

# Decreased RB1 mRNA, Protein, and Activity Reflect Obesity-Induced Altered Adipogenic Capacity in Human Adipose Tissue

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Retinoblastoma (Rb1) has been described as an essential player in white adipocyte differentiation in mice. No studies have been reported thus far in human adipose tissue or human adipocytes. We aimed to investigate the possible role and regulation of RB1 in adipose tissue in obesity using human samples and animal and cell models. Adipose RB1 (mRNA, protein, and activity) was negatively associated with BMI and insulin resistance (HOMA-IR) while positively associated with the expression of adipogenic genes (*PPAR $\gamma$*  and *IRSI*) in both visceral and subcutaneous human adipose tissue. BMI increase was the main contributor to adipose RB1 downregulation. In rats, adipose Rb1 gene expression and activity decreased in parallel to dietary-induced weight gain and returned to baseline with weight loss. RB1 gene and protein expression and activity increased significantly during human adipocyte differentiation. In fully differentiated adipocytes, transient knockdown of Rb1 led to loss of the adipogenic phenotype. In conclusion, Rb1 seems to play a permissive role for human adipose tissue function, being downregulated in obesity and increased during differentiation of human adipocytes. Rb1 knockdown findings further implicate Rb1 as necessary for maintenance of adipogenic characteristics in fully differentiated adipocytes.

**A**dipose tissue plays a central role in the management of systemic energy storage as well as in many other processes. This is due to its capacity to store triglycerides and to secrete many proteins that have a major impact on energy homeostasis. Several studies suggest that one of the key factors that link excess caloric intake and a positive energy balance with metabolic disturbance is the inability to appropriately expand the adipose tissue, which occurs in parallel to the decrease of its adipogenic capacity (1,2).

The retinoblastoma gene (*RB1*) was the first tumor suppressor gene to be recognized and cloned (3,4). The gene encodes the retinoblastoma protein (pRb), a central regulator of the cell cycle (5), which is required for several

cellular processes (6) including apoptosis, cell proliferation, and differentiation in skeletal myocytes, osteoblasts, and adipocytes (7–9).

The involvement of pRb in adipocyte differentiation was first suggested in 1994 (10) in connection with the ability of pRb to activate C/EBP-mediated transcription (10–13). Mouse embryonic fibroblasts and 3T3-L1 lacking the *Rb1* gene are unable to undergo adipose conversion in response to treatment with standard adipogenic inducers (11). The defective differentiation of *Rb1*<sup>-/-</sup> mouse embryonic fibroblasts, however, can be bypassed by administration of a peroxisome proliferator-activated receptor (PPAR) $\gamma$  ligand or by partial inhibition of extracellular signal-regulated kinase 1/2 activity (14,15).

In mice, pRb has been described as an essential player in white adipocyte differentiation and as a negative modulator of brown adipocyte differentiation (16,17). In fact, several studies in both mouse and rat models have shown that pRb inactivation leads to or associates with the acquisition of brown-like features (browning) in white fat depots (18–20).

To our knowledge, no previous studies have explored the possible role of Rb1 in fully differentiated 3T3-L1 adipocytes during human adipocyte differentiation or in whole human adipose tissue or the effects of diet-induced weight changes on adipose tissue *Rb1* gene expression.

In the current study, we aimed 1) to explore RB1 expression and activity in human adipose tissue in relation to obesity and during human adipocyte differentiation, 2) to study the effects of diet-induced weight gain and loss on Rb1 expression and activity in rat adipose tissue, and 3) to investigate the possible role of Rb1 in the adipogenic maintenance of fully differentiated 3T3-L1 adipocytes.

## RESEARCH DESIGN AND METHODS

**Subject recruitment.** Samples from two independent cohorts were analyzed: 152 adipose tissue samples (76 visceral and 76 subcutaneous) from a group of Caucasian men ( $n = 19$ ) and women ( $n = 57$ ) with BMI between 20 and 58 kg/m<sup>2</sup> and 64 adipose tissues (32 visceral and 32 subcutaneous) from a group of Caucasian morbidly obese men ( $n = 5$ ) and women ( $n = 27$ ) with different degrees of insulin sensitivity. Insulin sensitivity was measured using euglycemic-hyperinsulinemic clamp.

Both cohorts were recruited at the Endocrinology Service of the Hospital Universitari Dr. Josep Trueta (Girona, Spain). All subjects reviewed that their body weight had been stable for at least 3 months before the study and gave written informed consent after the purpose, nature, and potential risks of the study were explained to them.

Adipose tissue samples were obtained from subcutaneous and visceral depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia, and gastric bypass surgery), washed, fragmented, and immediately flash frozen in liquid nitrogen before being stored at  $-80^{\circ}\text{C}$ .

**Anthropometric measurements.** BMI was calculated as weight (in kilograms) divided by the square of height (in meters). According to this

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anthropometric parameter, subjects were classified as nonobese (BMI <30 kg/m<sup>2</sup>) and obese (BMI ≥30 kg/m<sup>2</sup>) following World Health Organization guidelines.

**Protein preparation.** Proteins were extracted from adipose tissue and adipocytes by using a Polytron PT-1200C homogenizer (Kinematica, Lucerne, Switzerland) directly in radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mmol/L NaCl, and 50 mmol/L Tris-HCl, pH 8.0), supplemented with protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride). Cellular debris and lipids were eliminated by centrifugation of the solubilized samples at 13,000 rpm for 60 min at 4°C, recovering the soluble fraction below the fat supernatant and avoiding the nonhomogenized material at the bottom of the centrifuge tube. Protein concentration was determined using the RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

**Total and phosphorylated (Thr821) pRb protein determination.** Total and phosphorylated pRb protein levels in RIPA extracts of human adipose tissue and adipocyte samples were measured using a human Rb total ELISA kit (KHO0011; Invitrogen) and a human Rb (pT821) ELISA kit (KHO0021; Invitrogen) following the manufacturer instructions or by immunoblotting after denaturing electrophoresis (see below), using the primary antibodies included in the ELISA kits.

**Effects of diet-induced weight gain and loss in rats.** Rb1 gene expression was analyzed by real time Q-PCR in retroperitoneal white adipose tissue (rWAT) of male Wistar rats (Charles River Laboratories España SA, Barcelona, Spain) fed for 4 months (from month 2 to month 6 of age) a normal-fat diet (10% of energy as fat), a high-fat diet (60% of energy as fat), or a cafeteria diet (19) and in a group of male Wistar rats fed the cafeteria diet followed by a normal-fat diet for 2 months (POST-CAF group). Six to seven animals were analyzed per group. Adiposity index was calculated by dividing the sum of the weight of all white adipose tissue depots by the body weight and multiplying by 100. Methodology used for analysis by immunoblotting of pRb in rat tissues is detailed as Supplementary Data.

