Improved metabolic regulation is associated with retinoblastoma protein gene haploinsufficiency in mice

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Retinoblastoma protein family members have been shown to be involved in nonredundant complex ways in adipogenic commitment and terminal adipogenesis in different model systems of murine and human origin (9–11, 34–36). pRb is unique in this context in that its activity favors terminal white adipogenesis (10, 11) and inhibits brown adipogenesis (19).

Changes in pRb function may impact energy metabolism and adiposity in vivo by impacting adipocyte biology and metabolic fate, among other mechanisms. In fact, studies in mouse models have shown that pRb inactivation leads to or associates with resistance to diet-induced obesity and the acquisition of brown adipose tissue (BAT)-like features in white adipose tissue (WAT), with increases in WAT capacity for oxidative metabolism, fatty acid oxidation, and thermogenesis (12, 29). An involvement of pRb in the control of energy metabolism is sustained by additional lines of evidence. The pRb-interacting transcription factor E2F1 represses the expression of oxidative genes in BAT and skeletal muscle in a pRb-dependent manner (4). Increases in cellular oxidative metabolism in response to physiological and pharmacological stimulus such as those seen in BAT upon cold exposure (4, 19), skeletal muscle upon fasting (4), and WAT or white adipocytes following retinoic acid treatment (28, 30) are accompanied by downregulation of the expression and/or inactivation of the pRb through its phosphorylation. More recently, pRb has been shown to be involved in neuronal regulation of feeding, and an antiobesity action of pRb activity in the hypothalamus has been proposed (26). Thus, pRb appears to be intimately linked to the control of different aspects of energy metabolism, acting in a tissue-specific manner.

As part of this background, we have previously shown that mice heterozygous for a null mutation in the Rb gene (Rb haploinsufficient mice) are protected against the development of obesity, insulin resistance, and hepatosteatosis when fed a high-fat diet (29). We noticed in that work that young Rb+/− mutants raised on a standard chow diet were indistinguishable from wild-type littermates in terms of body weight and fat content but already presented signs of increased metabolism. In the present work, we tested the hypothesis that partial deficiency in Rb may contribute to a healthy aging by improving the metabolic response to acute stress challenges under a normal diet.

MATERIALS AND METHODS

Animals and overall study design. Wild-type (WT, Rb+/+) and Rb1tm1Tyj (Rb+/−) C57BL/6J mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the University of the Balearic Islands animal house by mating Rb+/− females to WT males. Litters were genotyped by PCR of DNA extracted from tail biopsies using primer sets as indicated by the

THE CONCEPT THAT CELL CYCLE regulatory proteins modulate metabolism in addition to cell proliferation and survival is strengthened by recent research (14). One important protein in this context is the retinoblastoma protein (pRb), encoded by the Rb gene and best known for its role in the control of the cell’s proliferation/differentiation switch (25). pRb is the archetypical member of the retinoblastoma protein family, which also includes p107 and p130. In its active hypophosphorylated form, pRb functions as a cofactor for many transcription factors, including E2Fs and cell differentiation-related factors. Interaction with pRb represses the transactivating activity of E2F1 on genes encoding proteins required for entry into the DNA replication phase of the cell cycle while it stimulates the activity of cell differentiation inducers, including master transcription factors required for the differentiation of skeletal myocytes, osteoblasts, and adipocytes (8, 47).

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provider. From weaning throughout life, animals were fed a defined diet providing 15.9 kJ/g, with 20% energy from protein, 70% energy from carbohydrates, and 10% energy from fat (Research Diets, New Brunswick, NJ). The animals were housed at 22°C with a 12:12-h light-dark cycle (lights on at 0800) and free access to food and water. Rb+/− and WT mice were studied from weaning (day 21) until 31 wk of age; part of the animals was killed at a youngest age (11 wk). We did not extend the study to older ages because Rb+/− mice have been reported to be prone to develop pituitary tumors with age (22). Body composition was determined periodically using an Echo MRI body composition analyzer (EchoMRI, Houston, TX). Energy intake was estimated two times per week on a per-cage basis (2–3 animals/cage) from the actual amount of food consumed by the animals and its caloric equivalence. Functional tests as described below were performed from the actual amount of food consumed by the animals and its caloric equivalence. Functional tests as described below were performed in male animals between 1.5 and 2.5 mo of age (young adults) and between 6 and 7.5 mo of age (adult). Animals were killed by decapitation under fed conditions, within the first 2 h of the light cycle. Interscapular BAT, WAT depots, skeletal muscle (gastrocnemius), and liver were dissected in their entirety, weighed, snap-frozen in liquid nitrogen, and stored at −80°C until analysis. Serum was prepared from blood collected from the neck and stored at −80°C until analysis. A lengthways fragment of inguinal WAT and pituitary and liver biopsies were fixed for morphological and immunohistochemical analysis (see below). Animal protocols followed in this study were reviewed and approved by the Bioethical Committee of the University of the Balearic Islands, and guidelines for the use and care of laboratory animals of this university were followed.

