Identification of a Loss-of-Function Mutation in Ube2l6 Associated With Obesity Resistance

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We previously mapped a locus on BALB/c chromosome 2 associated with protection from leptin-deficiency–induced obesity. Here, we generated the corresponding congenic mouse strain by introgression of a segment of C57BL/6J chromosome 2 to the BALB/c background to confirm the genotype–phenotype associations. We found that the BALB/c alleles decreased fat mass expansion by limiting adipocyte hyperplasia and adipocyte hypertrophy. This was concomitant to an increase in adipocyte triglyceride lipase (ATGL) half-life in adipose tissue. In addition, BALB/c alleles on chromosome 2 exerted a cell-autonomous role in restraining the adipogenic potential caused by introduction of the gene coding for the ubiquitin-conjugating enzyme E2L6 (Ube2l6) of BALB/c mice, which showed that the BALB/c allele of Ube2l6 is a hypomorph leading to the lack of UBE2L6 protein expression. Ube2l6 knockdown in 3T3-L1 adipocytes repressed adipogenesis. Thus, altered adipogenic potential caused by Ube2l6 knockdown is likely critically involved in BALB/c obesity resistance by inhibiting adipogenesis and reducing adipocyte numbers. Overall, we have identified a loss-of-function mutation in Ube2l6 that contributes to the chromosome 2 obesity quantitative trait locus. Diabetes 62:2784–2795, 2013

The ability of white adipose tissue (WAT) depots to store energy as triglycerides (TGs) packed into lipid droplets represents a pivotal mechanism allowing for fuel storage and maintenance of organismal homeostasis in mammals. TG hydrolysis supplies energy for the whole organism during periods of negative energy balance, whereas lipogenesis is promoted during positive energy balance states (1). The ratio between lipolysis and lipogenesis controls fat build-up and is dynamically controlled by multiple hormonal signals. Catecholamines and leptin, among other regulators, maintain a catabolic mode by promoting lipolysis (2,3) and antagonizing lipogenesis (4), whereas insulin has an anabolic role (5,6). Thus, lipogenic pathways are markedly induced in the adipose tissue of C57BL6/J leptin-deficient animals (7), leading to increased fat mass attributable to the hypertrophy of preexisting adipocytes and the generation of new adipocytes from immature adipocytes or local precursor cells (8,9). Various lines of evidence indicate that adipocyte hyperplasia could be a factor in the development of obesity (10,11).

RESEARCH DESIGN AND METHODS

Animals. Congenic strain at the generation N4 (i.e., ~93.8% of BALB/c background) were produced by repeated backcrosses to an inbred BALB/c strain obtained from Jackson Laboratory, with selection for C57BL6/J-BALB/c heterozygous haplotype on chromosome 2. Allelic constitution of chromosome 2 was determined using microsatellites markers and analysis of single nucleotide polymorphism (SNP) as follows: D2Mit137 (74.4 Mb; forward: 5′-ttgctgaacgaagaaatggtg-3′; reverse: 5′-ggaggtctatgaataagtttc-3′); rs3007377 (70.18 Mb; forward: 5′-acgtgaaagaaagcaaccag-3′; reverse: 5′-ttgtagttgcgtgtg-3′; DpnI); rs2806667 (83.25 Mb; forward: 5′-gctctcttttcgttctactg-3′; reverse: 5′-getccgagtcgatccag-3′; HaelII); rs370636 (80.00 Mb; forward: 5′-tctctgatgctcataa-3′; reverse: 5′-gaataagagagcaacacta-3′;MspI); rs13459164 (94.35 Mb; forward: 5′-ctccctggttgcttcaata-3′; reverse: 5′-cttgctctgagctgatggtt-3′; HaeIII); rs1029561 (94.35 Mb; forward: 5′-ccagagagagagtgcct-3′; reverse: 5′-tgctgtgcggcagag-3′; MspI); D2Mit112 (102.90 Mb; forward: 5′-ctctctctcaggtctggg-3′; reverse: 5′-tccaagagagagcagacca-3′). D2Mit42 (104.41 Mb; forward: 5′-attacttgagagccagatg-3′; reverse: 5′-gcaagactaacctgcct-3′; D2Mit63 (117.68 Mb; forward: 5′-gctctctcagagcgagagc-3′; reverse: 5′-tgctgctctgagctgatggtt-3′).