**RB1 gene expression during human preadipocyte differentiation.** Isolated human preadipocytes from lean (BMI <25 kg/m<sup>2</sup>) and obese (BMI >30 kg/m<sup>2</sup>) subjects (Zen-Bio, Research Triangle Park, NC) were plated on T-75 cell culture flasks and cultured at 37°C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mix F-12 medium (1:1, v/v) supplemented with 10 units/mL penicillin/streptomycin, 10% FBS, 1% HEPES, and 1% glutamine (all from GIBCO, Invitrogen, Barcelona, Spain). One week later, the isolated and expanded human subcutaneous preadipocytes were cultured (~40,000 cells/cm<sup>2</sup>) in 12-well plates with preadipocyte medium (Zen-Bio) composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, penicillin, and streptomycin in a humidified 37°C incubator with 5% CO<sub>2</sub>. Twenty-four hours after plating, cells were checked for complete confluence (day 0) and differentiation was induced using differentiation medium (Zen-Bio) composed of preadipocyte medium, human insulin, dexamethasone, isobutylmethylxanthine, and PPAR $\gamma$  agonists (rosiglitazone). After 7 days, differentiation medium was replaced with fresh adipocyte medium (Zen-Bio) composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, biotin, pantothenate, human insulin, dexamethasone, penicillin, streptomycin, and amphotericin. Fourteen days after the initiation of differentiation, cells appeared rounded with large lipid droplets apparent in the cytoplasm. Cells were then considered mature adipocytes, harvested, and stored at -80°C for protein and RNA extraction for study of RB1 protein and gene expression levels after human adipocyte differentiation. The experiment was performed in triplicate for each sample. Adipogenic differentiation was verified by fatty acid synthase (*FASN*) (*Hs00188012\_m1*; Applied Biosystems, Madrid, Spain) and adiponectin (*Adipoq*) (*Hs00605917\_m1*; Applied Biosystems) gene expression.

**3T3-L1 preadipocyte differentiation and short hairpin RNA- and small interfering RNA-mediated knockdown of Rb1.** The embryonic fibroblast mouse cell line 3T3-L1 (American Type Culture Collection) was maintained in DMEM containing 20 mmol/L glucose, 10% FBS, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin. Two days after confluence, insulin (5  $\mu$ g/mL), dexamethasone (0.5  $\mu$ mol/L), and isobutylmethylxanthine (0.5 mmol/L) mixture was added for 2 days, followed by 5 days with insulin (5  $\mu$ g/mL) alone. Differentiation was verified by morphological assessment and adipogenic gene expression.

Permanent silencing was performed using Rb1-targeted and control short hairpin RNA (shRNA) lentiviral particles (sc-29468-V and sc-108080; Santa Cruz Biotechnology, Santa Cruz, CA) and following the manufacturer instructions. Positive 3T3-L1 preadipocytes harboring shRNA cassette for Rb1 were selected by puromycin (3  $\mu$ g/mL) selection 60 h after infection.

Transient silencing was performed in fully differentiated adipocytes using mouse Rb1 (ON-TARGETplus SMARTpool mouse Rb1, L-047474-00; Dharmacon) or control (ON-TARGETplus nontargeting pool, D-001810-01-05; Dharmacon) small interfering RNAs (siRNAs) introduced by electroporation

or lipid transfection. For electroporation, cells were suspended by mild trypsinization and electroporated using the BioRad kit according to the manufacturer's recommendations (GP Electrop.Buffer; BioRad). Approximately 2 million cells in 200  $\mu$ L GP Electrop.Buffer (BioRad) and 250 nmol/L siRNA were used, and the adipocytes were reseeded afterward into 12-well plates. In transfection using a lipid-based formulation (siPORT NeoFX; Life Technologies SA, Madrid, Spain), Rb1 siRNA (25 nmol/L) and the transfection reagent siPORT NeoFX (0.3%) were assayed, and a successful transfection was achieved using a reverse transfection protocol (21) with  $8 \times 10^4$  cells during 36 h. **Oil Red O staining and analysis.** For Oil Red O staining, cells were washed twice with PBS, fixed in 4% formaldehyde for 1 h, and stained for 30 min with 0.2% Oil Red O solution in 60% isopropanol. Cells were then washed several times with water, and excess water was evaporated by placing the stained cultures at ~32°C. For quantification of the extent of adipose conversion, 0.2 mL isopropanol was added to the stained culture dish. The extracted dye was immediately removed by gentle pipetting, and optical density was determined spectrophotometrically at 500 nm using a multiwell plate reader (Anthos Labtec 2010 1.7).

**Gene expression.** RNA was prepared from human adipose tissues and cell culture samples using RNeasy Lipid Tissue Mini Kit (QIAGEN; Izasa SA, Barcelona, Spain). The integrity of each RNA sample was checked by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was quantified by means of spectrophotometer (GeneQuant, GE Health Care, Piscataway NJ) and reverse transcribed to cDNA using a High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's protocol.

Real-time quantitative PCR was conducted with Light Cycler 480 Probes Master (Roche) using TaqMan technology suitable for relative genetic expression quantification. Commercially available and prevalidated TaqMan primer/probes sets were used, and they are described in Supplementary Data.

The reaction was performed in a final volume of 7  $\mu$ L. The cycle program consisted of an initial denaturing of 10 min at 95°C followed by 40 cycles of 15 s denaturing phase at 95°C and 1 min annealing and extension phase at 60°C. A threshold cycle ( $C_t$  value) was obtained for each amplification curve, and a  $\Delta C_t$  value was first calculated by subtracting the  $C_t$  value for human *PPIA* RNA from the  $C_t$  value for each sample. Fold changes compared with the endogenous control were then determined by calculating  $2^{-\Delta\Delta C_t}$ , so gene expression results are expressed as expression ratio relative to *PPIA* gene expression according to the manufacturers' guidelines.

For rat tissue samples, total RNA was extracted from frozen rWAT samples using TriPure reagent (Roche), purified using an E.Z.N.A. Total RNA Kit I (Omega Bio-Tek) and retrotranscribed to cDNA. Quantitative PCR was run using SybrGreen Master Mix on a StepOnePlus instrument essentially as previously described (22). Analysis of quantitative PCR data was done using the LinRegPCR (23) or the StepOnePlus software. *Eif4e*, *Eif2s1*, or both were used as reference genes (24). Primers for *Rb1*, *Eif4e*, and *Eif2s1* are provided in Supplementary Data.