Glucose tolerance test. Sterile glucose in saline (2 g/kg animal) was administered intraperitoneally after a 6-h fast (from 0600 to 1200), and glucose, insulin, and nonesterified fatty acid (NEFA) levels were measured in tail blood collected at the indicated times.

Insulin tolerance test. Sterile human recombinant insulin in saline (0.75 IU/kg animal) was administered intraperitoneally after a 6-h fast (from 0600 to 1200), and glucose levels were measured in tail blood collected at the indicated times.

Rectal temperature determination. Internal body temperature was determined by gently inserting the probe of a calibrated thermometer (RS 612–849; RS, Madrid, Spain) in the rectum, using Vaseline as a lubricant.

Surrogate indexes of insulin resistance/sensitivity. Indexes were derived from circulating levels of glucose, insulin, and NEFA in animals after a 6-h fast (from 0600 to 1200). The homeostatic model assessment for insulin resistance (HOMA-IR) score was calculated from fasting insulin and glucose concentrations as described (27): HOMA-IR = (in μU/ml) × glucose (in mmol/l)/22.5. The revised quantitative insulin sensitivity check index (R-QUICKI) was calculated as described (32): R-QUICKI = 1/[log glucose (in mg/dl) + log insulin (in μU/ml) + log NEFA (in mM)].

Assessment of the sensitivity to exogenous leptin. The anorectic effect of exogenous leptin relative to saline injection was studied essentially as previously described (40). Recombinant murine leptin (PeproTech, Rocky Hill, NJ) dissolved in saline was used. Mice from both genotypes were randomly assigned to one of the following groups: the intraperitoneal leptin group, which received an intraperitoneal injection of leptin (2 mg/kg body wt) just before lights off at 2000; or the vehicle group, which received saline. After injection, they were returned to their home cages and provided with their standard diet. Food intake was measured during the next 1, 2, 4, 12, and 24 h after leptin or saline injection, using red light during the dark phase to avoid disturbing the circadian rhythm of the animals. One week later, the experiment was repeated under the same conditions but reversing the assignment of animals to the intraperitoneal leptin or vehicle groups.

Oral fat tolerance test. Mice were fasted overnight for 16 h, and then a single dose of olive oil (10 ml/kg body wt) was orally administered by gavage. Plasma samples were obtained from the tail vein before and 1, 2, 3, and 4 h after the fat challenge for determination of plasma triglyceride levels.

Indirect calorimetry. The PhenoMaster Indirect Calorimetry System was employed (TSE Systems, Bad Homburg, Germany). At the indicated ages, subsets of animals were placed individually in metabolic chambers and let to adapt for 24 h before the measurement began. O2 consumption and CO2 production were simultaneously and continuously monitored for 24, h and data were collected every 20 min. Mean respiratory exchange ratio (RER) values were calculated over 24 h and distinctly over the light and dark phase. Spontaneous movement of the animals during the indirect calorimetry assay was automatically recorded.

Histology and immunohistochemistry. Inguinal WAT, liver, and pituitary samples from WT and Rb+/− mice were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C, dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin blocks. Five-micrometer-thick sections were stained with hematoxylin/eosin to assess morphology. No signs of neoplasia were found in any of the tissues from Rb+/− mice examined, including pituitary. Liver sections were classified into grades of steatosis according to Brunt et al. (6). Morphometric analysis of inguinal WAT sections was performed by digital acquisition of adipose tissue areas using AxioVision 40V 4.6.3.0 software and a Zeiss Axioskop 2 microscope equipped with an AxioCam iCCD camera (Carl Zeiss, Barcelona, Spain). Immunohistochemical demonstration of uncoupling protein (UCP) 1 was performed in inguinal WAT sections according to the avidin-biotin-peroxidase complex (ABC) method (21). Briefly, sections were incubated with normal goat serum 2% in PBS pH 7.3 to block unspecific sites and then overnight at 4°C with primary rabbit polyclonal UCP1 antibody (GeneTex, Irvine, CA) diluted 1:300 in PBS. Sections were then incubated with the corresponding biotinylated anti-rabbit IgG secondary antibody (Vector Labs, Burlingame, CA), diluted 1:200, and finally with ABC complex (Vectastain ABC kit; Vector Labs). Peroxidase activity was revealed by 3,3′-diaminobenzidine hydrochloride as chromogen (Sigma, St. Louis, MO) in water. Sections were counterstained with hematoxylin before image acquisition.

