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ARTICLE

Lipopolysaccharide-binding protein is a negative regulator of adipose tissue browning in mice and humans

Aleix Gavaldà-Navarro[1,2,3,4] · **José M. Moreno-Navarrete**[4,5] · **Tania Quesada-López**[1,2,3,4] · **Montserrat Cairó**[1,2,3,4] · **Marta Giralt**[1,2,3,4] · **José M. Fernández-Real**[4,5] · **Francesc Villarroya**[1,2,3,4]

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Abstract
Aims/hypothesis Adipocyte lipopolysaccharide-binding protein (LBP) biosynthesis is associated with obesity-induced adipose tissue dysfunction. Our purpose was to study the role of LBP in regulating the browning of adipose tissue.
Methods Adult mice were maintained at 4°C for 3 weeks or treated with the β_3-adrenergic agonist, CL316,243, for 1 week to induce the browning of white fat. Precursor cells from brown and white adipose tissues were cultured under differentiation-inducing conditions to yield brown and beige/brite adipocytes, respectively. In vitro, *Lbp* was knocked down in 3T3-L1 adipocytes, and cells were treated with recombinant LBP or co-cultured in transwells with control 3T3-L1 adipocytes. Wild-type and *Lbp*-null mice, fed a standard or high fat diet (HFD) for 15 weeks, were also used in investigations. In humans, subcutaneous and visceral adipose tissue samples were obtained from a cohort of morbidly obese participants.
Results The induction of white fat browning by exposure of mice to cold or CL316,243 treatment was strongly associated with decreased *Lbp* mRNA expression in white adipose tissue. The acquisition of the beige/brite phenotype in cultured cells was associated with downregulation of *Lbp*. Moreover, silencing of *Lbp* induced the expression of brown fat-related genes in adipocytes, whereas LBP treatment reversed this effect. *Lbp*-null mice exhibited the spontaneous induction of subcutaneous adipose tissue browning, as evidenced by a remarkable increase in *Ucp1* and *Dio2* gene expression and the appearance of multivacuolar adipocyte clusters. The amount of brown adipose tissue, and brown adipose tissue activity were also increased in *Lbp*-null mice. These changes were associated with decreased weight gain in *Lbp*-null mice and protection against HFD-induced inflammatory responses, as shown by reduced IL-6 levels. However, rather than improving glucose homeostasis, these effects led to glucose intolerance and insulin resistance.
Conclusions/interpretation LBP is identified as a negative regulator of the browning process, which is likely to contribute to the obesity-promoting action of LBP. The deleterious metabolic effects of LBP deletion are compatible with the concept that the appropriate regulation of inflammatory pathways is necessary for a healthy systemic metabolic profile, regardless of body weight regulation.

Keywords Brown adipose tissue · Browning · High-fat diet · Lipopolysaccharide-binding protein · Obesity

Abbreviations

BAT	Brown adipose tissue
eWAT	Epididymal white adipose tissue
HFD	High fat diet

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✉ Francesc Villarroya
fvillarroya@ub.edu

[1] Department of Biochemistry and Molecular Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Catalonia, Spain

[2] The Institute of Biomedicine of the University of Barcelona (IBUB), Barcelona, Spain

[3] Institut de Recerca Pediàtrica Hospital Sant Joan de Déu, Barcelona, Spain

[4] Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Spain
http://www.ciberobn.es/en

[5] Section of Diabetes, Endocrinology and Nutrition, Institut d'Investigació Biomèdica de Girona (IdIBGi), Hospital of Girona "Dr Josep Trueta", Girona, Spain

iBAT	Interscapular brown adipose tissue
ITT	Insulin tolerance test
iWAT	Inguinal white adipose tissue
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
SAT	Subcutaneous adipose tissue
shControl	Control small hairpin RNA
shLBP	Small hairpin RNA-mediated LBP knockdown
shRNA	Small hairpin RNA
UCP1	Uncoupling protein 1
VAT	Visceral adipose tissue
WAT	White adipose tissue
WT	Wild-type

Introduction

Obesity is characterised by lipid accumulation in white adipose tissue (WAT) as a consequence of a disturbed energy balance due to increased food energy intake and/or lowered energy expenditure. Importantly, it is associated with an increased incidence of metabolic disorders, especially type 2 diabetes mellitus [1].

Brown adipose tissue (BAT) is characterised by the presence of multivacuolar adipocytes with a thermogenic capacity that reflects the natural uncoupling of their mitochondria, as mediated by uncoupling protein 1 (UCP1). BAT is the main site of adaptive energy expenditure in response to cold and possibly to diet [2]. In experimental models, enhanced BAT activity is associated with protection against obesity, whereas impaired BAT activity favours an obese phenotype. Moreover, BAT is a major site of lipid breakdown and glucose uptake; therefore, BAT activation is associated with improvements in hyperlipidaemia and hyperglycaemia [3, 4]. In humans, it was previously thought that BAT is only present in neonates. However, more recent research has established that adult humans retain substantial BAT activity [5, 6]. In addition to the presence of BAT at defined anatomical locations, recent studies have attached special relevance to the WAT browning process [7]. This phenomenon consists of the appearance of functionally thermogenic brown adipocyte-like cells (so-called beige or brite adipocytes) in WAT depots. Several studies in mice have indicated that the capacity to activate this process may protect against diet-induced obesity and systemic metabolic disturbances [8, 9], although the actual relevance of adipose tissue browning to metabolism is still a matter of debate [10, 11].

Lipopolysaccharide (LPS)-binding protein (LBP) is a type I acute-phase reactant protein, thought to be mainly produced by the liver [12]. LBP facilitates the binding of the lipid A component of LPS to CD14 and toll-like receptor 4 (TLR4) [13], thus modulating the immunostimulatory capacity of LPS in bacterial infections [14]. LBP is also present in adipose tissues and high levels of LBP synthesis in white adipocytes are associated with WAT dysfunction in obesity [15]. LBP expression is associated with inflammatory markers and is increased with metabolic deterioration and insulin resistance in obese patients [16]. Here, we sought to establish the role of LBP in the browning of WAT. We investigated the association of *Lbp* expression with browning and studied alterations in browning and metabolism in *Lbp*-null mice.