N2 ob/+Agpr− (generated from F1 ob/+Agpr−/ N5 BALBc ob/+Agpr−) were backcrossed three times with BALB/c mice and N4 ob+/Agpr−/ were intercrossed to compare N4 BALB/c ob/+Agpr−/ littermates fed with chow diet (Labdiet 5001; kcal %: protein, 28.5%; fat, 13.5%; carbohydrates, 58%). Lean N4 BALB/c2/2 and N4 BALB/c2 ob+ or ob+/Agpr−/ were fed a high-fat diet (Research diet DI2451; kcal % protein, 20%; fat, 45%; carbohydrates, 35%). All procedures were reviewed and approved by the institution's animal care committee.

Body composition and indirect calorimetry. Body composition was measured by magnetic resonance spectroscopy using an EchoMRI (Echo Medical Systems). Fat mass percentage was calculated as the ratio between fat mass and body weight. Metabolic measurements (oxygen consumption, respiratory exchange ratio, locomotor activity, food intake) were obtained continuously using a CLAMS (Columbus Instruments) open-circuit indirect calorimetry system.

Histological analysis of adipose tissue and estimation of adipocytes number per fat pad. Adipocyte sizes and number were determined as previously described (13,14).

Ex vivo lipolysis. Glycerol and fatty acids released from fat explants were determined as previously described (12).

RNA quantification by RT-PCR. RNA was prepared from C57BL/6J ob/ob and BALB/c ob/+ adipose tissue using QiagenRNeasy tissue kit (Qiagen). cDNA was synthesized using SuperScript III and random hexamers (Invitrogen). Amplification of whole Ube2l6 cDNA was performed with (forward) 5′-atgatggcagccagcagagatg-3′ and (reverse) 5′-ttaagaggctcgctcactaca-3′. Quantitative PCR was performed with the following primer sets: Atp1 (forward: 5′-ggctttctgttagctcctc-3′; reverse: 5′-gccggctctgttagctcctc-3′); Pyr4 (forward: 5′-ggagtctactcagctccag-3′; reverse: 5′-ggagtctactcagctccag-3′); Ap2 (forward: 5′-ggagtctactcagctccag-3′; reverse: 5′-ggagtctactcagctccag-3′); Hsl

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associated with protection from leptin-de

We previously mapped a locus on BALB/c chromosome 2 forward: 5'-gtccgtagaggacaggtgttt-3' and reverse: 5'-ccgcctctaggcgaactgg-3'. Ube2bF (forward: 5'-aagcgaggtcgcaagagtgaag-3', reverse: 5'-ttgagcctatcaggaagc-3') and β-actin (forward: 5'-ctgcttgggctgtgctgc-3' and reverse: 5'-ctcttcgctgctacatcgtg-3').

Cultures of adipose tissue explants. Organ culture was performed as previously described (15,16). Briefly, freshly dissected subcutaneous fat pad from ob/ob mice were minced and placed in Mil-09 medium supplemented with insulin (1 μM) and dexamethasone (30 nM) and incubated in a humidified incubator maintained at 37°C with 5% CO2. To analyze adipocyte TG lipase (ATGL) protein turnover, cycloheximide (CHX) with or without MG-132 was added to the culture medium. After 5 h of culture, samples were processed for protein analysis.

Primary stromal vascular cell differentiation to adipocytes. Adipose stromal vascular (SV) cells were isolated from WAT of lean wild-type male mice and digested with 1 mg/mL collagenase D (Roche). SV cells were amplified in DMEM supplemented with 10 ng/mL basic fibroblast growth factor and 10% calf serum. After 72 hours, adipose SV cells were seeded at the same density and grown to confluence. After adipogenic induction (DMEM supplemented with 10% FBS, 0.25 μM/mL Dexamethasone, 0.5 mmol/L isobutylmethylxanthine, and 1 μg/mL insulin) for 72 h, cultures were maintained in DMEM 10% FBS and insulin.

Metabolites and hormone concentration determination. Samples were collected from mice either fed ad libitum or fasted overnight (~15 h). Glycemia was determined using AccuCheck (Abbott), serum insulin levels using ELISA (Lineo Mouse Insulin kit), plasma free fatty acids (FFA, Wako) and serum glyceraldehyde (Cayman) using a colorimetric assay.

Immunoblot analysis. Protein extracts were prepared as previously described (12). Immunoblots were incubated with primary antibodies against hormone-sensitive lipase (HSI), peroxisome proliferator–activated receptor PPARγ, ATGL, β-actin (Cell Signaling), AP2 (Rkd) and UBE2L6 (Santa Cruz).