Immunoblotting analysis. UCP1, peroxisome proliferator-activator receptor (PPAR)-α, UCP3, and peroxisome proliferator-activated receptor coactivator 1 (PGC-1α) protein levels in tissues were analyzed by immunoblotting using specific primary anti-mouse antibodies [from, respectively, GeneTex for UCP1; Abcam (Cambridge, UK) for PPARα; and Santa Cruz Biotechnology (Dallas, TX) for UCP3 and PGC-1α] and correspondent IRDye secondary antibodies (LI-COR, Lincoln, NE); blots were developed and scanned at an Odyssey CLX Near-Infrared Western Blot Scanner (LI-COR) following the manufacturer’s protocol. Carnitine palmitoyltransferase 1b (CPT1b) was analyzed by immunoblotting as described previously (1), using an anti-mouse CPT1b primary antibody (Santa Cruz Biotechnology), stabilized horseradish peroxidase-conjugated secondary antibody (Thermo Fisher Scientific, Rockford, IL), and an enhanced chemiluminescence detection system “SuperSignal West Dura” (Thermo Fisher Scientific) to reveal the immunocomplexes. Unspecific bands or amido black-stained bands were used to control for equal loading and transfer of proteins during immunoblotting.

Gene expression analysis. Total RNA was extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, CA) following the supplier’s instructions. Aqueous phase containing total RNA was mixed with one volume of 70% ethanol and treated with DNAse using an E.Z.N.A. total RNA kit (Omega Bio-Tek, Norcross, GA). RNA was further purified by standard ethanol/sodium acetate precipitation. The concentration and purity of RNA were estimated using a NanoDrop 1000 instrument (Thermo-Fisher Scientific, Waltham, MA). RNA was analyzed for integrity on a 1% agarose gel stained with SYBRsafe (Life Technologies, Carlsbad, CA) and stored at −80°C until analysis. Total tissue RNA (250 ng/reaction) was retrotranscribed using an MuLV Reverse Transcriptase-based dedicated kit (Life Technologies, E173 AJP-Endocrinol Metab • doi:10.1152/ajpendo.00308.2014 • www.ajpendo.org

http://ajpendo.physiology.org/ by 10.20.31.110 on July 7, 2017
Grand Island, NY), using random hexamers priming. mRNA expression levels of genes of interest were analyzed by real-time PCR using the StepOnePlus system with SYBR Green I sequence nonspecific detection (Life Technologies). For each reaction, Power SYBR Green Master mix (Life Technologies), 250 nM of forward and reverse primers, and 4-ng cDNA equivalents were used. Following amplification, melt curve analysis was performed in each plate. The sequences of the primer sets used are available upon request. The identity of the amplicons was confirmed by resolving the qPCR reaction products on 2–4% agarose gel and by comparing the observed melting temperature with the predicted ones (by Poland algorithm; see Ref. 44). Raw amplification data (Rn) were exported and used to determine the efficiency of the amplification and the cycle of quantification by the LinRegPCR software (39). Relative gene expression was calculated using the Pfaffl method, correcting for the different efficiencies (33).

Other parameters. Serum insulin and leptin levels were measured using commercial ELISA kits (from, respectively, Merckodia, Uppsala, Sweden; and R&D Systems, Minneapolis, MN); blood glucose levels using an Accu-Chek Aviva system (Roche Diagnostics); and triacylglycerol, NEFA, and β-hydroxybutyrate in serum enzymatically using commercial kits (from, respectively, Sigma, Madrid, Spain; Wako Chemicals, Neuss, Germany; and Ben S.r.l. Biochemical Enterprise, Milano, Italy). Total tissue lipids were extracted and quantified following a previously published procedure (37). Triacylglycerol content was measured in the lipid extract using a commercial enzymatic kit (Sigma).

Statistical analysis. Data are presented as means ± SE. Statistical significance of differences between groups was assessed by two-tailed Student’s t-test using SPSS 14.0 for windows (SPSS, Chicago, IL). Threshold of significance was set at \( P < 0.05 \).