**Immunoblotting analysis.** RIPA protein extracts of human adipose tissues and cell culture samples (25–50  $\mu$ g) were separated by SDS-PAGE and transferred to nitrocellulose membranes by conventional procedures. Membranes were immunoblotted with primary antibodies against p<sup>Thr821</sup>Rb1 and Rb1 (KHO0021 and KHO0011, respectively; Invitrogen); Rb1, FASN, PPAR $\gamma$ , Adipoq, Ucp1, and  $\beta$ -actin (sc-74562, sc-20140, sc-7273, sc-26497, sc-28766, and sc-47778, respectively; Santa Cruz Biotechnology); and Prdm16 (AP18024PU-N; Acris Antibodies). Anti-rabbit IgG and anti-mouse IgG coupled to horseradish peroxidase were used as secondary antibody. Horseradish peroxidase activity was detected by chemiluminescence, and quantification of protein expression was performed using scion image software.

**Statistics.** Statistical analyses were performed using SPSS 12.0 software for Windows (SPSS, Chicago, IL). All assays were performed at least in duplicate and are reported as means  $\pm$  SD. The relation between variables was analyzed by ANOVA (using minimum significant difference [DMS] and Bonferroni post hoc tests), simple correlation (Pearson or Spearman test), and multiple linear regression models (using stepwise method). One-way ANOVA was used to compare the effects of weight gain and loss and differences between groups.

## RESULTS

### RB1 gene expression in human adipose tissue

**First cohort.** Mean anthropometric, clinical, and adipose gene expression data of participants from the first human cohort are described in Table 1. *RB1* gene expression in adipose tissues decreased significantly in association with obesity, together with expression of adipogenic/lipogenic genes. No differences were found among obese subjects depending on the presence of type 2 diabetes.

TABLE 1  
Anthropometric, clinical, and adipose gene expression features of the first human cohort

	Nonobese subjects	Obese subjects	Obese subjects with type 2 diabetes	<i>P</i>
<i>N</i>	31	25	20	
Sex (male/female)	9/22	4/21	6/14	
Age (years)	51.1 ± 13.27	43.9 ± 8.76	48.2 ± 11.3	<b>0.05</b>
BMI (kg/m <sup>2</sup> )	25.4 ± 2.7	43.5 ± 8.38*	43.58 ± 5.16*	< <b>0.0001</b>
Fasting glucose (mg/dL)	91.3 ± 11.5	92.4 ± 14.1	158.1 ± 69.4*##	< <b>0.0001</b>
Fasting insulin (uU/mL)	8.4 ± 4.6	9.7 ± 3.7	31.48 ± 21.2*##	<b>0.001</b>
HOMA-IR	2.49 ± 2.3	2.28 ± 1.0.3	8.86 ± 5.78*##	<b>0.002</b>
HDL cholesterol (mg/dL)	59.7 ± 16.7	60.0 ± 40.8	48.9 ± 11.0 *	<b>0.042</b>
Fasting triglycerides (mg/dL)	108.5 ± 48.5	96.15 ± 36.2	170.8 ± 108.8*##	<b>0.002</b>
Gene expression (RU)				
<i>RBI</i>				
VAT	0.016 ± 0.004	0.014 ± 0.005*	0.013 ± 0.006*	<b>0.029</b>
SAT	0.015 ± 0.005	0.011 ± 0.005*	0.012 ± 0.004*	<b>0.031</b>
Leptin				
VAT	0.12 ± 0.11	0.27 ± 0.20*	0.34 ± 0.14*	<b>0.039</b>
SAT	0.62 ± 0.39	0.93 ± 0.35	0.84 ± 0.35	0.133
<i>GLUT4</i>				
VAT	0.058 ± 0.029	0.022 ± 0.014*	0.017 ± 0.009*	< <b>0.0001</b>
SAT	0.066 ± 0.021	0.043 ± 0.026*	0.025 ± 0.023*	<b>0.016</b>
<i>IRS1</i>				
VAT	0.016 ± 0.012	0.010 ± 0.004*	0.007 ± 0.004*	<b>0.001</b>
SAT	0.015 ± 0.011	0.012 ± 0.006	0.009 ± 0.003*	<b>0.030</b>
<i>PPARγ</i>				
VAT	0.030 ± 0.019	0.008 ± 0.006*	0.006 ± 0.004*	< <b>0.0001</b>
SAT	0.043 ± 0.009	0.010 ± 0.005*	0.010 ± 0.004*	< <b>0.0001</b>
<i>FASN</i>				
VAT	0.18 ± 0.13	0.08 ± 0.10*	0.06 ± 0.06*	< <b>0.0001</b>
SAT	0.59 ± 0.93	0.08 ± 0.06*	0.06 ± 0.05*	< <b>0.0001</b>
<i>ACC</i>				
VAT	0.050 ± 0.044	0.017 ± 0.011*	0.015 ± 0.011*	< <b>0.0001</b>
SAT	0.044 ± 0.044	0.023 ± 0.021*	0.018 ± 0.011*	<b>0.003</b>

Data are means ± SD unless otherwise indicated. RU, relative units. \**P* < 0.05 compared with nonobese subjects after performing DMS and Bonferroni post hoc tests. #*P* < 0.05 compared with obese subjects after performing DMS and Bonferroni post hoc tests. Boldface data indicates statistical significance (*P* < 0.05).

In both subcutaneous (SAT) and visceral (VAT) adipose tissues, *RBI* gene expression was negatively associated with BMI ( $r = -0.269$ ,  $P = 0.019$ , and  $r = -0.332$ ,  $P = 0.003$ , respectively) and positively associated with *PPARγ* ( $r = 0.460$ ,  $P < 0.0001$ , and  $r = 0.414$ ,  $P = 0.001$ ) and *IRS1* ( $r = 0.413$ ,  $P = 0.001$ , and  $r = 0.358$ ,  $P = 0.004$ ) gene expression (Table 2). In addition, adipose *RBI* gene expression was negatively associated with *LEP* ( $r = -0.295$ ,

TABLE 2  
Correlations of *RBI* gene expression with anthropometric and clinical characteristics of first human cohort

	<i>RBI</i> gene expression (RU)			
	VAT ( <i>n</i> = 76)		SAT ( <i>n</i> = 76)	
	<i>R</i>	<i>P</i>	<i>R</i>	<i>P</i>
Age (years)	0.041	0.718	0.288	<b>0.012</b>
BMI (kg/m <sup>2</sup> )	-0.332	<b>0.003</b>	-0.269	<b>0.019</b>
Fasting glucose (mg/dL)	-0.221	0.060	-0.023	0.845
Fasting insulin (μU/mL), <i>n</i> = 34	-0.471	<b>0.005</b>	-0.446	<b>0.009</b>
HOMA-IR, <i>n</i> = 34	-0.454	<b>0.007</b>	-0.404	<b>0.018</b>
HDL cholesterol (mg/dL)	0.210	0.070	0.032	0.801
Fasting triglycerides (mg/dL)	-0.182	0.120	-0.103	0.397
Gene expression (RU)				
Leptin	-0.148	0.337	-0.295	<b>0.010</b>
<i>GLUT4</i>	0.392	<b>0.002</b>	0.192	0.187
<i>IRS1</i>	0.358	<b>0.004</b>	0.413	<b>0.001</b>
<i>PPARγ</i>	0.414	<b>0.001</b>	0.460	< <b>0.0001</b>
<i>FASN</i>	0.059	0.606	0.251	<b>0.036</b>
<i>ACC</i>	0.062	0.591	0.240	<b>0.042</b>

Boldface data indicates statistical significance (*P* < 0.05). RU, relative units.

