

# Genetic Ablation of the c-Cbl Ubiquitin Ligase Domain Results in Increased Energy Expenditure and Improved Insulin Action

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Casitas b-lineage lymphoma (c-Cbl) is a multiadaptor protein with E3-ubiquitin ligase activity residing within its RING finger domain. We have previously reported that c-Cbl-deficient mice exhibit elevated energy expenditure, reduced adiposity, and improved insulin action. In this study, we examined mice expressing c-Cbl protein with a loss-of-function mutation within the RING finger domain (*c-Cbl<sup>Δ</sup>* mice). Compared with control animals, *c-Cbl<sup>Δ</sup>* mice display a phenotype that includes reduced adiposity, despite greater food intake; reduced circulating insulin, leptin, and triglyceride levels; and improved glucose tolerance. *c-Cbl<sup>Δ</sup>* mice also display elevated oxygen consumption (13%) and are protected against high-fat diet-induced obesity and insulin resistance. Unlike *c-Cbl<sup>Δ</sup>* mice, mice expressing a mutant c-Cbl with the phosphatidylinositol (PI) 3-kinase binding domain ablated (*c-Cbl<sup>Δ/Δ</sup>* mice) exhibited an insulin sensitivity, body composition, and energy expenditure similar to that of wild-type animals. These results indicate that c-Cbl ubiquitin ligase activity, but not c-Cbl-dependent activation of PI 3-kinase, plays a key role in the regulation of whole-body energy metabolism. *Diabetes* 55:3411–3417, 2006

**T**he increased incidence of obesity and its consequences (type 2 diabetes and cardiovascular disease) has led to increased efforts to understand the molecular control of energy balance and fat mass. We have recently reported a novel lean mouse model that may provide new insights into the

molecular regulation of energy expenditure (1). Mice with a homozygous deletion of the Casitas b-lineage lymphoma (*c-Cbl*) gene possessed 50% less adipose tissue than control animals, despite a 30% increase in food intake, due in part to increased whole-body energy expenditure. This was accompanied by enhanced peripheral insulin sensitivity and improved blood glucose clearance, even when the animals were challenged with a high-fat feeding regime (2).

The exact molecular mechanism(s) by which c-Cbl regulates energy metabolism is still unclear. This may be due to the complexity of the c-Cbl protein itself. c-Cbl is a multiadaptor protein with numerous protein-protein interaction domains (3–5). The NH<sub>2</sub> terminus of c-Cbl contains a tyrosine kinase-binding domain (TKB), a calcium-binding EF-hand, and an atypical SH2 domain. This functional domain allows the binding of c-Cbl to phosphorylated tyrosine residues. After a short linker region, c-Cbl has a RING finger domain that contains intrinsic E3 ubiquitin ligase activity. The COOH-terminal half of c-Cbl contains a proline-rich region that acts as a binding motif for proteins containing SH3 domains, and it also contains a ubiquitin-associated domain (UBA) through which c-Cbl dimerizes with itself or with other Cbl family members (6,7). It is also important to note that several tyrosine residues within c-Cbl are the target of protein tyrosine kinases. Once phosphorylated, these residues form binding sites for SH2 domain-containing proteins, such as the p85 subunit of phosphatidylinositol (PI) 3-kinase.

By virtue of its E3 ubiquitin ligase activity located in the RING finger domain, c-Cbl is probably best known as a negative regulator of growth factor action. c-Cbl binds to phosphorylated tyrosines on activated growth factor receptor tyrosine kinases, and it promotes the ubiquitylation of these receptors, targeting them for lysosomal degradation (4,8). In addition to catalyzing the ubiquitin-mediated downregulation of receptor tyrosine kinase, c-Cbl has been reported to act as an adaptor protein in a range of intracellular signaling cascades. Within this category falls the proposed role for c-Cbl in the activation of an alternative signaling cascade involved in insulin-regulated movement of GLUT4 to the cell surface of 3T3-L1 adipocytes (6,9–11). The physiological relevance of c-Cbl function in this pathway is, however, controversial because the depletion of c-Cbl in either cultured cells or animals is not associated with impaired insulin action (1,12).

In this study, we investigated whether specific functional domains within c-Cbl were required to control whole-body energy homeostasis in mice. To address this

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J.C.M. and N.T. contributed equally to this work. ACC, acetyl-CoA carboxylase; AMPK, AMP kinase; c-Cbl, Casitas b-lineage lymphoma; PI, phosphatidylinositol; PGC, peroxisome proliferator-activated receptor- $\gamma$  coactivator; UCP, uncoupling protein.