RESULTS

**Rb**\(^{+/−}\) mice display improved blood lipids and accumulate less body fat than WT mice with age. Energy intake, body weight, and whole body composition were monitored in Rb\(^{+/−}\) mice and WT littermates from weaning up to 7.5 mo of age. There were no significant differences between genotype groups in cumulative food intake (data not shown) or body weight (Fig. 1A) measured throughout the whole experimental period. However, mature adult Rb\(^{+/−}\) mice displayed reduced percent body fat mass (by 23%) and increased percent lean body mass (by 7%) compared with age-matched WT mice (Fig. 1, B and C). Differences in body composition between genotype groups began to show up by 5 mo of age and were absent up to 2.5 mo of age. These results were obtained in two independent cohorts of male mice, and similar results were found in female mice (data not shown). The data indicate that the increase in percent body fat and concomitant decline in percent lean body mass that normally occur as the animals age are attenuated in the setting of partial Rb deficiency.

Consistent with the results of body composition analysis, there were significant reductions in WAT depot mass in mature adult but not young Rb\(^{+/−}\) mice (Fig. 1D). The adiposity index, defined as the sum of the mass of all WAT depots dissected expressed as percentage of body weight, was reduced by 36% in mature adult Rb\(^{+/−}\) mice compared with WT controls (3.44 ± 0.28 vs. 5.39 ± 0.57, \( n = 9–10 \) animals/group). Increased visceral WAT appears to exert a higher metabolic risk than subcutaneous WAT (48), and, interestingly, reduced fat depot mass in mature adult Rb\(^{+/−}\) mice was particularly evident for the visceral WAT depots (40 and 50% reduction for the epididymal and retroperitoneal depots, respectively, vs. 25% reduction for the subcutaneous (inguinal) WAT depot (Fig. 1D). Nevertheless, morphometric analysis revealed a significant 25% reduction of inguinal WAT adipocyte diameter in mature adult Rb\(^{+/−}\) mice (35.7 ± 0.95 vs. 47.7 ± 1.4 \( \mu \)m in WT, \( P = 0.013, n = 4 \) animals/group). BAT mass was unaffected by the Rb genotype at either age (Fig. 1D). Likewise, liver mass and levels of hepatic lipids (triacylglycerols and total lipids) were equal in WT and Rb\(^{+/−}\) mice (data not shown). However, histological examination of liver sections of mature adult (31-wk-old) mice revealed the presence of grade 3 steatosis (corresponding to >66% of hepatocytes presenting with fat vacuoles) in three out of nine WT mice and none out of nine Rb\(^{+/−}\) mice studied, suggesting a protective effect of Rb haploinsufficiency against hepatic lipid accumulation with age (Fig. 1E).

Plasma free fatty acids and triacylglycerols are elevated in obesity and cardiovascular disease (23). Both at young and mature adult age, Rb\(^{+/−}\) mice displayed lower serum NEFA and triacylglycerol levels than WT mice after a 6-h fast, indicating a healthier blood lipid profile (Fig. 1, F and G). Mature adult Rb\(^{+/−}\) mice also displayed reduced serum triacylglycerol levels in the fed state compared with WT controls (0.434 ± 0.039 vs. 0.595 ± 0.048 mM, \( n = 9/\)group, \( P = 0.019 \)).

Rb\(^{+/−}\) mice display increased insulin sensitivity. Excess fat mass and high circulating free fatty acids and triacylglycerols induce insulin resistance (23). Reduced fat mass at mature adult age and improved blood lipids led us to hypothesize increased insulin sensitivity in the Rb\(^{+/−}\) mice. To test this, plasma insulin and glucose levels were analyzed, and insulin (ITT) and glucose (GTT) tolerance tests were performed on young (2–2.5 mo) and mature adult (6–6.5 mo) Rb\(^{+/−}\) and WT mice following a previously published procedure (37). Triacylglycerol content was measured in the lipid extract using a commercial enzymatic kit (Sigma).

Statistical analysis. Data are presented as means ± SE. Statistical significance of differences between groups was assessed by two-tailed Student’s t-test using SPSS 14.0 for windows (SPSS, Chicago, IL). Threshold of significance was set at \( P < 0.05 \).
age in the Rb+/− mice (Fig. 2G). NEFA suppression during GTT showed no differences between Rb+/− and WT groups at either age (~40% suppression), indicating similar sensitivity of WAT to the antilipolytic effect of insulin (Fig. 2H). Absolute NEFA values during GTT were, however, consistently lower in the Rb+/− mice, both at young and adult age (data not shown).