TABLE 3  
Anthropometric and clinical features and adipose RB1 gene expression of second cohort

	IS <3.3 [mg/(kg · min)]	IS >3.3 [mg/(kg · min)]	P
N	16	16	
Age (years)	49.29 ± 7.9	48.65 ± 10.14	0.5
BMI (kg/m <sup>2</sup> )	44.8 ± 4.7	42.2 ± 7.1	0.3
IS*	2.19 ± 0.61	5.33 ± 1.51	<0.0001
<b>RB1 gene expression (RU)</b>			
VAT	0.0077 ± 0.0038	0.0066 ± 0.0036	0.4
SAT	0.0074 ± 0.0046	0.0069 ± 0.0025	0.6

Data are means ± SD unless otherwise indicated. Boldface data indicates statistical significance ( $P < 0.05$ ). RU, relative units. \*Insulin sensitivity (IS) was measured using euglycemic-hyperinsulinemic clamp.

$P = 0.01$ ) and positively associated with *FASN* ( $r = 0.251$ ,  $P = 0.036$ ) and *ACC* ( $r = 0.240$ ,  $P = 0.042$ ) gene expression in SAT and positively associated with *GLUT4* ( $r = 0.392$ ,  $P = 0.002$ ) gene expression in VAT (Table 2). In a multiple linear regression model, *PPARγ* ( $\beta = 0.44$ ,  $P = 0.02$ ) gene expression contributed independently to *RB1* gene expression variance after BMI and *IRS1*, *FASN*, *ACC*, and *LEP* gene expressions in SAT were controlled for. In VAT, *IRS1* ( $\beta = 0.44$ ,  $P = 0.02$ ) gene expression contributed independently to *RB1* gene expression variance after BMI and *PPARγ* and *GLUT4* gene expressions were controlled for.

In a subgroup of 34 participants in whom fasting insulin was measured, *RB1* gene expression in both SAT and VAT was negatively associated with fasting insulin ( $r = -0.446$ ,  $P = 0.009$ , and  $r = -0.471$ ,  $P = 0.005$ , respectively) and homeostasis model assessment of insulin resistance (HOMA-IR) ( $r = -0.404$ ,  $P = 0.018$ , and  $r = -0.454$ ,  $P = 0.007$ ) (Table 2). In the multiple linear regression model, BMI ( $\beta = -0.42$ ,  $P = 0.01$ , in SAT and  $\beta = -0.36$ ,  $P = 0.03$ , in VAT) contributed independently to *RB1* gene expression variance after fasting insulin or HOMA-IR was controlled for.

**Second cohort.** A second cohort made up of morbidly obese subjects with different degrees of insulin sensitivity (measured using euglycemic-hyperinsulinemic clamp) was used to investigate the possible relationship between *RB1* gene expression in adipose tissues and insulin sensitivity excluding the effects of obesity. Mean anthropometric and clinical characteristics as well as adipose *RB1* gene expression levels in this second cohort are described in Table 3. No correlation was found between *RB1* gene expression in SAT or VAT and insulin sensitivity ( $r = -0.205$ ,  $P = 0.30$ , in SAT and  $r = 0.161$ ,  $P = 0.42$ , in VAT).

**pRb activity in human adipose tissue.** The activity of pRb was analyzed in 18 SAT and 28 VAT human samples measuring by ELISA the levels of pRb protein phosphorylated at Thr 821 and of total pRb protein and calculating the ratio between them. Typically, more phosphorylation indicates less Rb1 protein activity (i.e., less capacity of pRb to interact with transcription factors and other interacting proteins [25]). In concordance with *RB1* mRNA levels, in both SAT and VAT total pRb protein levels