Additional information for this article can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

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TABLE 1

Body mass, lean mass, fat mass, and oxygen consumption in the different c-Cbl mouse strains

	<i>c-Cbl</i> <sup>+/-</sup>	<i>c-Cbl</i> <sup>A/-</sup>	<i>c-Cbl</i> <sup>+/+</sup>	<i>c-Cbl</i> <sup>F/F</sup>
Body mass (g)	30.6 ± 0.3	28.4 ± 0.6*	30.6 ± 0.5	29.9 ± 0.3
Lean mass (g)	26.1 ± 0.3	25.1 ± 0.5	25.9 ± 0.5	24.9 ± 0.4
Fat mass (g)	4.5 ± 0.2	3.3 ± 0.1*	4.9 ± 0.3	5.0 ± 0.2
Epididymal fat mass (g)	0.34 ± 0.02	0.21 ± 0.01†	0.39 ± 0.04	0.43 ± 0.05
Brown fat mass (mg)	86 ± 4	63 ± 3*	83 ± 5	80 ± 4
VO <sub>2</sub> (ml · g <sup>-1</sup> · h <sup>-1</sup> )	3.12 ± 0.06	3.52 ± 0.06†	3.08 ± 0.06	3.12 ± 0.06

Data are the means ± SE of 10–24 animals per group. Lean and fat mass was determined by dual X-ray absorptiometry scanning. VO<sub>2</sub> represents the average oxygen consumption over a 24-h period. \**P* < 0.002, †*P* < 0.0001 vs. *c-Cbl*<sup>+/-</sup> mice.

issue, we examined the phenotype of genetically manipulated knock-in mice expressing c-Cbl proteins where either the RING finger domain (*c-Cbl*<sup>A/-</sup> mice) or the PI 3-kinase binding motif (*c-Cbl*<sup>F/F</sup> mice) have been ablated, retaining other functional domains within the protein.

## RESEARCH DESIGN AND METHODS

*c-Cbl*<sup>+/+</sup> and *c-Cbl*<sup>+/-</sup> mice were generated as described in detail previously (13). Ablation of the ubiquitin ligase activity of c-Cbl was achieved with a single mutation (C379A) located within the RING finger domain (14–17). The majority of mice containing this mutation in both c-Cbl alleles (*c-Cbl*<sup>AA</sup> mice) died in utero or during the first 24 h after birth (17). For this reason and to obtain the number of animals required to conduct the study, we examined animals containing only one mutant copy of c-Cbl (*c-Cbl*<sup>A/-</sup> mice) and compared these mice with c-Cbl heterozygous littermates (*c-Cbl*<sup>+/-</sup> mice). Immunoblotting with an antibody specific for c-Cbl showed that both of these lines expressed ~50% the amount of total c-Cbl protein found in wild-type animals, whereas the protein was undetectable in the *c-Cbl*<sup>-/-</sup> animals, consistent with previous findings (17) (supplemental Figure, available at <http://diabetes.diabetesjournals.org>).

*c-Cbl*<sup>F/F</sup> mice were generated by Ozgene (Perth, Australia). Relevant portions of the c-Cbl gene amplified from 129X1/SvJ genomic DNA were targeted by PCR-directed mutagenesis to alter the 5'-GTACCTATGAAGCGA TGT-3' sequence in exon 13 to 5'-GTACCTTCGAAGCGATGT-3', which encodes a Tyr→Phe substitution at amino acid 737 (Y737F). The targeting vector, consisting of 5' mutant and 3' wild-type c-Cbl homology arms cloned on either side of a loxP-flanked pGKneo gene, was electroporated into 129S1/SvImJ-derived W9.5 embryonic stem cells. Correctly targeted embryonic stem clones were injected into C57BL/6 blastocysts to produce chimeric males, which were then mated to C57BL/6 females. Germline transmission of litters born was confirmed by Southern blotting. All subsequent genotyping of mice was carried out by PCR using 5'-GAAGAGGACACAGAATATATGACT C-3' with 5'-CTGGATGTTATACATCGCTTCAT-3' (wild type) or 5'-CTGGATG TTATACATCGCTTCGA-3' (Y737F) c-Cbl-specific primers. The Y737F substitution (Y731F in the human c-Cbl protein) ablates the binding site of the p85 subunit of PI 3-kinase to c-Cbl, as well as the c-Cbl-mediated activation of this lipid kinase in several cell types (18–20). Expression of c-Cbl (Y737F) mutant forms did not significantly affect the fertility or the viability of the animals (data not shown), and these animals were compared with wild-type (*c-Cbl*<sup>+/+</sup>) controls.

All experiments were performed on male mice maintained on the hybrid 129/SvJ × C57BL/6 background. The animals were kept on a 12-h light/dark cycle with free access to food and water. Animals were fed ad libitum with standard lab chow or with a high-fat diet for 4 weeks (60% of caloric intake from fat [70% saturated fat], 20% from carbohydrates, and 20% from protein) based on rodent diet #D12492 (Research Diets, New Brunswick, NJ). All experiments were carried out in 18- to 24-week-old male mice with the approval of the Garvan Institute/St. Vincent's Hospital animal experimentation ethics committee, following guidelines issued by the National Health and Medical Research Council.