Together considered, the aforementioned results suggested that Rb+/− mice have enhanced insulin sensitivity and a better maintenance of insulin sensitivity as they age. To get further insight into the molecular basis for this phenotype, expression of key genes in the insulin signaling pathway was compared. Mature adult Rb+/− mice displayed increased gene expression of glucos transporter 4 (Glut4) in retroperitoneal WAT (RWAT) (44% increase, \( P = 0.010 \)) and of insulin receptor substrate 1 in both RWAT (23% increase, \( P = 0.049 \)) and epididymal WAT (27% increase, \( P = 0.04 \)) (data not shown). Similar trends were present in the young Rb+/− mutants in which gene expression of phosphatidylinositol 3-kinase in the RWAT depot was also increased (by 37.5%, \( P = 0.046 \)) (data not shown). Other genes examined in WAT depots [insulin receptor (Insr), suppressor of cytokine signaling 3, and adiponectin] were similarly expressed regardless of the Rb genotype. In skeletal muscle, no gross differences between genotype groups regarding the expression of genes involved in the insulin signaling pathway were found, although Rb+/− mice displayed slightly higher (by 8%, \( P = 0.023 \)) muscle Insr mRNA levels at mature adult age and, unexpectedly, slightly lower (by 15%, \( P = 0.009 \)) muscle Glut4 mRNA levels at young age (data not shown).

Rb+/− mice have increased leptin sensitivity. Leptin resistance has been implicated in the pathogenesis of obesity- and aging-related abnormalities of lipid metabolism (49, 50), and it
might be a predisposing factor for obesity (41). Furthermore, insulin and leptin sensitivity are often coregulated (18, 24). Reduced fat mass at mature adult age, increased insulin sensitivity, and resistance to diet-induced obesity in Rb<sup>−/−</sup> mice (this work and Ref. 29) led us to hypothesize that Rb haploinsufficiency might associate with increased leptin sensitivity. To test this, we compared the anorectic response to a single dose of exogenous leptin (2 mg/kg ip) in WT and Rb<sup>−/−</sup> mice, both at young and mature adult age (2 and 6 mo). The anorectic response was maximally evident when considering the 24-h period following leptin administration in the young adult animals (Fig. 3A, top), and when considering the first 12 h (corresponding to the active dark period) in the mature adult animals (Fig. 3A, bottom). In any case, both at young and mature adult age, the reduction in food intake following leptin administration was greater in the Rb<sup>−/−</sup> mice and statistically significant in this genotype only, indicating an increased sensitivity of Rb<sup>−/−</sup> mice to leptin.

**Mature adult Rb<sup>−/−</sup> mice have increased fat tolerance.** Postprandial lipemia is considered an important contributor to cardiovascular disease risk and progression (45). We performed oral fat tolerance tests (OFTT) on young (1.5 mo) and mature adult (7.5 mo) Rb<sup>−/−</sup> mice and corresponding WT littersmates. At young age, no significant differences were found in OFTT: both Rb<sup>−/−</sup> and WT mice showed a 3- to 3.5-fold increase in blood triacylglycerols 2 h after the fat load and a 30–35% blood triacylglycerol clearance 2 h after the peak (Fig. 3B, top). At mature adult age, Rb<sup>−/−</sup> and WT mice showed again a similar blood triacylglycerol peak 2 h after the fat load (~2.2-fold increase), but blood triacylglycerol clearance 2 h after the peak was greater in the Rb<sup>−/−</sup> mice (37 ± 9 vs. 15 ± 7%, n = 9–10/genotype group, P = 0.064), indicating a faster clearance in the Rb<sup>−/−</sup> mice (Fig. 3B, bottom). Comparison of OFTT curves at the two ages evidenced a better maintenance of oral fat tolerance with age in the Rb<sup>−/−</sup> mice.

**Rb<sup>−/−</sup> mice display an increased use of fatty acids as a fuel.** Rb-deficient mice resist the development of obesity when fed a high-fat diet because of increased metabolism (12, 29). Rb<sup>−/−</sup> mice maintained on a normal fat diet displayed higher rectal temperature than WT controls after a 6-h fast or 3-h cold exposure, suggesting increased metabolism under nonchallenging dietary conditions as well (Fig. 4, A–C, and Ref. 29). Consistent with this suggestion, body weight gained per energy consumed (i.e., food efficiency) tended to be reduced in mature adult Rb<sup>−/−</sup> mice compared with WT controls (1.3 ± 0.14 vs. 2.3 ± 0.40 g/MJ, measures taken over a 15-day period, between days 204 and 219 of age, n = 4–5 cages/group, P = 0.076). Reduced food efficiency was not due to increases in spontaneous locomotor activity, since no differences in this
oxidation and brown-like character in RWAT of Rb mice. These results point to an increased capacity for fatty acid oxidation and reliance on lipids as energy source in the Rb mutant mice. Gene expression analysis in adipose tissues and skeletal muscle was undertaken to study underlying molecular mechanisms. Mature adult Rb+/− mice displayed increased peroxisome proliferator-activated receptor-α (Ppara) mRNA levels and trends (P < 0.1) toward increased Cpt1b (muscle isometric) and Ucp1 mRNA levels in RWAT compared with WT controls (Fig. 5A). These results point to an increased capacity for fatty acid oxidation and brown-like character in RWAT of Rb+/− mice.