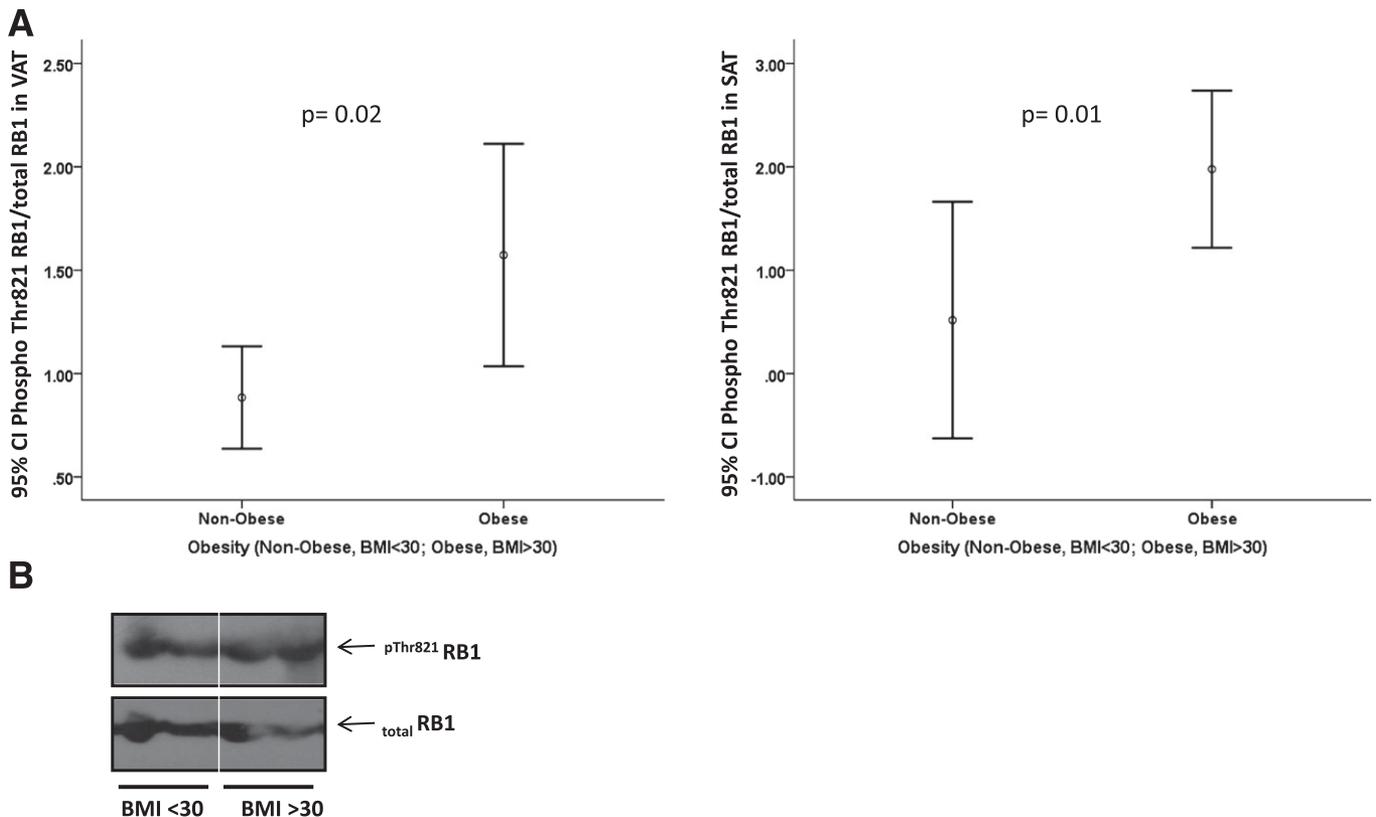


FIG. 1. A: <sup>pThr821</sup>RB1-to-total RB1 protein ratio in SAT and VAT of nonobese and obese subjects. <sup>pThr821</sup>RB1 and total RB1 were measured using specific ELISA kits. B: Representative immunoblots of <sup>pThr821</sup>RB1 and total RB1 protein in VAT of nonobese and obese subjects measured by immunoblotting using the ELISA antibodies. Phospho, phosphorylated.

decreased ( $r = -0.46$ ,  $P = 0.05$ , and  $r = -0.41$ ,  $P = 0.03$ , respectively) and levels of phosphorylated pRb increased ( $r = 0.55$ ,  $P = 0.03$ , and  $r = 0.39$ ,  $P = 0.04$ ) in association with obesity, resulting in an increased phosphorylated pRb-to-total pRb ratio indicative of reduced pRb activity (Fig. 1A). The ELISA measurements were validated by immunoblotting (Fig. 1B).

#### Effects of diet-induced weight gain and loss in rats.

To gain further insight into the effects of obesity on *Rb1* gene expression in adipose tissue, we explored the impact of diet-induced weight changes on *Rb1* mRNA levels in rats fed with a cafeteria-style high-fat diet. Confirming the association found in human obesity, adipose *Rb1* gene expression decreased significantly in response to cafeteria diet-induced weight gain in rats. Furthermore, when the cafeteria diet was replaced by a normal diet and body weight returned toward normality, adipose *Rb1* gene expression was reestablished (Fig. 2A). In fact, adipose *Rb1* gene expression was negatively correlated with the adiposity index of the animals ( $r = -0.587$ ,  $P = 0.003$ ) (Fig. 2B). In keeping with the mRNA data, adipose pRb activity decreased significantly with cafeteria diet-induced weight

gain, as indicated by increased  $\text{pSer}^{780}\text{pRb-to-pRb}$  ratio, and returned to basal levels after weight loss (Fig. 2C).

#### Changes in RB1 during human adipocyte differentiation.

RB1 gene and protein expression and activity were monitored during differentiation of primary preadipocytes derived from human VAT and SAT. In both cases, *RB1* gene expression decreased transiently at day 2 and then increased gradually in parallel to adipogenic gene expression (Fig. 3A) and lipid droplet formation. Similar to gene expression, pRb total protein quantity increased significantly during human adipocyte differentiation (Fig. 3B), as did pRb activity (Fig. 3B [reduced Thr821 phosphorylation indicative of increased activity]). ELISA measurements were validated by immunoblotting analyses (Fig. 3C and D).

#### Effects of Rb1 knockdown on adipocyte gene expression.

Rb1 permanent knockdown in preadipocytes using shRNA lentiviral particles decreasing Rb1 expression by 50% led to decreased expression of adipogenic genes (*Ppar $\gamma$* , *Adipoq*, and *Fasn*) and increased expression of brown adipocyte-related genes (*Prdm16* and *Ucp1*) during differentiation, measured both at the mRNA and