Expression profiling. RNA quality was assessed with Agilent Bioanalyzer 2100. The amplification and hybridization on the Affymetryx Mouse Gene 1.0 ST chip were performed by the core facility of Albert Einstein College of Medicine. Raw data were normalized using the robust multi-array average algorithm using the Affymetryx Expression Console Software 1.1 and data from two independent replicates (each replicate represents a pool of three individuals) were used to assess expression differences between the two strains. Differentially expressed genes were considered to be with a coefficient of variation <0.5 and P < 0.05 (t-test). We used uncorrected t-test to err on the side of false-positive results rather than risk false-negative results. We filtered out low expression signal for genes with signal below the detection error (indicating no low expression) and, cut-off of 32 (mean ± 2 SD) was calculated from negative controls.

In silico assessment of nonsynonymous polymorphism functionality. SIFT scores (http://sift.bii.a-star.edu.sg/) classify amino acid substitution as deleterious (0.00–0.05), potentially intolerant (0.0051–0.10), borderline (0.101–0.20), or tolerant (0.201–1.00). (17)

Cell transfection and allele-specific protein stability assay. The vector containing Ube2bF cDNA was obtained from Addgene (#12440) (18) and targeted mutagenesis, verified by sequencing, was used to produce the BALB/c congenic strain by introgression of a subregion from C57BL6/J chromosome 2 extending from 74.5 to 122.2 Mb into BALB/c (BALB.2Cc) mice. BALB.2Cc (heterozygous haplotype) and BALB.2Cc mice (homozygous BALB/c haplotype) were compared in the context of leptin deficiency and diet-induced obesity. As previously described, mice were Agrp−/− (20) to lower glucose and insulin levels, which are confounding factors in obesity studies (12).

At 2 months of age, BALB.2Cc ob/ob males and females displayed a significant increase in body weight compared with BALB.2Cc ob/ob littermates because of increased fat mass deposition (+40%) (Fig. 1A). Similar effects were observed in BALB.2Cc diet-induced obesity congenic mice with enhanced fat deposition (10.2 ± 1.36 vs. 13.8 ± 0.79 g of fat mass [P = 0.04] in BALB.2Cc and BALB.2Cc males, respectively) mirrored by decreased lean mass growth (27.7 ± 1.33 vs. 24.0 ± 0.83 g of lean mass [P = 0.035] in BALB.2Cc and BALB.2Cc males, respectively) compared with diet-induced obesity BALB.2Cc mice.

Indirect calorimetry revealed that BALB.2Cc ob/ob leannes was associated with decreased respiratory exchange ratio values (Fig. 1B), indicating increased use of FFA relative to carbohydrates. VO2 consumption (Fig. 1C), activity (Fig. 1D), and caloric intake (Fig. 1E) were similar between BALB.2Cc ob/ob and BALB.2Cc ob/ob.

Concerning glucose homeostasis, we found comparable fasting glucose levels in BALB.2Cc and BALB.2Cc ob/ob mice (231.3 ± 34.97 vs. 218.0 ± 21.06 mg/dL of glucose [P = 0.78] in BALB.2Cc and BALB.2Cc ob/ob males, respectively) and increased insulin levels in BALB.2Cc ob/ob mice (25.0 ± 3.02 vs. 6.38 ± 1.48 ng/mL of insulin [P = 0.0008] in BALB.2Cc and BALB.2Cc males, respectively). A glucose tolerance test revealed impaired glucose clearance in BALB.2Cc ob/ob compared with BALB.2Cc ob/ob mice (Fig. 1F). Accordingly, an insulin tolerance test showed that BALB.2Cc ob/ob mice were more insulin-resistant (Fig. 1G). Thus, BALB.2Cc mice displayed an increased propensity for development of obesity associated with glucose intolerance.

BALB/c alleles on Lipg1 enhanced TG mobilization and ATGL protein stability. Histological analysis of WAT (Fig. 2A) showed decreased fat mass in BALB.2Cc ob/ob mice, we have measured the levels of FFA and glyceraldehyde in mice fed ad libitum to characterize the metabolic changes underlying BALB/c obesity resistance. Both FFA (Fig. 2E) and glyceraldehyde (Fig. 2G) were elevated in BALB.2Cc ob/ob. When fasted, only FFA levels were elevated in BALB.2Cc (Fig. 2F and 2H). Moreover, basal glyceraldehyde release from WAT explants indicated that BALB.2Cc ob/ob mice displayed higher lipolysis rates compared with BALB.2Cc ob/ob mice (Fig. 2D).