Methods

Exposure of mice to chronic cold and treatment with CL316,243 The care and use of mice was carried out in accordance with the European Community Council Directive 86/609/EEC and approved by the Institutional Animal Care Committee of the University of Barcelona. C57BL/6 mice (Harlan Laboratories; Indianapolis, IN, USA) were maintained under standard conditions (12 h light/12 h dark cycle, 21 ± 1°C). Where indicated, 3-month-old mice were exposed to 4°C for 3 weeks, or 2-month-old mice received daily i.p injections with 1 mg/kg CL316,243 for 8 days (see electronic supplementary material [ESM] Methods; exposure of mice to chronic cold, and treatment of mice with CL316,243, for further details).

Adipocyte culture For primary adipocyte cultures, the stromal–vascular fraction was isolated from the inguinal WAT (iWAT) and interscapular BAT (iBAT) depots of 1-month-old mice. Pre-adipocytes were induced to differentiate into beige/brite and brown adipocytes, respectively, as previously reported [17, 18]. Differentiated beige/brite and brown adipocytes were treated with 0.5 μmol/l noradrenaline (norepinephrine) for 6 h. Differentiation of 3T3-L1 fibroblasts (ATCC; LGC Standards, Barcelona, Spain) was induced by treatment with 0.86 μmol/l insulin, 0.5 μmol/l dexamethasone and 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX) for 2 days, followed by 0.86 μmol/l insulin alone for 5 days. See ESM Methods for further details.

LBP knockdown and co-culture experiments Stable silencing of *Lbp* was achieved by transfecting 3T3-L1 fibroblasts with small hairpin (interfering) RNA (shRNA) targeting mouse *Lbp* (shLBP)or control shRNA (shControl) (Sigma Mission shRNA; Sigma-Aldrich, St Louis, MO, USA) as previously reported [16], generating transfected cells described in this paper as shRNA-mediated LBP knockdown (shLBP) or control (shControl) cells, respectively. Where indicated, shLBP adipocytes were incubated with 10 ng/ml LBP (R&D Systems; Minneapolis, MN, USA) during differentiation. Differentiated shLBP or shControl 3T3-L1 adipocytes were placed on one side of a Transwell plate (VWR-

International Eurolab, Barcelona, Spain) and co-cultured with shLBP adipocytes on the other side. See ESM Methods for further details.

***Lbp*-null mouse studies** *Lbp*-knockout mice (C.129P2-Lbptm1Jack/J, Balb strain background; obtained from The Jackson Laboratory, Bar Harbor, ME, USA) were maintained at 21 ± 1°C. Five-week-old male *Lbp*-null and wild-type (WT) littermate mice were fed standard chow (CTRL) or a high fat diet (HFD) for 15 weeks. For GTTs, 2.5 g glucose/kg was administered i.p. to mice starved for 6 h. For insulin tolerance tests (ITTs), 0.75 IU/kg insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was administered i.p. Plasma glucose (Accutrend; Roche Diagnostics, Mannheim, Germany), plasma insulin (BioVendor, Brno, Czech Republic); adiponectin (Life Technologies, Foster City, CA, USA); leptin, resistin and IL-6 (Millipore; Billerica, MA, USA); NEFA and 3-hydroxybutyrate (Wako Chemicals; Neuss, Germany); and triacylglycerols (Sigma-Aldrich) were assessed. Samples of iBAT or iWAT (25–50 mg) were incubated with 55.5 kBq/ml [U-^{14}C]D-glucose (Hartmann Analytic, Braunschweig, Germany) for 3 h. Disintegrations per minute (DPM) from $^{14}CO_2$ retained in a CO_2 trap and [^{14}C]tissue lipid extracts [19] were measured (Packard Instrument Company, Meriden, CT, USA). See ESM Methods for further details.

Human study Paired subcutaneous and visceral adipose tissue (VAT) samples (n = 38) from a cohort of morbidly obese (BMI >35 kg/m^2) participants were studied. These participants were recruited, gave written informed consent, and were validated and approved by the ethical committee at the Hospital of Girona 'Dr Josep Trueta'. Samples were obtained from subcutaneous adipose tissue (SAT) and VAT depots during elective surgical procedures. See ESM Methods for further details.

RNA isolation, cDNA synthesis and real-time PCR Total RNA was isolated (Macherey-Nagel, Düren, Germany) from BAT, iWAT, eWAT and liver samples from mice, and SAT and VAT samples from humans and retrotranscribed using TaqMan Reverse Transcription Reagents (Life Technologies). For the quantitative analysis of mRNA, TaqMan quantitative real-time PCR was performed on a 7500 Real-Time PCR System (Life Technologies) using the specific primer pair/probe sets, as described in ESM Table 1. See ESM Methods for further details.

Western blot assays Western blot analysis was performed on BAT and iWAT samples following standard procedures, using primary anti-UCP1 (1:1000; Abcam, Cambridge, UK) and anti-β-actin (1:5000; Sigma-Aldrich) antibodies. Both antibodies were diluted in 1× PBS containing 0.1% Tween-20, as per manufacturer's instructions. See ESM Methods for further details.

Statistical methods Results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA, USA). The statistical significance of differences was assessed using unpaired Student's t tests, one-way ANOVA with Tukey's multiple comparison test, or two-way ANOVA with Bonferroni post-testing, as appropriate.

Results

Induction of browning in subcutaneous WAT is associated with reduced *Lbp* expression We subjected mice to long-term cold exposure (4°C for 3 weeks) and then examined *Lbp* gene expression in adipose tissue depots. As expected, cold exposure increased expression of the thermogenesis-related genes *Ucp1*, *Sirt3*, *Fgf21* and *Ppargc1a* in subcutaneous WAT (i.e. iWAT; Fig. 1d–h); it also induced the browning process, indicated by the appearance of large amounts of multilocular adipocytes (Fig. 1a,b). These changes were associated with a marked reduction in *Lbp* expression (Fig. 1c). *Lbp* expression in visceral WAT (i.e. epididymal WAT [eWAT]), a WAT site less prone to browning, was also decreased in mice exposed to chronic cold, albeit to lesser extent (ESM Fig. 1a). In contrast, chronic cold exposure did not modify *Lbp* expression in iBAT (ESM Fig. 1h), even though we observed significant induction of thermogenesis-related genes in this tissue (ESM Fig. 1i–n).