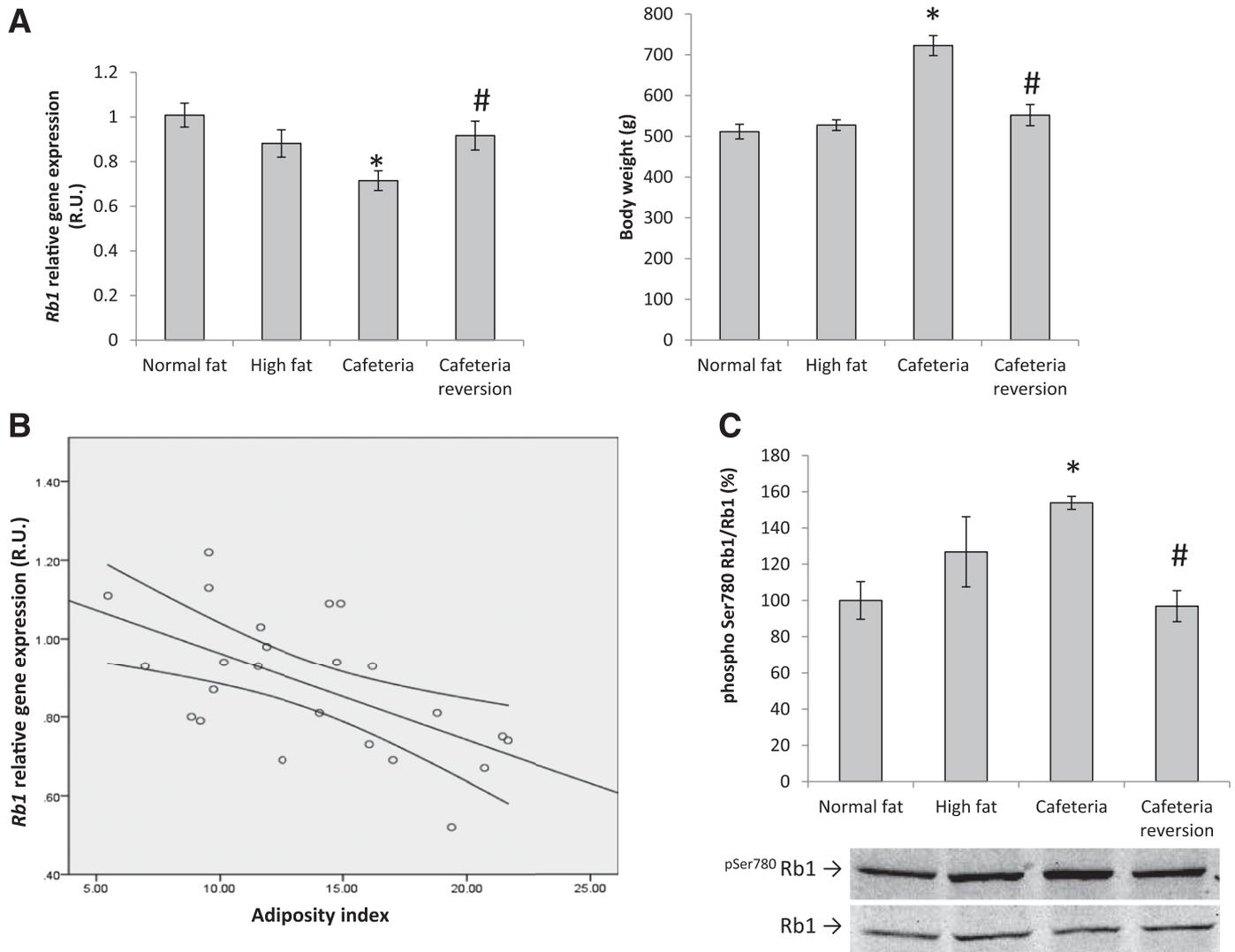
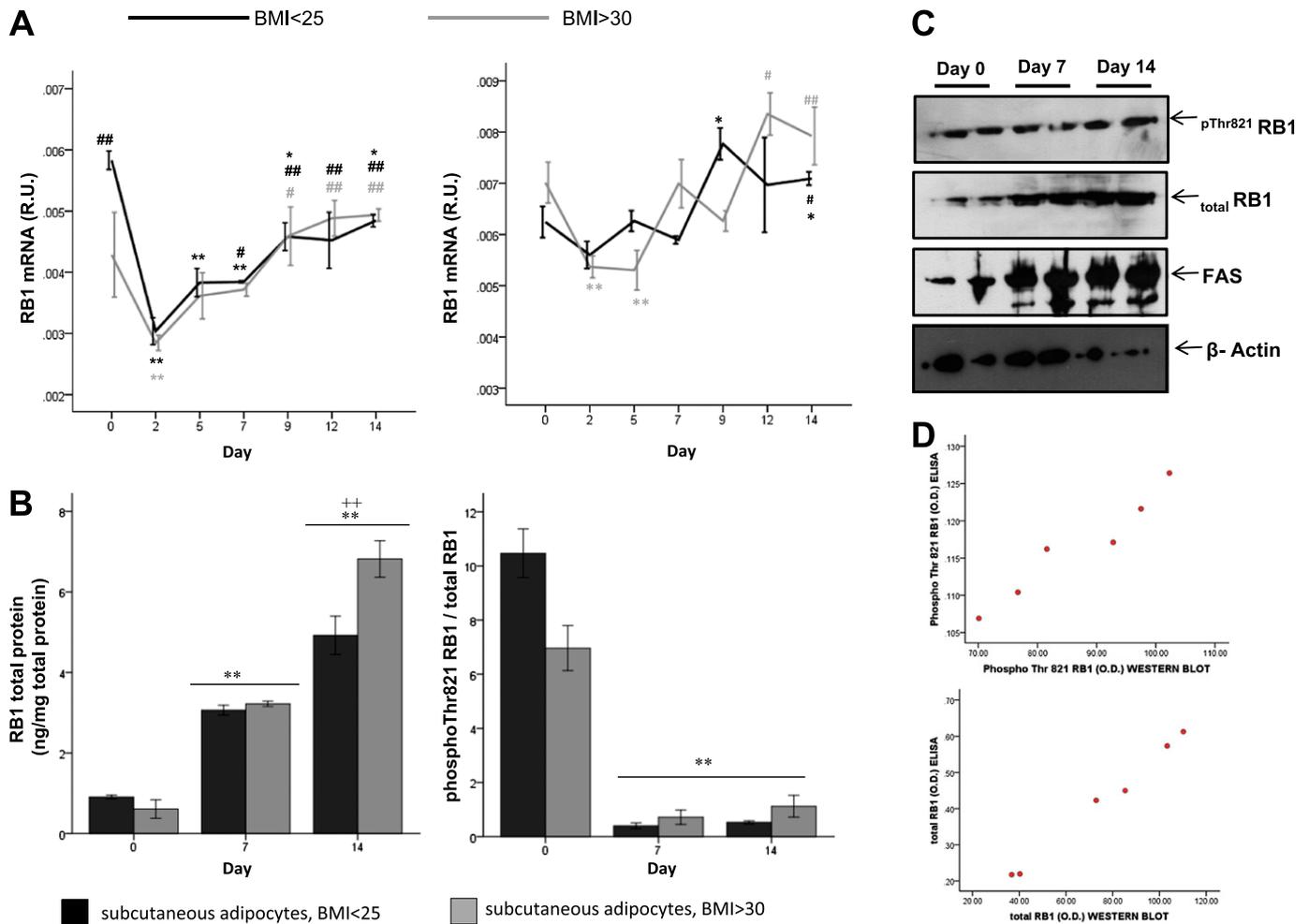


FIG. 2. **A:** Effect of high-fat and cafeteria diet on *Rb1* gene expression in rWAT and body weight. **B:** Correlation between *Rb1* gene expression in rWAT and adiposity index. **C:** Effect of high-fat and cafeteria diet on  $\text{pSer}^{780}\text{Rb1/Rb1}$  protein in rWAT. \* $P < 0.05$  in comparison with normal-fat diet; # $P < 0.05$  in comparison with cafeteria diet. Phospho, phosphorylated.



**FIG. 3.** *A* and *B*: *RB1* mRNA (*A*), *RB1* total protein (*B*) (left panel), and *RB1* activity (*B*) (right panel) during time course of differentiation of human preadipocytes isolated from the denoted fat depots of lean (BMI <25 kg/m<sup>2</sup>) or obese (BMI >30 kg/m<sup>2</sup>) subjects. Phosphorylated (Phospho) Thr821 *RB1* and total *RB1* were measured by ELISA. \**P* < 0.05 in comparison with day 0; \*\**P* < 0.005 in comparison with day 0; #*P* < 0.05 in comparison with day 2; ##*P* < 0.005 in comparison with day 2; ++*P* < 0.005 in comparison with day 7. *C*: Representative immunoblots of p<sup>Thr821</sup>*RB1* and total *RB1* protein during time course of differentiation of human subcutaneous preadipocytes. Immunoblots for fatty acid synthase as an adipocyte marker and  $\beta$ -actin as an internal control in the same cultures are also shown. *D*: Bivariate correlation between ELISA and Western blotting measurements of p<sup>Thr821</sup>*RB1* and total *RB1* protein (*r* > 0.8, *P* < 0.01). O.D., optical density. (A high-quality color representation of this figure is available in the online issue.)