With regard to the lower respiratory exchange ratio values and adipocyte hypothrophy observed in the BALB.2Cc ob/ob mice, we have measured the levels of FFA and glyceraldehyde in mice fed ad libitum to characterize the metabolic changes underlying BALB/c obesity resistance. Both FFA (Fig. 2E) and glyceraldehyde (Fig. 2G) were elevated in BALB.2Cc ob/ob. When fasted, only FFA levels were elevated in BALB.2Cc (Fig. 2F and 2H). Moreover, basal glyceraldehyde release from WAT explants indicated that BALB.2Cc ob/ob mice displayed higher lipolysis rates compared with BALB.2Cc ob/ob mice (Fig. 2D).

In adipocytes, ATGL and HSL activity account for 95% of the TG hydrolysis activity (21), and we have further investigated the relative contribution of each enzyme. Lipolysis in WAT explants isolated from BALB.2Cc ob/ob and BALB.2Cc ob/ob mice was determined in the absence and presence of the HSL inhibitor CAY104099 (22), which allowed us to measure total and ATGL-mediated lipolysis, respectively. FFA release by BALB.2Cc ob/ob explants was higher in both conditions (Fig. 2F). However, the extent of the difference in lipolysis between BALB.2Cc ob/ob and BALB.2Cc explants (i.e., ∆FFA release) was greater in the basal condition than after CAY104099 treatment (496.4 ± 91.2
vs. 245.8 ± 44.4 in basal and CAY10499-treated conditions; \( P = 0.0307 \), indicating that the increased TG breakdown observed in BALB.2\textsuperscript{CC} \textit{ob/ob} adipose tissue relied on enhanced activity of HSL and ATGL. In addition, ATGL expression was upregulated in BALB.2\textsuperscript{CC} \textit{ob/ob} mice (Fig. 3D), whereas total HSL concentration was unchanged despite increased phospho Ser660-HSL in BALB.2\textsuperscript{CC} versus BALB.2\textsuperscript{BC} \textit{ob/ob} (data not shown). However, with regard to the expression of these two lipases in BALB/c \textit{ob/ob} mice (12), we reasoned that the pathways that are under direct control of genetic determinants on chromosome 2 should be replicated in congeneric lines and in parental strains (23).

FIG. 1. BALB.2\textsuperscript{CC} \textit{ob/ob} mice displayed increased obesity susceptibility associated with altered glucose clearance and increased fatty acid oxidation. A: Body composition (fat mass and fat-free mass) were analyzed in leptin-deficient BALB.2\textsuperscript{CC} and BALB.2\textsuperscript{BC} males and females at age 2 months \(( n = 7–10)\). Respiratory exchange ratios (RER; B), oxygen consumption normalized to fat-free mass (C), spontaneous locomotor activity (D), and daily food intake (E) were determined during light and dark cycles in BALB.2\textsuperscript{CC} and BALB.2\textsuperscript{BC} \textit{ob/ob} mice with free access to chow diet \(( n = 4)\). F: Glucose tolerance test (GTT) was performed by measuring blood glucose concentration after an overnight fast at the indicated times after intraperitoneal injection of glucose (1 mg/g body weight) in BALB.2\textsuperscript{CC} and BALB.2\textsuperscript{BC} \textit{ob/ob} mice and the glucose area under the curve (AUC) was calculated \(( n = 4)\). G: Insulin tolerance test was performed after 6 h of fast with 3 U/kg insulin in BALB.2\textsuperscript{CC} and BALB.2\textsuperscript{BC} \textit{ob/ob} mice \(( n = 4)\). Glucose concentration is expressed as % of basal glycemia. Data are expressed as average ± SEM. Unpaired \( t \) tests \((A, D, E, F)\) and two-way ANOVA tests \((B, C)\) were performed. *\( P < 0.05\). ***\( P < 0.0005\).
Because only ATGL, and not HSL, was increased in parental (12) and in congenic lines, we assumed that at least ATGL might participate in BALB/c obesity resistance.

With RT quantitative PCR, we did not detect any difference in \textit{Atgl} mRNA expression between BALB and B6 \textit{ob/ob} (Fig. 3A), whereas ATGL protein was upregulated in WAT of BALB \textit{ob/ob} (Fig. 3B)(12). In the adipose tissue of the congenic BALB.2\textsuperscript{BC} \textit{ob/ob} strain, \textit{Atgl} mRNA was downregulated by \textasciitilde30\% (Fig. 3C), whereas the ATGL protein level was decreased by \textasciitilde70\% (Fig. 3D). Unchanged or subtle alteration of \textit{Atgl} mRNA suggests that a degradative mechanism could be involved in the regulation of ATGL protein expression (24).