Chronic treatment of mice with the β_3-adrenergic agonist CL316,243 (a model of induction of browning in iWAT and eWAT [20]), triggered the appearance of multilocular adipocytes (Fig. 1l, m), induced thermogenesis-related gene expression (Fig. 1o–v) and strongly repressed *Lbp* gene expression (Fig. 1n) in iWAT and eWAT (ESM Fig. 2a–i). Although iBAT also showed signs of activation following CL316,243 treatment (e.g. the induction of *Dio2* and *Bmp8b*), *Lbp* expression was not significantly altered in this tissue (ESM Fig. 2l–r). Overall, our results suggest that the downregulation of LBP is more strongly associated with WAT browning than with the induction of thermogenesis in iBAT.

***Lbp* expression is involved in the repression of browning in adipocytes** In order to determine whether the inverse relationship between *Lbp* expression and browning is a cell-autonomous phenomenon, we used rosiglitazone-induced browning (i.e. acquisition of the beige/brite phenotype) in precursor cells obtained from iWAT [17, 21]. Incubation of iWAT precursor cells with rosiglitazone during differentiation induced the expression of several marker genes for browning (Fig. 2b–f), and repressed *Lbp* expression (Fig. 2a), without

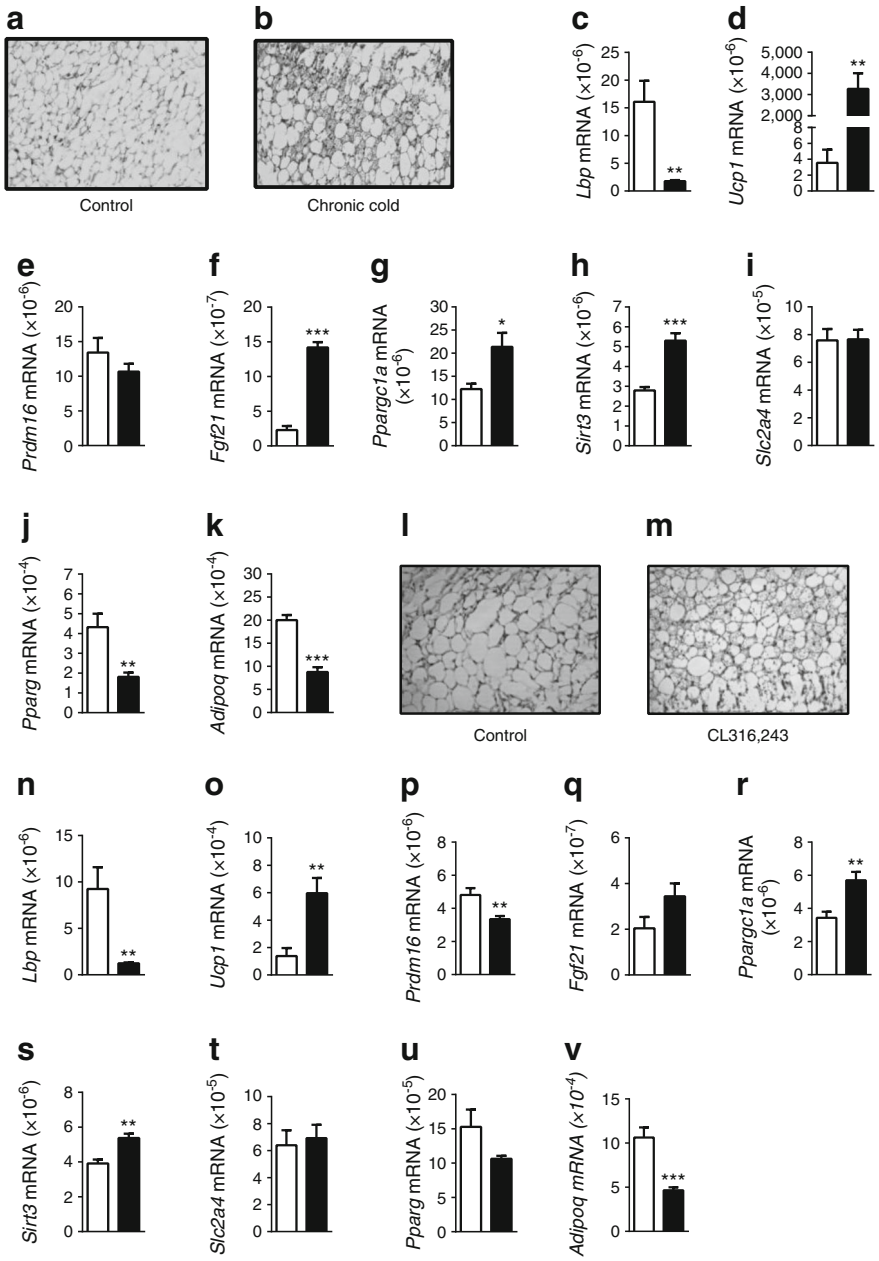

Fig. 1 Cold and β_3-adrenergic induction of iWAT browning is associated with reduced *Lbp* expression. (**a**, **b**) Micrographs (magnification × 20) of iWAT from (**a**) control mice or (**b**) mice maintained at 4°C for 3 weeks (chronic cold). (**c**–**k**) Transcript levels of (**c**) *Lbp*, (**d**) *Ucp1*, (**e**) *Prdm16*, (**f**) *Fgf21*, (**g**) *Ppargc1a*, (**h**) *Sirt3*, (**i**) *Slc2a4*, (**j**) *Pparg* and (**k**) *Adipoq* from control mice (white bars) or mice maintained at 4°C for 3 weeks (black bars). (**l**, **m**) Micrographs (magnification × 20) of iWAT from (**l**) control mice or (**m**) mice injected daily with 1 mg/kg CL316,243 for 8 days. (**n**–**v**) Transcript levels of (**n**) *Lbp*, (**o**) *Ucp1*, (**p**) *Prdm16*, (**q**) *Fgf21*, (**r**) *Ppargc1a*, (**s**) *Sirt3*, (**t**) *Slc2a4*, (**u**) *Pparg* and (**v**) *Adipoq* from control mice (white bars) or mice injected with CL316,243 (black bars). mRNA levels are normalised to 18S rRNA levels. Data are presented as mean ± SEM of six independent samples per group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

affecting the expression of the general adipogenesis-related genes *Slc2a4* (also known as *Glut4*), *Adiponectin* (*Adipoq*) and *Pparg* (Fig. 2g–i). These data confirm that negative regulation of LBP expression is associated with the adipocyte browning. Moreover, treatment of differentiated beige/brite adipocytes with noradrenaline repressed *Lbp* and induced *Ucp1* gene expression (ESM Fig. 3a,b); however, noradrenaline did not modify *Lbp* expression in brown adipocytes (ESM Fig. 3c).

