janus kinase 2 (human residues 893–898) (ref. 48), STAT3 (human residues 1,142–1,145) (ref. 49), SOCS3 (human residue 1,142; 27), STAT5 (human residues 1,079–1,082) (refs. 23,50), SHP-2 (human residues 987–990 including Tyr 987 involved in the generation of mLEPRb autoinhibitory signals) (27,51) activities are conserved between mouse and human proteins. Similarly, the extracellular domain responsible for leptin binding (mouse residues 323–640) is 85% identical between mouse and human LEPR (34). Specifically, residues within sites I–III modeled to bind leptin are conserved in both mouse and human (36,52).

The LEPR Q223R substitution is located in the loop connecting the cytokine receptor (CK) and the fibronectin type III (F3) domains of CRH1 (**Figure 1a**). Residues in the Ig-like (36), CRH2 (ref. 35), and membrane-proximal F3 (ref. 53) domains have been implicated in LEPR function. No role has yet been assigned to CRH1 because it is not necessary for leptin binding or LEPR activity (36). CRH1 and CRH2 are 23% identical at the amino acid level. Despite the conservation of critical CRH2 residues in CRH1, the Q223R polymorphism lies in a region unique to CRH1 (**Figure 6**), that is not conserved in a number of related cytokine receptors (34). In contrast, the mutation that underlies the leptin insensitivity of *fatty* rats (Q269P) lies within an important structural subdomain of CRH1, and the Q269P substitution presumably destabilizes the structure of CRH1 and thus the entire LEPR molecule (34,54,55). Whatever the functional role (if any) of the LEPR Q223R polymorphism may be, it does not appear to affect leptin signaling as reflected by *in vitro* activation of STAT3, or the body composition/metabolic performance of mice studied over relatively long periods of time. We conclude from the present analysis that LEPR Q223R is unlikely to play a significant role in risk of human obesity.

The previous association detected in the human association studies may be due to another polymorphism in linkage disequilibrium with *LEPR* Q223R, or could simply be a spurious finding. There is an important caveat to our conclusions regarding the likely absence of functional consequences of the Q223R alleles of LEPR. We examined the effects of these alleles in a single mouse strain (129P3/J). It is well known that the phenotypic "penetrance" of spontaneous and engineered mutations varies widely depending upon the strain(s) in which the mutation is segregated. The striking differences in diabetes-related phenotypes of $Lepr^{db}$ mutations on the C57BLKS/J and C57BL/6J backgrounds (56) is a relevant example. This sort of effect may also account for apparent racial differences in the phenotypic consequences of genetic variation in humans (e.g., *TCF7L2*) (ref. 57). Clearly, studies of the sort described here must be interpreted in the context of such considerations. In this instance, given the discordant results in human studies, our *in vivo* results in 129 mice, and our *in vitro* analysis of signaling by the Q223R alleles, we conclude that Q223R allelic variation in *LEPR* plays a small role (if any) in human adiposity.

The approach used here was designed to provide a prototype for biological assessment of potentially small effects of allelic variants in candidate genes for complex convergent phenotypes such as human obesity. This approach can be used to examine alleles of several genes at one time, to recapitulate implicated haplotypes without confounding due to other genetic variation, or variable environments.

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DISCLOSURE

The authors declared no conflict of interest.

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