To assess ATGL protein stability, inguinal fat explants were cultured with or without CHX, an inhibitor of protein translation (25), and degradation of ATGL was followed by Western blot. In B6 \textit{ob/ob} explants, ATGL was decreased by 50\% after 5 h of incubation with CHX, whereas in BALB \textit{ob/ob} adipose tissue the ATGL levels were unaffected (Fig. 3E). Similarly, in the congenic fat explants, inhibition of protein translation showed that ATGL was more rapidly degraded in BALB.2\textsuperscript{BC} \textit{ob/ob} WAT as compared with BALB.2\textsuperscript{CC} (Fig. 3F). This difference in stability is likely to be involved in the difference in ATGL protein levels between BALB versus B6 \textit{ob/ob} mice and between BALB.2\textsuperscript{CC} versus BALB.2\textsuperscript{BC} \textit{ob/ob}. Then, we selectively inhibited the proteasome-mediated degradation in B6 fat explants using MG-132 (26) and found that the degradation of ATGL was suppressed in B6 and in BALB.2\textsuperscript{BC} \textit{ob/ob} WAT explants in the absence of MG-132 (Fig. 3G–H). Together, our data highlight that BALB/c alleles on chromosome 2 participate in ATGL protein levels in adipose tissue of leptin-deficient mice through a post-translational control involving the proteasomal degradation system.

BALB alleles exert a cell-autonomous role and limit the adipogenic competency. Decreased adipocyte number per fat pad in BALB.2\textsuperscript{CC} \textit{ob/ob} mice indicated that hypoplasia contributed to the decreased fat mass. Consequently, we examined whether the BALB/c genome modulated adipogenesis. Because new adipocytes can arise from a committed population of cells residing within the adipose tissue, we cultured and differentiated SV preadipocytes isolated from adipose tissue of lean BALB/c and B6 mice, as well as lean BALB.2\textsuperscript{CC} and BALB.2\textsuperscript{BC} congenic mice (Fig. 4). In response to the adipogenic cocktail, B6
and BALB/c preadipocytes underwent morphological conversion into adipocytes, as evidenced by the accumulation of lipid stained with oil red O (Fig. 4A). However, adipogenesis of BALB/c preadipocytes was less efficient than in B6, because we counted fewer mature adipocytes (Fig. 4C). Similarly, BALB.2CC preadipocytes also displayed reduced adipogenic potential (Fig. 4B and D).

Accordingly, gene expression analysis showed that Pparγ and the differentiation markers (i.e., not expressed in preadipocytes) Ap2, Atgl, and Hsl were induced to higher levels in B6 relative to BALB/c cells (Fig. 4E), reflecting the increased B6 adipocyte count. These same observations were made for the cells derived from the congenic strains, with higher expression of PPARγ, Ap2, Atgl, and Hsl in BALB.2BC cells relative to BALB.2CC cells (Fig. 4F). When we compared the expression of the adipogenic regulator PPARγ in adipose tissue of BALB and B6 ob/ob mice, we did not find a trend toward decreased protein levels (1 ± 0.03 vs. 0.77 ± 0.08; P = 0.08). However, PPARγ also can be influenced by multiple factors such as the increased insulin concentration we observed in serum of BALB.2CC congenic. In rodents, PPARγ expression is decreased by fasting and by insulin deficiency in adipose tissue, and treatment of diabetic mice with insulin leads to a partial restoration of PPARγ levels (27). Consequently, BALB/c alleles on chromosome 2 exert a cell-autonomous role to limit the adipogenic propensity that may contribute to decreased fat mass.
Identification of a nonsynonymous coding SNP in Ube2l6 located in the Lipq1 critical interval. The generation of subcongenic mice enabled us to narrow the position of the genes responsible for the obesity QTL on chromosome 2. We obtained subcongenic strains that contain a small introgressed region from the B6 chromosome 2 in the BALB/c background by crossing BALB.2BCob/− and BALB.2CCob/+. Then, for each subcongenic strains, we determined the B6/BALB boundaries as well as the body fat fraction. Using this approach, we redefined the location of the genes responsible for obesity resistance and narrowed the region to a 9.8-Mb interval on chromosome 2. The obese congenic strains BALB.2BC79–123 and BALB.2BC74–114 that carried the 9.8-Mb chromosome 2 segment derived from the C57BL6/J strain exhibited increased adiposity when compared with the obese congenic strains BALB.2BC102–123, BALB.2BC74–79, and BALB.2BC89–123 (Fig. 5A). Because the subcongenic lines were at the fourth back-cross generation, some uncertainties regarding the remaining B6 contribution remained in the unmapped areas. Nevertheless, the probabilities of these results being observed because of the unmapped regions are very low because the unmapped regions would not be consistently inherited in the N4 cohorts, unlike the mapped introgressed region on chromosome 2.