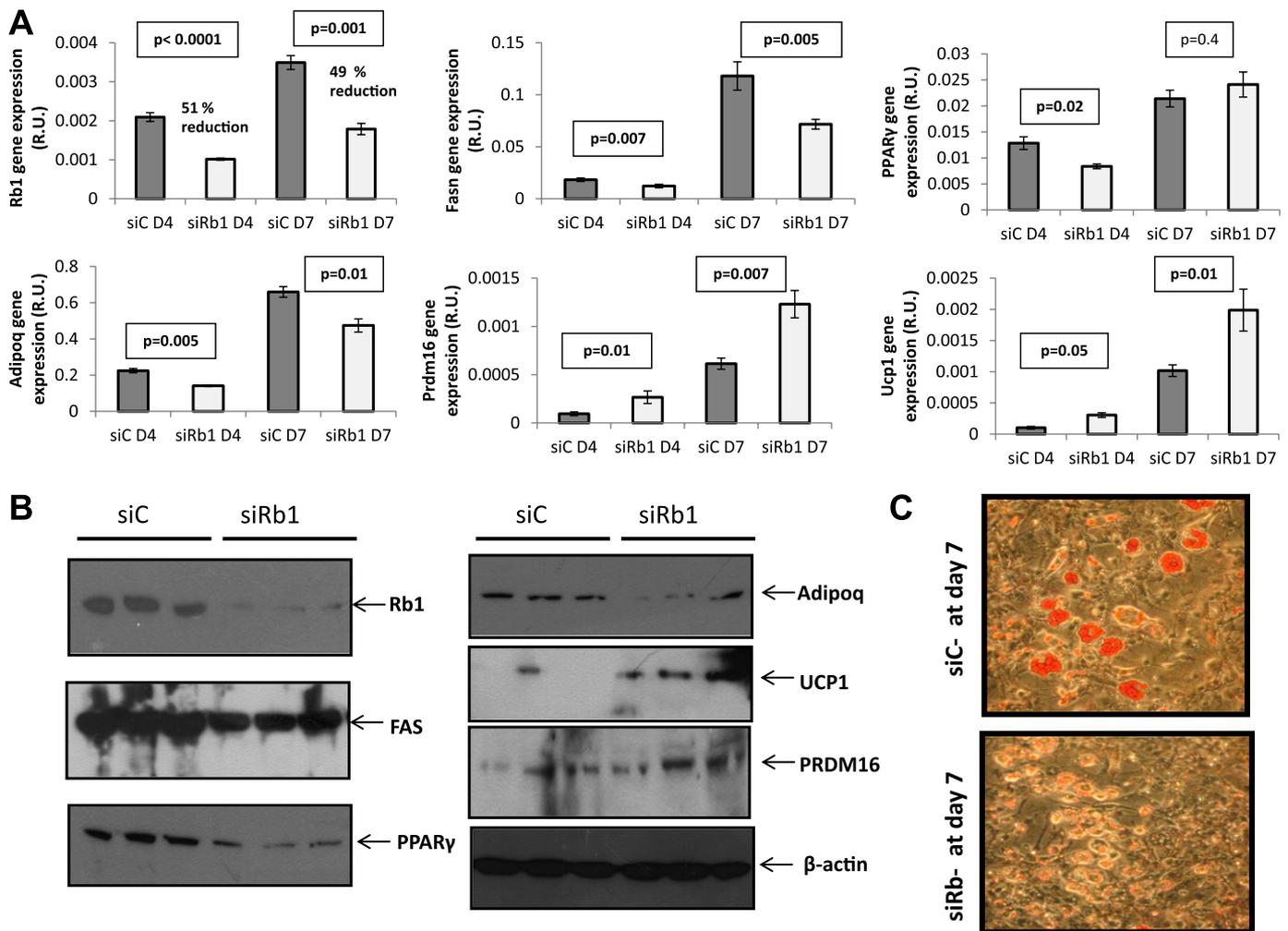
the protein levels (Fig. 4*A* and *B*). In line with the gene expression results, decreased accumulation of intracellular lipid droplets was apparent in the *Rb1*-silenced cells after 7 days of adipogenic conditions (Fig. 4*C*). In fully differentiated adipocytes, transient siRNA-induced *Rb1* knockdown (36 h) (through electroporation or lipid-based formulation, decreasing *Rb1* mRNA by 68 and 41%, respectively), led to loss of the adipocyte phenotype, as indicated by decreased adipogenic/lipogenic gene and protein expression (Fig. 5 and Supplementary Fig. 1). Although no effects were observed on the mRNA expression levels of the brown adipocyte-related genes *Ucp1* and *Prdm16*, UCP1 protein was weakly detected after transient siRNA-induced *Rb1* knockdown (Fig. 5).

**DISCUSSION**

*Rb1* has been described as a player in white adipocyte differentiation mainly on the basis of studies using immortalized murine cell lines, such as 3T3-L1. Studies dealing with pRb in human adipogenesis and with the regulation of adipose pRb in connection with human or

rodent obesity are lacking. Moreover, most studies thus far have evaluated the role of *Rb1* in adipogenesis but not in the mature adipocyte. To the best of our knowledge, this is the first study showing that adipose *RB1* gene and protein expression and activity are significantly associated with human adipose tissue function and modulated in obesity and during human adipocyte differentiation. It is also the first to evidence a previously unappreciated role for the *Rb1* protein in the maintenance of the mature adipocyte phenotype.

We observed an inverse association between adipose *RB1* levels/activity and human obesity in cross-sectional analysis, with this finding confirmed longitudinally in the rat diet interventional study, which indicated reciprocal changes in *Rb1* gene expression/activity with weight gain and loss. Importantly, in both subcutaneous and visceral human adipose tissue, *RB1* gene expression was negatively associated with BMI and positively associated with adipogenic gene expression (mainly, *PPAR $\gamma$*  and *IRS1*). Obesity is well-known to be characterized by a hypertrophied, dysfunctional adipose tissue, with an increased expression of proinflammatory cytokines (26) in parallel to