To investigate whether LBP has a direct role in the browning process, we studied the effects of shRNA-mediated LBP knockdown in 3T3-L1 adipocytes. After 7 days of differentiation, shLBP exhibited increased mRNA levels of several thermogenesis-related genes, including *Ucp1*, *Ppargc1a* and *Sirt3* (Fig. 2k–o). Treatment of shLBP cells with recombinant LBP rescued the induction of *Ucp1* mRNA expression induced by loss of *Lbp* (Fig. 2s). Moreover, shLBP adipocytes subjected to transwell co-culture with control adipocytes showed lower levels of *Ucp1* mRNA expression compared with those co-cultured with shLBP cells (Fig. 2t). Overall, these data suggest that the downregulation of *Lbp* represses browning in a cell-autonomous manner.

***Lbp*-null mice exhibit reduced body weight gain accompanied by an induction of the browning process in iWAT** To study the role of LBP in vivo, *Lbp*-null mice and WT littermate controls were fed standard chow (CTRL) or an

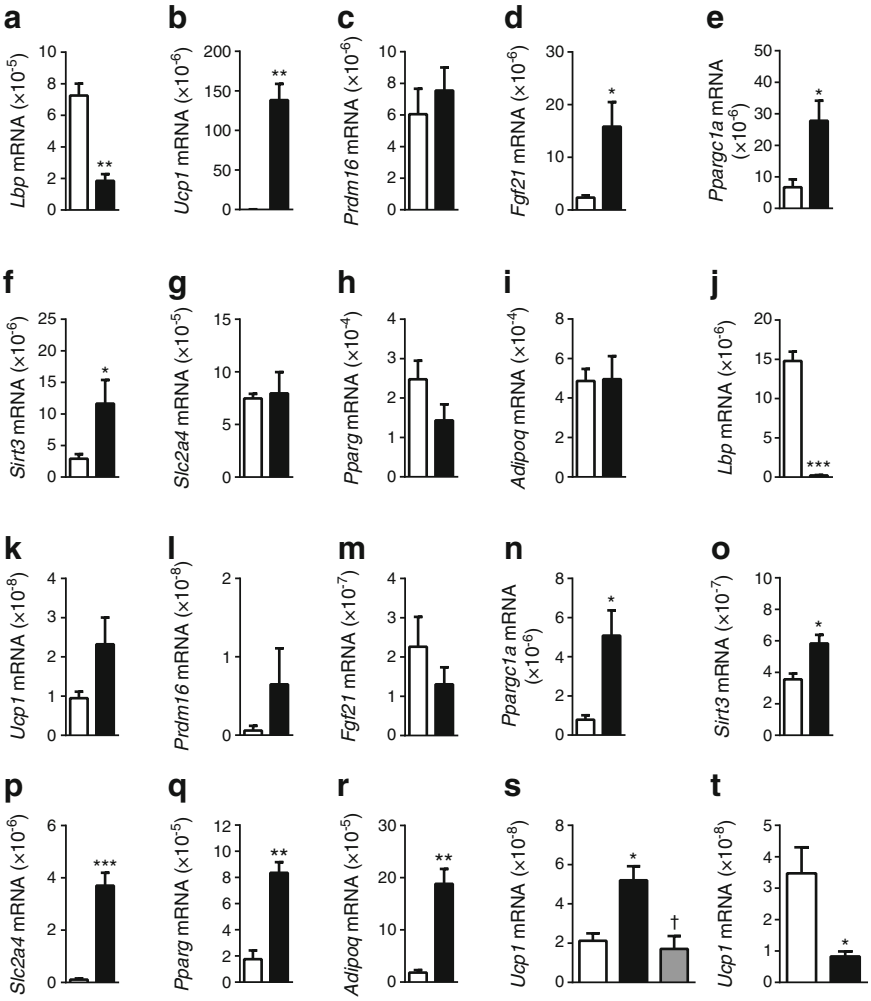

Fig. 2 *Lbp* and the expression of browning-related genes are inversely regulated in a cell-autonomous manner. (**a**–**i**) Transcript levels of (**a**) *Lbp*, (**b**) *Ucp1*, (**c**) *Prdm16*, (**d**) *Fgf21*, (**e**) *Ppargc1a*, (**f**) *Sirt3*, (**g**) *Slc2a4*, (**h**) *Pparg* and (**i**) *Adipoq*, in WAT-derived primary white adipocytes (white bars) or rosiglitazone-induced beige/brite adipocytes (black bars). (**j**–**r**) Transcript levels of (**j**) *Lbp*, (**k**) *Ucp1*, (**l**) *Prdm16*, (**m**) *Fgf21*, (**n**) *Ppargc1a*, (**o**) *Sirt3*, (**p**) *Slc2a4*, (**q**) *Pparg* and (**r**) *Adipoq* in 3T3-L1 adipocytes transfected with a control shRNA (shControl; white bars) or an shRNA targeting LBP (black bars). (**s**) Transcript levels of *Ucp1* in control 3T3-L1 adipocytes (white bars) or LBP-knockdown adipocytes untreated (black bar) or treated (grey bar) with 10 ng/ml LBP. (**t**) Transcript levels of *Ucp1* in LBP-knockdown 3T3-L1 adipocytes co-cultured with LBP-knockdown (white bar) or control 3T3-L1 adipocytes (black bar). mRNA levels are normalised to 18S rRNA levels. Data are presented as mean ± SEM of 4–7 independent samples per group. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs control $^{\dagger}p < 0.05$ for treated vs non-treated LBP-knockdown)

HFD for 15 weeks. At the start of the experiment (5-week-old mice), body weight was similar between WT and *Lbp*-null mice. The body weight of WT mice increased to 144% of the initial weight under standard feeding conditions and to 162% when fed an HFD (Fig. 3a,b). When fed a standard (CTRL) diet, the increase in body weight was significantly lower in *Lbp*-null mice (135%) than in WT mice on the same diet. On an HFD, the body weight of *Lbp*-null mice increased to only 145% of their initial weight; this increase was not significantly different from the body weight increase in *Lbp*-null mice on a standard diet. *Lbp*-null mice did not show significant changes in food intake relative to WT mice under standard diet (105 ± 4% kJ/day in *Lbp*-null vs WT) or HFD (112 ± 8% kJ/day in *Lbp*-null vs WT) conditions. Oxygen consumption was not significantly different among the different experimental groups (Fig. 3c).