We next used the complete genome sequence database (www.sanger.ac.uk) of C57BL6/J and BALB/c parental strains to identify putative candidates. There are 254 genes in the segment between 79.18 and 89 Mb on chromosome 2. Among them, we excluded from our analysis 37 putative genes and two microRNAs. We also excluded 186 genes (including 174 Olfr genes) that are not expressed in the adipose tissue of the ob/ob mice. Finally, we searched for expression variants between BALB/c and B6 among the 29 genes expressed in the adipose tissue and located on the chromosome 2 QTL. We observed only one gene Frzb with differential expression (Fig. 5B), but this was not confirmed by RT quantitative PCR in the obese congenic WAT (data not shown). We further investigated nonsynonymous coding SNPs (nsSNP) in genes expressed in WAT. Each nsSNP was categorized based on cross-species conservation and physicochemical properties, and a SIFT score (17) was assigned (Table 1). Interestingly, only Ube2l6 was identified with a deleterious nsSNP between BALB and B6 strain. UBE2L6 is an ubiquitin-conjugating enzyme with a sequence that is well-conserved between E2 enzymes (28). In the B6 transcript, the guanine (codon #29 GAT) was replaced by a thymidine in the BALB/c sequence (rs28011451) (Figs. 5C and 6A). BALB/c polymorphism encodes a tyrosine in place of an aspartate conserved in...
mammals (Fig. 5C). Thus, this substitution is likely to damage UBE2L6 protein.

**The BALB/c allele of UBE2L6 is a hypomorphic variant.** UBE2L6 protein is severely diminished in WAT and in confluent SV preadipocytes of BALB compared with B6 ob/ob, as well as in WAT of BALB.2CC relative to BALB.2BC ob/ob (Fig. 6B). We were able to amplify the full-length Ube2l6 cDNA from BALB/c WAT (Fig. 6C), indicating that the coding sequence was unaltered. We subsequently assayed the consequence of the nsSNP on UBE2L6
protein stability. We transfected 3T3-L1 fibroblasts with the BALB or the B6 variant of Ube2l6 fused to a FLAG tag. The BALB Y28-UBE2L6 protein was markedly less expressed than the B6 D28-UBE2L6 (Fig. 6D) despite the same transfection efficiency (data not shown). Then, the stability of Y28-UBE2L6 and D28-UBE2L6 was determined in B16 cells treated with CHX. After 2 h, Y28-UBE2L6 stability of Y28-UBE2L6 and D28-UBE2L6 was determined in BALB or the B6 variant of C57BL6/J mice.‡

**TABLE 1**

| Genes with nonsynonymous coding SNP in the narrowed Lipq1 |
|----------------------------------------------------------|
| **Gene symbol** | **Gene name** | **Position (Mb)** | **SNP** | **Residues aaB6→aaBALB (position in protein)** | **Residue conserved†** | **Polymorphism conserved between mammals‡** | **SIFT score‡** |
|----------------|--------------|-----------------|--------|-----------------------------------------------|------------------------|---------------------------------------------|---------------|
| SScone2a | Sperm-specific antigen 2 | 79.48 | A → G | Thr→ Ala (319) | Low | Yes | 0.76 |
| Frzb | Frizzled-related protein | 79.50 | T → G | Ile→ Met (1071) | Yes | Yes | 1 |
| Dusp19 | Dual-specificity phosphatase 19 | 80.25 | G → A | Ala→ Val (321) | Yes | No | 0.3 |
| Itig | Integrin α/V | 80.47 | G → C | Gly→ Asp (218) | No | - | - |
| Serping1 | Serine (or cysteine) peptidase inhibitor, clade G, member 1 | 80.47 | G → A | Gly→ Arg (218) | No | - | - |
| Ube2l6 | Ubiquitin-conjugating enzyme E2L 6 | 84.64 | G → T | Ala→ Gly (872) | No | - | - |
| Tnks1bp1 | Tankyrase 1-binding protein 1 | 84.64 | C → G | Ala→ Ser (921) | Yes | No | 0.11 |
| Tnks1bp1 | Tankyrase 1-binding protein 1 | 84.64 | G → T | Ala→ Gly (872) | No | - | - |
| Tnks1bp1 | Tankyrase 1-binding protein 1 | 84.64 | C → T | Ala→ Val (619) | Yes | Yes | 0.43 |
| Tnks1bp1 | Tankyrase 1-binding protein 1 | 84.90 | A → G | Asn→ Asp (941) | Low | Yes | 0.69 |