Mature adult Rb+/− mice also displayed reduced lipoprotein lipase mRNA levels and increased hormone-sensitive lipase (Lipe) mRNA levels in RWAT (Fig. 5A), together suggestive of a reduced potential for tissue expansion. In good concordance with the scenario found in the RWAT depot, an increased gene expression of Ppara (P = 0.05), Pdk4 (P = 0.04), Lipe (P = 0.01) and, although nonsignificant, Cpt1b (P = 0.13) was found in mature adult Rb+/− mice in a second WAT depot examined, namely the epididymal (Fig. 5B). Interestingly, the mRNA levels of Ppara, Cpt1b, Pdk4, Ucp1, and Lipe were already elevated or tended so in the RWAT of young Rb+/− mice at an age (11 wk) at which there were no differences in body composition relative to WT littermates (Fig. 5A, top). The young Rb+/− mutants also displayed an increased gene expression of PPARγ in RWAT (Fig. 5A, top). Other genes analyzed in RWAT and epididymal WAT (Pgc-1α, PR domain containing 16, nuclear receptor interacting protein 1, cytochrome c oxidase subunit Va, and patatin-like phospholipase domain containing 2I) were similarly expressed between WT and Rb+/− genotypes at either age (data not shown). Brown fat-like changes in WAT of mature adult Rb+/− mice were further evidenced after examination of the inguinal WAT, a depot particularly prone to browning (43). Morphological and immunohistochemical analysis of this depot revealed the presence of multilocular adipocytes that immunostained positively for UCP1 in three out of four Rb+/− mice but none out of four WT mice analyzed (Fig. 5C). Increased UCP1 protein levels in inguinal WAT of mature adult Rb+/− mice was confirmed by immunoblotting, which also revealed increased expression of PPARα and CPT1b protein (Fig. 5D), in line with the mRNA expression results found in both RWAT and epididymal WAT of these animals.
Selected gene expression in BAT and skeletal muscle was also analyzed. Differences in BAT gene expression between WT and Rb\(^{+/\text{H11001}}\)/H11002 mice were found at young age only, when the Rb\(^{+/\text{H11001}}\)/H11002 mice demonstrated higher Pgc-1\(_\alpha\), Cpt1b, and Ucp1 mRNA levels (Fig. 6A, top), as well as increased PGC-1\(_\alpha\) protein levels (Fig. 6C), indicative of an increased potential for fatty acid oxidation and thermogenesis. In contrast, no such signs of BAT activation were present in the mature adult Rb\(^{+/\text{H11001}}\)/H11002 mice in which lack of induction of UCP1 and PGC-1\(_\alpha\) protein levels in BAT was confirmed by immunoblotting (Fig. 6, A and C). In skeletal muscle, mature adult Rb\(^{+/\text{H11001}}\)/H11002 mice presented increased Ucp3 and Pdk4 gene expression compared with WT littermates, in line with enhanced muscle fatty acid oxidation, and similar trends for these two genes were found at young age.

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**Fig. 4.** Rb\(^{+/\text{H11001}}\) mice have increased metabolism and preference for fat as a fuel. A: rectal temperature in 2-mo-old WT and Rb\(^{+/\text{H11001}}\) female mice following a 6-h fast (from 0600 to 1200); \(n = 5–6\)/group. B: rectal temperature in 5-mo-old WT and Rb\(^{+/\text{H11001}}\) male mice following a 6-h fast as in A; \(n = 5\)/group. C: rectal temperature in 3.5-mo-old WT and Rb\(^{+/\text{H11001}}\) female mice following 3 h exposure to the cold (6°C); \(n = 9–10\)/group. D and E: respiratory exchange ratio (RER) in 2 (young) and 6 (adult)-mo-old WT and Rb\(^{+/\text{H11001}}\) male mice. Reduced values in the Rb\(^{+/\text{H11001}}\) mutants are indicative of an increased use of fatty acids as a fuel. Panels on right show the average RER (means ± SE) during the light and dark cycles; \(n = 8\)/group (adult) or 4/group (young). Different from WT (*), different from young age (#), and different from daytime (§), \(P < 0.05\), t-test.
and the presence of BAT-like features in WAT are in line with previous results (12, 29) and would reflect an intimate coupling between cell cycle events, including senescence, whose control is the primary role of pRb, and the regulation of cell energetics (14).