The RQ was significantly decreased in WT mice fed HFD (Fig. 3d). In *Lbp*-null mice, RQ did not change significantly in response to an HFD, indicating a somewhat altered metabolic flexibility to adapt to HFD as a consequence of *Lbp* invalidation.

Lbp-null mice showed no statistically significant alterations in blood glucose levels (Fig. 3e), whereas *Lbp*-null mice had higher plasma triacyclglycerol levels when maintained on an HFD diet (Fig. 3f). Insulinaemia was strongly increased in *Lbp*-null mice relative to WT mice on an HFD (Fig. 3g). There were no differences in plasma NEFA, 3-hydroxybutyrate, leptin, adiponectin or resistin levels between WT and *Lbp*-null

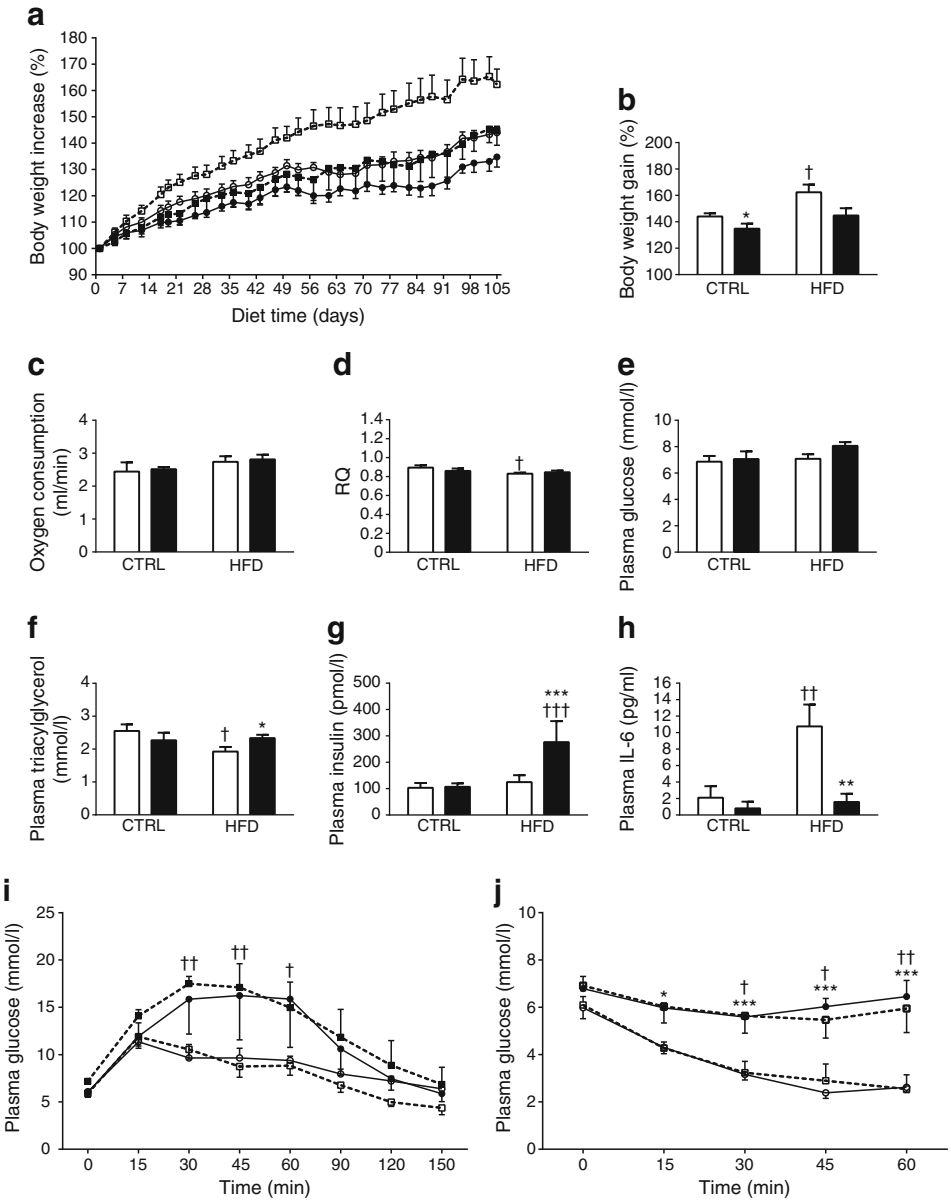

Fig. 3 *Lbp*-null mice have diminished body weight gain, with impaired glucose homeostasis. (**a**) Time course of mouse body weight, (**b**) final body weight gain, (**c**) oxygen consumption and (**d**) RQ. (**e**–**h**) Plasma levels of (**e**) glucose, (**f**) triacylglycerols, (**g**) insulin and (**h**) IL-6. White bars, WT mice; black bars, *Lbp*-null mice. (**i**) Glucose tolerance and (**j**) insulin tolerance tests in WT and *Lbp*-null mice after 15 weeks on a standard diet (CTRL) or HFD. White circles, WT mice, standard diet; White squares, WT mice, HFD; black circles, *Lbp*-null mice, standard diet; black squares, *Lbp*-null mice, HFD. Data are presented as mean ± SEM of 6–7 mice per group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, WT vs LBP-null mice; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$, CTRL vs HFD

mice under CTRL or HFD conditions (ESM Table 2). Of the proinflammatory factors, HFD strongly induced circulating IL-6 levels in WT mice; this effect was totally ablated in *Lbp*-null mice (Fig. 3h). Consistent with the Balb genetic background of these mice, HFD did not affect glucose tolerance or the insulin response of WT mice, as determined by GTTs and ITTs (Fig. 3i,j) [22]. However, *Lbp*-null mice exhibited marked glucose intolerance and reduced insulin sensitivity on both a standard diet and HFD.