*Mouse Genomes Project, www.sanger.ac.uk. †Protein sequences at www.ensembl.org and conservation analysis with Jalview software (50). ‡SIFT score predicts whether an amino acid substitution will affect protein function. SIFT scores are classified as damaging if the score ranges from 0.00–0.05, as potentially intolerant if score ranges from 0.051–0.10, as borderline if score ranges from 0.101–0.2, or as tolerant if score ranges from 0.201–1.00 (17)."

**DISCUSSION**

In this report, we used congenic mice to uncover the genetic determinants located at the Lipq1 locus on mouse chromosome 2 that protect BALB/c mice from obesity and glucose intolerance.

At the phenotypic level, our data revealed that the allelic variation in Lipq1 limited fat mass expansion via two mechanisms. First, we observed heightened lipolysis rates that would control adipocyte TG content (29,30) and adipocyte size. This was concomitant with an increase in ATGL-mediated TG breakdown and prolongation of ATGL half-life in adipose tissue. This process is likely relevant to human obesity because obese individuals have been shown to present increased ATGL mRNA expression in subcutaneous adipose tissue, whereas ATGL protein was decreased in this depot compared with lean individuals (31). Thus, discrepancy between mRNA and protein expression may reflect posttranslational mechanisms such as
modification of protein stability between lean and obese human adipocytes as we observed in our model. Second, we found impaired adipogenic potential in preadipocytes bearing Lipq1, which would explain the decrease in mature adipocytes numbers we measured. Because altering the amount of ATGL activity does not alter adipocyte differentiation either in cell culture models or in vivo (29,30,33), it is unlikely that adipocyte numbers would be affected by the downregulation of ATGL we observed. The importance of adipocyte number and changes in adipose cellularity can be a major factor regulating fat pad size and obesity. Restraining adipogenic potential is associated with obesity resistance (34,35) and, conversely, adipocyte hyperplasia favors obesity (11,36).

In search of the putative candidate genes limiting obesity susceptibility in our model, we refined the obesity QTL and identified a missense coding polymorphism in Ube2l6 encoding an E2 ubiquitin-conjugating enzyme. UBE2L6 is involved in ubiquitination of multiple substrates (37,38) and, like all E2 enzymes, it acts via selective interactions with E3 enzymes that confer specificity to ubiquitination by recognizing target substrates (39). Furthermore, UBE2L6 serves as the E2 enzyme for posttranslational addition of an ubiquitin-like protein ISG15 (interferon-stimulated gene 15) important for antiviral immunity (40,41), although we have not found ISG15 expression in WAT (data not shown). The critical role of UBE2L6 in BALB/c obesity resistance was strongly supported by several lines of evidence. First, Ube2l6 is located in the 9.8-Mb minimal interval and harbors a polymorphism in BALB/c mice coding for a defective allele. This is in line with BALB/c obesity resistance, which is a recessive inherited