Greater capacity for lipolysis and fatty acid oxidation in visceral WAT depots of Rb+/− mice was indicated by increased expression of Lipe, Ppara, Pdk4, Cpt1b, and Ucp1. Furthermore, paramount signs of browning, namely multilocularity in the distribution of intracellular lipids, expression of UCP1, and enhanced expression of PPARα and CPT1b, were present in the inguinal WAT of Rb+/− mice at mature adult age. Differences between Rb+/− and WT mice regarding the expression of lipid metabolism-related genes in WAT were in general better demonstrated at mature adult age yet already present at young age, at least in the retroperitoneal depot, suggesting that fat progenitor cells might be affected, perhaps through epigenetics mechanisms. Interestingly, no signs of BAT activation were found in BAT of adult Rb+/− mice, suggesting dissociation between WAT browning and BAT

DISCUSSION

In this work, we show that Rb haploinsufficiency provides metabolic advantages in front of acute metabolic stressors and as the animals age under a normal nonobesogenic diet. Both at young and mature adult age, Rb+/− mice displayed increased insulin and leptin sensitivity and a trend toward increased glucose tolerance compared with WT littermates. Moreover, age-related body fat (particularly visceral fat) gain and age-related reductions in insulin sensitivity and fat tolerance were all ameliorated in the Rb+/− mice. These beneficial traits associated with increased fatty acid usage, enhanced capabilities for fat catabolism in WAT and skeletal muscle,
activation in these animals. However, as in WAT, changes in line with increased fatty acid oxidation were present in skeletal muscle of the Rb+/−/− mice, where the expression of UCP3, CPT1b, and Pdk4 was upregulated. UCP3 activity can help mitochondrial fatty acid handling (42), and CPT1b activity is considered the limiting factor for long-chain fatty acid import and oxidation by mitochondria. PDK4 activity can also enhance fat catabolism, since it tips the balance between glucose and fat usage toward fat by promoting pyruvate dehydrogenase inhibition through phosphorylation and hence decreased mitochondrial glucose oxidation (46). Of note, upregulation of the Pdk4 gene was consistently found in WAT depots and skeletal muscle of Rb+/−/− mice. This result might be in keeping with a previous demonstration that E2F1 transactivates the Pdk4 gene promoter in an effect that is suppressed by pRb and of induction of the Pdk4 gene in cell models of pRb deficiency/inactivation (20).

Changes in tissue gene expression translated into an increased use of fatty acids as a fuel, as indicated by reduced RER in the Rb+/−/− mice. This trait was already present at young age and may explain reduced blood lipids and the leaner phenotype and better fat tolerance exhibited by Rb+/−/− mice later in life. In humans, a low RER predicts a healthy metabolic phenotype in overweight, sedentary men (38), and a high RER (low fat oxidation) has been implicated as a risk factor for weight gain and obesity (3). Animal studies have specifically...
highlighted the importance of oxidative metabolism and fat oxidation in WAT deposits, with or without UCP1 expression, for a lean phenotype (5, 17). Rb°/+ mice displayed improved blood lipids compared with WT mice, both at young and mature adult age. Decreased circulating NEFA levels in the Rb°/+ mice are unlikely to reflect hypersensitivity in these mutants to the suppressive effect of insulin on lipolysis in WAT, since NEFA suppression during GTT was similar regardless of the Rb genotype. We attribute the lower circulating NEFA levels in Rb°/+ mice to enhanced fatty acid oxidation in tissues of these animals, which is indicated by the indirect calorimetry results and supported by the gene and protein expression data.

Metabolic flexibility is defined as the capacity of the organism to adapt fuel selection to fuel availability, is reflected in the diurnal pattern of RER (in rodents, lower at daytime, when stored fat is used, and higher at nighttime, when the animals eat and glucose becomes highly available), and is often used as an indicator of insulin sensitivity. Our results point to reduced RER in the daytime only, and thus increased metabolic flexibility, in the young Rb°/+ mice. On the contrary, mature adult Rb°/+ mice appear to preferentially oxidize fatty acids during the whole cycle and especially the dark phase, despite still exhibiting a greater response to insulin than WT mice in ITT. Our results underscore that metabolic inflexibility toward fat oxidation is not necessarily linked to insulin resistance and an unhealthy phenotype. They also suggest that enhanced metabolic flexibility at young age might represent an early predictive biomarker of future metabolic health.