Under both standard diet and HFD conditions, *Lbp*-null mice showed a marked induction of the browning phenotype markers *Ucp1* and *Dio2* in iWAT (Fig. 4a,b). Other marker genes, including *Prdm16*, *Fgf21* and *Ppargc1a* (Fig. 4c–e), showed a similar trend that did not reach statistical significance between groups, although multifactorial ANOVA analysis revealed a significant increase in *Fgf21* ($p = 0.03$) for the *Lbp*-null factor. iWAT depots showed clusters of multivacuolar adipocytes (with beige/brite morphology) in *Lbp*-null mice, consistent with the gene expression data (Fig. 4j–m). Expression of genes involved in overall adipogenesis (*Pparg*, *Leptin* [*Lep*], and *Adipoq*) did not change (Fig. 4g–i), whereas multifactorial ANOVA analysis revealed a significant increase in *Slc2a4* ($p = 0.04$) for the *Lbp*-null factor. The amount of UCP1 protein per iWAT depot was higher in *Lbp*-null mice than in WT mice under both standard diet and HFD conditions (Fig 4p). Under conditions that promoted the highest browning, the amount of UCP1 protein in iWAT was around 8% of the levels observed in iBAT. Explants of iWAT from *Lbp*-null mice had an increased rate of glucose oxidation (Fig. 4q), but no change

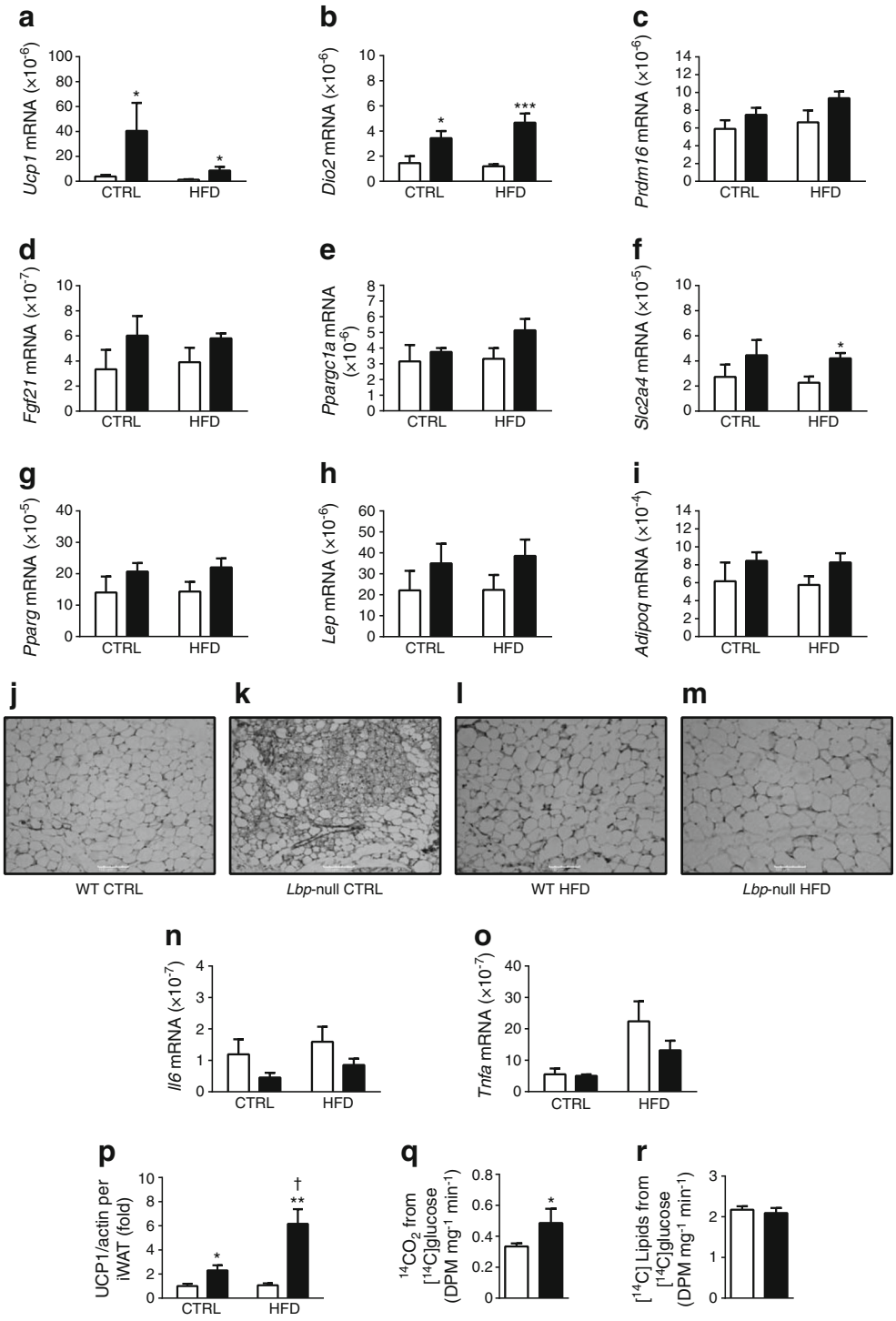

Fig. 4 *Lbp*-null mice exhibit induction of iWAT browning. (**a**–**i**, **n**, **o**) Transcript levels of (**a**) *Ucp1*, (**b**) *Dio2*, (**c**) *Prdm16*, (**d**) *Fgf21*, (**e**) *Ppargc1a*, (**f**) *Slc2a4*, (**g**) *Pparg*, (**h**) *Lep*, (**i**) *Adipoq*, (**n**) *Il6* and (**o**) *Tnfa* in iWAT from WT (white bars) and *Lbp*-null (black bars) mice fed a standard diet (CTRL) or an HFD for 15 weeks. (**j**–**m**) Micrographs (magnification × 20) of iWAT sections. (**p**) UCP1 protein per iWAT depot. (**q**) Glucose oxidation and (**r**) glucose incorporation into lipids in iWAT explants from WT (white bars) and *Lbp*-null (black bars) mice. mRNA levels are normalised to 18S rRNA levels. Data are presented as mean ± SEM of 4–6 independent samples per group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, WT vs *Lbp*-null mice; $^{\dagger}p < 0.05$, CTRL vs HFD. DPM, disintegrations per minute

in the incorporation of glucose into lipids (Fig. 4r). This result indicates that oxidative pathways are enhanced in iWAT from *Lbp*-null mice, which is consistent with the molecular and morphological signs of browning in iWAT.