FIG. 6. BALB/c allele of UBE2L6 is a hypomorph and UBE2L6 expression is repressed during obesity and 3T3 adipogenesis. A: BALB/c and B6 polymorphism detection in DNA sequence electropherogram. The aspartate (D) substitution to tyrosine(Y) is indicated. B: Immunoblot and densitometry analysis of UBE2L6 in inguinal fat from BALB/c and B6 ob/ob (n = 4), in inguinal fat from BALB.2yc and BALB.2yn ob/ob (n = 3), and in SV preadipocytes from lean BALB/c and B6 mice, at day 0, before addition of the differentiating medium (n = 3). C: Cartoon representing the exon organization of Ube2l6 transcript. Open boxes (□) show the 5’ and 3’ UTR and filled boxes (■) show the coding sequence. Whole Ube2l6 transcript can be amplified from BALB and B6 ob/ob inguinal fat samples shown by gel electrophoresis. D: Immunoblots of UBE2L6 in 3T3 fibroblasts protein extracts prepared 48 h after transfection with BALB (Y28) or B6 (D28) FLAG-UBE2L6. E: Protein expression analysis of BALB (Y28) or B6 (D28) FLAG-UBE2L6 after incubation with the CHX for the indicated time (n = 3). Immunoblots and densitometry analysis of UBE2L6 (F) in inguinal fat from (+/+) lean and ob/ob B6 mice (n = 3). G: UBE2L6 protein expression during differentiation of 3T3-L1 adipocytes (n = 3). Data are expressed as average ± SEM. Unpaired t tests were performed. *P < 0.05. ***P < 0.0005.
trait. Also, the increased expression of UBE2L6 protein in lean versus obese adipose tissue indicated that UBE2L6 levels are regulated in response to metabolic changes. Finally, Ube2l6 knockdown in 3T3-L1 adipocytes revealed that UBE2L6 was critically involved in adipocyte differentiation mirroring the impaired BALB/c and BALB.2CC SV adipogenic potential. Consequently, UBE2L6 appeared to be a key regulator of adipocyte biology that likely determines obesity susceptibility in our model. It is intriguing that UBE2L6 expression and proteasomal degradation correlated with decreased ATGL stability in B6 adipose tissue. However, we were unable, so far, to detect any ubiquitination of ATGL using in vivo and in vitro ubiquitinylation assays (data not shown). In cells in which Ube2l6 was knocked-down, inhibition of adipogenesis did not enable us to measure ATGL protein expression because of the differential degree of differentiation. Thus, further studies remain necessary to evaluate the link between UBE2L6 and ATGL protein regulation.

BALB/c obesity resistance was associated with improved glucose tolerance and insulin sensitivity, despite increased circulating levels of FFA. This result contrasted with the ATGL knockout mice characterized by decreased FFA and improved glucose tolerance and insulin sensitivity (42). However, we can hypothesize that decreased adiposity may counterbalance the effects of increased FFAs, as in transgenic mice overexpressing ATGL in the adipose tissue (29). In addition, BALB.2CC ob/ob mice are characterized by increased insulin level. Although the reason why insulinemia increased is currently unknown, we can hypothesize that elevated FFA levels in the BALB.2CC strain may contribute to increased insulin secretion (43).

The BALB/c-Ube2l6 allele is found in several strains such as A/J, AKR/J, C3H/HeJ, CBA/J, DBA/2J, and LP/J, whereas the B6-Ube2l6 allele is found in three 129/SV substrains, NOD strains, and NZO strains (www.sanger.ac.uk). Because BALB/c and DBA/2J, the first inbred mouse strain to be developed (44), share the same allele, it is likely that Ube2l6 variants existed before the development of inbred mouse strains and its widespread representation would suggest that it is a functional variant that is advantageous under conditions of domestication (reliable food supply) to limit obesity. Of note, the wild strains CAST/EiJ, PWK/PhJ, Spretus/EiJ, and WSB/EiJ all have the C57BL6/J variant, suggesting that B6-Ube2l6 variant promotes efficient storage in times of food abundance, providing a survival advantage during times of food shortage (45,46).

Interestingly, the human UBE2L6 gene encodes the obesegenic polymorphism (D28) encoded by the C57BL/6 allele. However, the SNP database lists genetic variation at the human UBE2L6 locus. Notably, rs140725358 located in the same domain as the mouse BALB/c polymorphism (Fig. 5C) is predicted to be highly deleterious by SIFT score, as was predicted for the BALB/c-Ube2l6 allele, although the very low frequency of this allele makes it a relatively rare allele. Thus, it would be interesting to investigate such variants as an obesity-protective polymorphism in humans.

Overall, our data support the implication of UBE2L6 in BALB/c obesity resistance. In vivo, BALB/c alleles
functions to decrease adipocyte size and number, and in vitro we found that BALB/c alleles repressed SV adipogenic potential. Similarly, limited adipogenesis led to decreased TG accumulation in 3T3-L1 adipocytes with Ube216 knockdown. Thus, repressed adipogenic potential after Ube216 knockdown would explain adipocyte hypoplasia in BALB/c mice. UBE2L6 is expressed in 3T3-L1 preadipocytes and its expression is suppressed early after addition of the differentiation medium, suggesting its important role during the very early stage of preadipocytes differentiation during growth arrest and commitment steps (48,49). Thus, using genetics, we identified a new gene controlling adipocyte commitment; however, future studies will be needed to identify UBE2L6 targets.

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G.M. researched the data and wrote the manuscript. S.-M.L. researched the data. G.J.S. reviewed and edited the manuscript and contributed to discussion. S.C.C. reviewed and edited the manuscript and contributed to discussion. S.C.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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