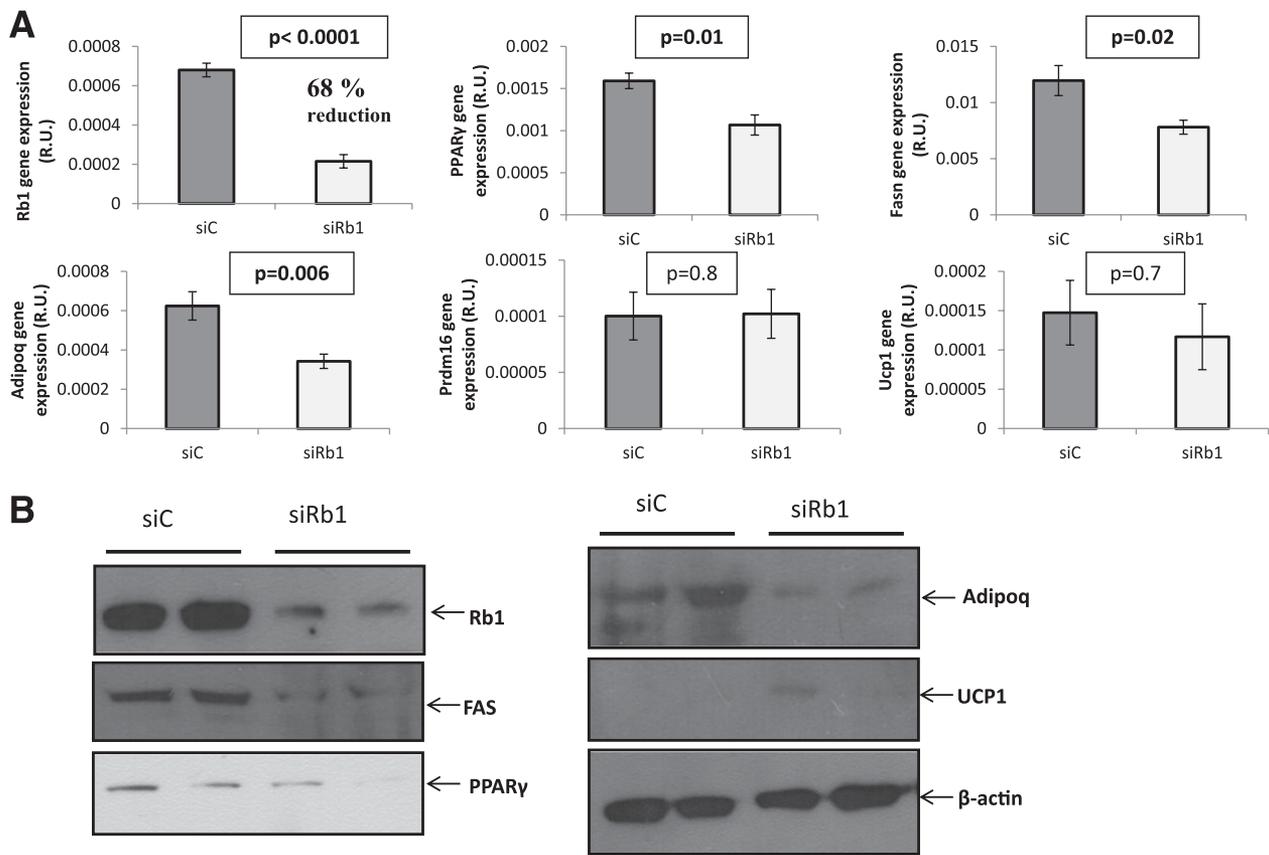
**FIG. 4. A:** 3T3-L1 preadipocytes were treated with a nontargeted shRNA (siC) (control cells) or with a shRNA targeted to silence Rb1 (siRb1). Control cells and cells with permanent (~50%) Rb1 knockdown were induced to differentiate using a standard adipogenic cocktail, and the mRNA expression levels of the denoted genes were measured at day 4 (D4) and day 7 (D7) of adipogenic differentiation. **B:** Same genes as in A were measured at the protein level in control and Rb1-silenced cells after 7 days of adipogenic differentiation; representative immunoblots are shown. **C:** Oil Red O staining of control and Rb1-silenced cells after 7 days of differentiation to test the accumulation of lipid droplets. R.U., relative units.

a decreased expression of adipogenic/lipogenic genes (27–31). Changes in obesity are to some extent reminiscent of those occurring during dedifferentiation of isolated human adipocytes and adipose tissue explants—a process known to be promoted by cytokines (such as tumor necrosis factor- $\alpha$ ) and prevented by adipogenic inducers (such as isobutylmethylxanthine) (32–34). Results in this study strongly suggest that RB1 is required for an optimal adipose tissue function, since Rb1 knockdown favored adipocyte dedifferentiation and, additionally, decreased RB1 levels correlated with decreased expression of adipogenic genes in the adipose tissue of obese subjects. Considering the well-known antiproliferative role of RB1 as a cell cycle break, reduced RB1 and adipogenic gene expression in human obesity may also be linked to and favor the hyperplastic component of obesity.

Dual effects of Rb1 on murine adipogenesis have been described. Thus, whereas a proadipogenic role of Rb1 is well established (10–12,35), some studies have shown that the activation of Rb1 during mitotic clonal expansion (at the first stage of adipocyte differentiation) leads to blunted adipogenesis (36–38). In fact, pRb is known to be hyperphosphorylated (inactive form) during clonal expansion of

3T3-L1 cells, corresponding to previously quiescent cells reentering the cell cycle. Later in adipogenesis, pRb becomes hypophosphorylated (active form) at the same time that its expression is induced (12,39,40). Similar changes in RB1 expression and activity have been demonstrated here during differentiation of human preadipocytes, including a decrease in *RB1* gene expression at an early stage (day 2) followed by a gradual increase as adipogenesis further progresses.

Permanent knockdown of Rb1 in preadipocytes using shRNA lentiviral particles led to impaired white adipogenesis and promoted a brown-like adipocyte differentiation pattern with increased *Prdm16* and *Ucp1* gene expression—in agreement with previous studies using *Rb1*<sup>-/-</sup> knockout models (16–18). Transient Rb1 knockdown in fully differentiated adipocytes, in its turn, resulted in markedly reduced expression of adipogenic/lipogenic genes, suggesting that Rb1 is necessary, and not just permissive, for maintenance of the adipogenic/lipogenic capacity of mature adipocytes, and in good concordance with our findings in human adipose tissue samples. Browning due to Rb1 reduction in this model cannot be discarded, since a weak UCP1 protein band could be



**FIG. 5.** Effects of transient Rb1 knockdown (~70% reduction for 36 h) in fully differentiated 3T3-L1 adipocytes on the expression of the denoted genes at the mRNA (A) and the protein (B) level. Treatments: SiC, control siRNA; siRb1, Rb1 siRNA. siRNAs were introduced in the mature adipocytes by electroporation.

detected only after transient siRNA-induced Rb1 knockdown. However, *Ucp1* and *Prdm16* gene expression in the cells was not affected by Rb1 silencing. Additionally, no correlation was found between *RB1* and *UCP1* gene expression in human adipose tissue (data not shown).

In summary, RB1 expression and activity seem to be essential for maintenance of the adipogenic/lipogenic capacity of adipose tissue in the human, as they are reduced in parallel with adipogenic gene expression in the dysfunctional adipose tissue of obese subjects and increased during primary human preadipocyte differentiation. Rb1 knockdown findings further implicate Rb1 as necessary for maintenance of adipogenic characteristics in fully differentiated fat cells.

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J.M.M.-N., P.P., and M.S. researched data and wrote the manuscript. F.O., E.G.-R., and P.O. researched data. J.R. and W.R. contributed to discussion and reviewed the manuscript. A.P., M.L.B., and J.M.F.-R. researched data

and wrote, reviewed, and edited the manuscript. M.L.B. and J.M.F.-R. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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