No evidence of significant browning due to *Lbp* gene ablation was detected in eWAT under any feeding condition, whether determined by gene expression analysis (with the exception of *Dio2*; ESM Fig. 4) or microscopic examination (data not shown).

iBAT in *Lbp*-null mice iBAT weight tended to be higher in *Lbp*-null mice relative to controls (Fig. 5a) and total iBAT protein content was significantly higher in *Lbp*-null mice (Fig. 5b), indicating functional hypertrophy of iBAT in

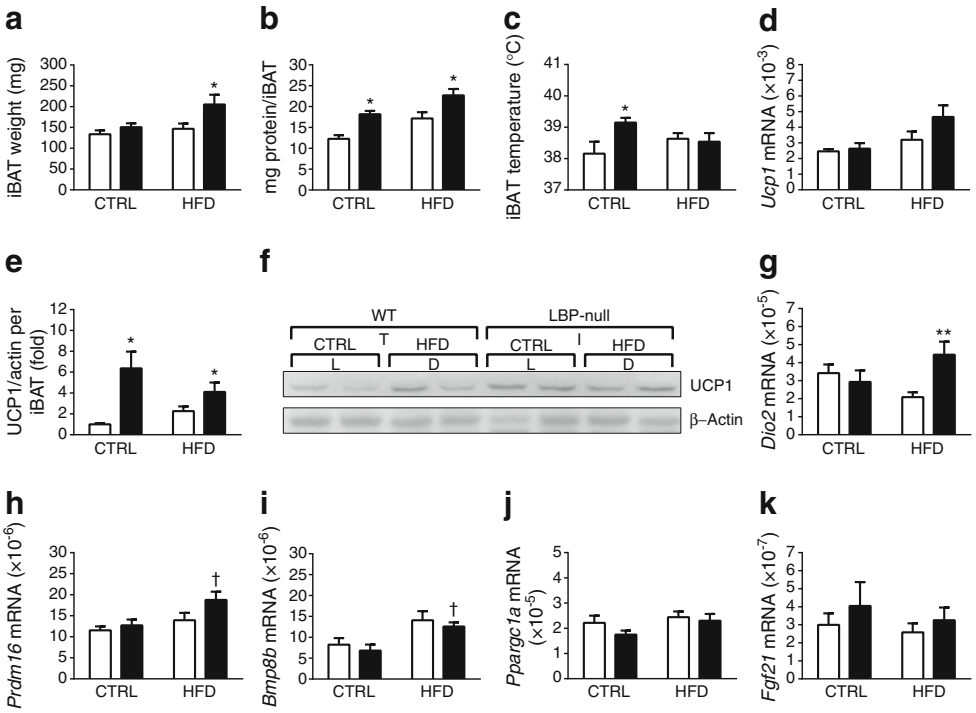

Fig. 5 iBAT in *Lbp*-null mice. (**a**) iBAT weight, (**b**) iBAT protein content and (**c**) surface temperature at the iBAT site in WT (white bars) and *Lbp*-null (black bars) mice fed a standard diet (CTRL) or an HFD. (**d**) *Ucp1* transcript levels and (**e**) UCP1 protein content per iBAT depot. (**f**) Representative immunoblot showing UCP1 protein levels in iBAT (40 μg protein/lane). (**g**–**k**) Transcript levels of (**g**) *Dio2*, (**h**) *Prdm16*, (**i**) *Bmp8b*, (**j**) *Ppargc1a* and (**k**) *Fgf21* in iBAT. mRNA levels are normalised to 18S rRNA levels. Data are presented as mean ± SEM of 4–6 independent samples per group. *$p < 0.05$ and **$p < 0.01$, WT vs *Lbp*-null mice; †$p < 0.05$, CTRL vs HFD

response to the lack of LBP. Consistent with this, the local temperature in the iBAT region was higher in *Lbp*-null mice than in WT mice, at least under standard diet conditions (Fig. 5c).

Total UCP1 per iBAT depot was increased in *Lbp*-null mice (Fig. 5e,f), although relative *Ucp1* transcript levels were not significantly altered (Fig. 5d). This scenario is consistent with a long-term BAT recruitment process [23]. Transcript levels for other thermogenesis-related genes showed no major changes due to LBP ablation, except for a significant induction of *Dio2* in the HFD group (Fig. 5g–k).

Hepatic alterations in *Lbp*-null mice Liver size and hepatic triacylglycerol content were unaltered in *Lbp*-null mice (data not shown). Expression of genes encoding gluconeogenesis-related enzymes (*G6pase* and *Pepck*) tended to be lower in *Lbp*-null mice (ESM Fig. 5a,b). The hepatic expression of *Srebp*, a master regulator of lipid synthesis, as well as *Fas*, *Scd1* and *Acaca*, was significantly reduced in *Lbp*-null mice under CTRL conditions (ESM Fig. 5c–f). *Il6* gene expression was undetectable under CTRL conditions in both WT and *Lbp*-null mice, but was strongly induced under HFD. This induction was impaired in *Lbp*-null mice, a profile that paralleled circulating IL-6 levels (ESM Fig. 5i).

LBP expression negatively correlates with the expression of browning marker genes in human subcutaneous adipose tissue We analysed the expression of *LBP* and browning marker genes in SAT and VAT biopsies from morbidly obese patients. Anthropometrical and clinical variables included age (48.3 ± 9.1 years), BMI (43.8 ± 6.9 kg/m^2), percentage fat mass (56.1 ± 10.1%), fasting glucose (5.33 ± 0.67 mmol/l) and *M* value (23.5 ± 13.3 μmol kg^{-1} min^{-1}). We found significant negative correlations between *LBP* transcript levels and the levels of transcripts for the browning marker genes *UCP1*, *PRDM16*, *PPARGC1A* and *TMEM26* (the latter being a putative marker of beige/brite adipocytes [24]) in SAT (Table 1). No such correlation was found between *LBP* expression and general markers of WAT such as *PLIN1*, *LEP* or *DGAT1*. In VAT, *LBP* mRNA levels negatively correlated with *TMEM26* mRNA levels and positively correlated with *LEP* mRNA levels (Table 1).