The coexistence in young Rb°/+ mice of an improved response to insulin as measured in ITT, on the one hand, and increased fasting insulinemia and HOMA-IR index meaning less insulin sensitivity, on the other, is intriguing. Nevertheless, the calculated R-QUICKI, which has been described as more accurate than HOMA-IR as a surrogate marker of insulin sensitivity by incorporating the levels of fasting NEFA together with fasting insulin and glucose levels (32), showed no differences between Rb genotype groups. Moreover, both ITT results and glucose and insulin data during GTT support the conclusion that insulin sensitivity as for glucose control was increased in the Rb°/+ mice already at young age, before differences in body composition relative to WT mice became evident. Reduced circulating NEFA owing to increased tissue fatty acid usage is likely to be a key factor for the increased insulin sensitivity of Rb°/+ mice, considering the close relationship between NEFA levels and insulin resistance/sensitivity (23). Other explanations may relate to increased expression of key proteins in the insulin signaling pathway in tissues such as WAT and skeletal muscle (which was better demonstrated for the adult Rb°/+ mice), lower levels of “toxic lipid intermediates” as a consequence of increased complete fatty acid oxidation, or a combination of several of these factors. Increased fasting insulinemia in young Rb°/+ mice (Fig. 2B) and better maintenance of glucose-stimulated insulin secretion with age in these mutants (Fig. 2G) could relate to previous findings linking Rb deficiency to a potentiation of pancreatic β-cell mass and function (2, 7). Tissue-specific knockout of the Rb gene in pancreatic islet progenitors results in mice with increased β-cell mass and function and increased insulinemia in the absence of insulin resistance (7). Thus, increased insulinemia could be an intrinsic characteristic of the Rb°/+ model, rather than a response to insulin resistance.

Whereas targeted deletion of Rb in adipose tissues protects mice against dietary obesity (12), the selective deletion of Rb in proopiomelanocortin (POMC) neurons leads to a hyperphagia-obesity-diabetes syndrome by reducing the production of anorexigenic signals (26). In this seemingly contradictory scenario, Rb°/+ mice with generalized partial deficiency of Rb throughout development constitute an interesting model to test the relevance of Rb in the regulation of overall energy balance in the long term. We found Rb°/+ mice to be resistant to diet-induced obesity (29) and age-associated body fat gain (this work), and no differences in cumulative food intake relative to WT mice, either on a high-fat diet (29) or a normal fat diet. It would appear, therefore, that the effect of deletion of Rb in peripheral tissues prevails in vivo. Nevertheless, because Rb°/+ mice demonstrated greater sensitivity to exogenous leptin, it cannot be ruled out that increased central sensitivity to endogenous leptin compensates for eventual deficiencies in POMC neurons in these animals. Increased sensitivity to leptin could also conceivably contribute to the metabolic phenotype of Rb°/+ mice, since leptin signaling favors fat oxidation and thermogenesis in peripheral tissues (13).

The mechanisms underlying the impact of Rb deficiency on WAT metabolic fate can be multiple. pRb has been described as proadipogenic (10, 11), and it is expressed in terminally differentiated adipocytes (35). A relative lack of pRb may alter the activity of pRb-interacting transcription factors known to be involved in adipogenesis and the control of mature white adipocyte metabolism such as C/EBPs (10, 34, 35), PPARα (15), and E2F1 (16) in such a way that WAT expandability is impaired, and enhanced capabilities for tissue fatty acid oxidation develop secondarily. In fact, decreased pRb mRNA, protein, and activity reflects obesity-induced altered (reduced) adipogenic capacity in human adipose tissue (31), and silencing of Rb in fully differentiated murine 3T3-L1 adipocytes downregulates adipogenic/lipogenic markers while weakly inducing UCP1 (31). Nevertheless, our results in skeletal muscle indicate that the connection between Rb status and tissue fatty acid oxidation capacity extends to nonadipose tissues.

Findings in this work may be relevant for humans, since recently a permissive role of Rb for human adipose tissue function has been proposed (31). Because Rb°/+ mice resist diet-induced obesity and age-associated body fat gain and maintain a better metabolic profile than WT mice at mature adult age, the identification and development of nutritional and physiological factors capable of regulating Rb expression along life would be of major interest to enhance metabolic robustness and contribute to a healthy phenotype.

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