Table 1 Bivariate correlations between *LBP* gene expression and the expression of browning marker genes in SAT and VAT from obese individuals ($n = 38$)

Gene	SAT	VAT
PRDM16	−0.32*	−0.21
PPARGC1A	−0.51**	−0.28
UCP1	−0.35*	−0.08
TMEM26	−0.33*	−0.49**
PLIN1	−0.06	−0.22
LEP	0.21	0.55***
DGAT1	0.22	0.27

Data are Spearman's *r* coefficients

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

Discussion

In addition to its immunostimulatory functions [14], LBP has been identified as an adipokine associated with obesity-induced metabolic and proinflammatory disorders [15, 16]. The promotion of BAT activity and, especially, of WAT browning are active research areas that hold promise for strategies aimed at protecting against obesity and associated metabolic abnormalities (e.g. hyperglycaemia and hyperlipidaemia) [24, 25]. Here, we report that both cold exposure and chronic treatment with a β_3-adrenergic agonist (two validated models for inducing WAT browning [20, 26]) were associated with dramatic decreases in *Lbp* gene expression in iWAT. This phenomenon occurred in a cell-autonomous manner: *Lbp* expression was strongly repressed in cultures of adipocytes induced to acquire a beige/brite phenotype. Moreover, silencing of *Lbp* expression in adipocytes enhanced the expression of browning marker genes, whereas LBP treatment reversed this effect. As LBP is secreted by adipocytes, these data suggest that LBP may negatively regulate browning, apparently in an autocrine manner.

We observed that *Lbp*-null mice were protected against body weight increases, which is consistent with the relationship observed between obesity and *Lbp* expression in the adipose tissues of human patients and mouse models [15]. In rodents, the thermogenic activity in BAT accounts for a significant proportion of whole body energy expenditure [27]; therefore, the observed activation of BAT in *Lbp*-null mice may be involved in reducing weight gain. Moreover, although the estimated total thermogenic capacity of 'browned' WAT is much lower than that of BAT [23], browning of WAT is proposed to be relevant to protection against HFD-induced obesity [8, 9]. Our results indicate that a lack of LBP in mice strongly enhances the browning of WAT, thus indicating that the in vitro function of LBP as negative regulator of browning also occurs in vivo. These findings support the concept that LBP, possibly acting in an autocrine manner, negatively regulates the browning process. The negative correlation between *Lbp* expression and marker genes of browning in human adipose samples is consistent with this scenario. The signs of BAT activation in mice lacking LBP appear to contrast with the minor association of BAT activation with *Lbp* downregulation in iBAT. It is possible that the effects of LBP deficiency on body weight in mice occur through alterations in *Lbp* expression in other tissues (e.g. nervous system) that indirectly contribute to regulation of BAT activity, and that the cell-autonomous effects of LBP deficiency are minor in the context of the whole animal. Furthermore, the lack of an overt increase in oxygen consumption (despite activated BAT and WAT browning) and unchanged food intake in *Lbp*-null mice could be due to changes in these variables that are too small to be measurable but have long-term consequences on body weight. In any case, these observations warrant further research to determine whether impaired absorption of food might be different in *Lbp*-null mice than in WT mice, given evidence for a role for LBP in gut biology [28]. In summary, the *Lbp*-null model supports a role for LBP in the browning process, but the pleiotropic phenotype in these mice (see below) precludes definitive conclusions on the impact of browning induction on whole-body energy balance.

The obesity resistance, promotion of browning and increased BAT activity observed in *Lbp*-null mice were accompanied by glucose intolerance and insulin insensitivity. Although seemingly unexpected, these findings can be viewed in relation to our current understanding that complex relationships exist between inflammatory pathways (*Lbp*-null mice also showed a decrease in systemic and local inflammatory markers, such as IL-6) and metabolic regulation. Numerous studies have demonstrated a positive association between serum LBP concentration and obesity-associated metabolic disturbances, including insulin resistance, in humans [29–33]. However, a discrepancy between circulating concentrations of innate immune system proteins in obese patients and the metabolic phenotype of the corresponding knockout mice has also been reported for lipocalin-2 [34, 35] and IL-6 [4, 36]. Other mouse models with loss of function of proinflammatory factors leading to impaired inflammation, such as the dominant-negative *Tnfa* transgenic mouse [37] and *Fsp27*-deficient mice [38], show reduced inflammation in adipose tissue associated with weight loss, but also insulin resistance. In fact, mice lacking LPS-binding capacity due to *Cd14* knockout show reduced proinflammatory responses to LPS, but with significantly impaired glucose tolerance [39]. Thus, although current concepts tend to support the hypothesis that adipose tissue expansion in obesity is associated with local inflammation, which contributes to insulin resistance, recent research indicates that a certain degree of inflammatory response is essential for healthy adipose tissue expansion [37]. Our findings suggest the possibility that total impairment of the LPS signalling pathway limits an appropriate inflammatory response, abolishing the improvement in glucose tolerance that might be expected as a consequence of protection against obesity.

In conclusion, LBP appears to be a negative regulator of WAT browning and possibly of BAT activation. This action of LBP may be relevant to its role in favouring obesity and fat accretion. However, a total lack of LBP, even if protective against obesity, does not result in a systemic healthy phenotype. The complexities of homeostatic vs deleterious roles of inflammatory pathways in metabolic regulation are highlighted in the mouse model of *Lbp* gene ablation. Fine-tuning LBP levels should be considered a part of any prospective use of this factor in promoting healthy metabolism.

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Contribution statement AG-N, TQ-L and MC performed experiments in rodent models; JMM-N and JMF-R performed the studies involving human tissue; AG-N, TQ-L, MG and JMM-N performed the cell culture experiments; all authors contributed to the study design and participated in data analysis; AG-N and FV wrote the manuscript; all authors contributed to drafting the manuscript or revised it critically for intellectual content; and all authors gave final approval of the version to be published. FV is the guarantor of this work.

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