**Metabolic assays.** Glucose tolerance tests (2 g/kg glucose i.p.) were performed in overnight-fasted mice. Blood samples were obtained from the tail tip at the indicated times, and glucose levels were measured using a glucometer (AccuCheck II; Roche). Insulin concentrations were measured using an ultrasensitive enzyme-linked immunosorbent assay kit (Merckodia, Uppsala, Sweden). Other plasma measurements were performed on blood collected from the chest cavity into tubes containing EDTA and centrifuged at 14,000g for 10 min to obtain the plasma. Insulin and leptin concentrations were assayed by radioimmunoassay (Linco Research, St. Louis, MO). The concentrations of nonesterified fatty acids (Wako Pure Chemical Industries,

Osaka, Japan) and triglycerides (Roche Diagnostics) were determined using a colorimetric kit.

**Indirect calorimetry studies.** The oxygen consumption rate (VO<sub>2</sub>) was measured using an eight-chamber indirect calorimeter (Oxymax series; Columbus Instruments, Columbus, OH) with an air flow of 0.6 l/min. Studies were commenced after 2 h of acclimation to the metabolic chamber (20 cm × 10 cm × 12.5 cm). VO<sub>2</sub> was measured in individual mice at 27-min intervals over a 24-h period under a consistent environmental temperature (22°C). During the study, mice had ad libitum access to food and water.

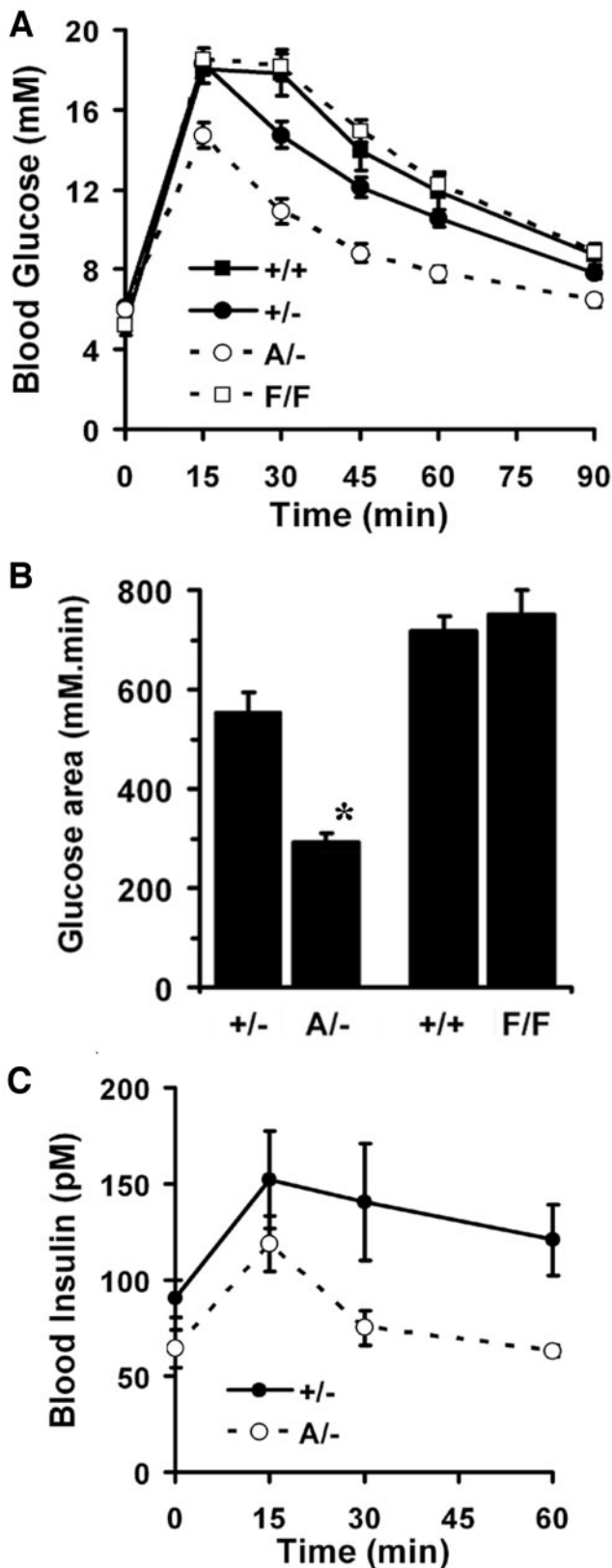
**Western blotting.** Powdered quadriceps muscles and brown adipose tissue from the different strains of c-Cbl mice were resuspended in RIPA buffer (PBS, pH 7.5, 1% Nonidet NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 10 mmol/l NaF, and 10 mmol/l Na PPI) and solubilized for 2 h at 4°C. Aliquots of tissue homogenates were resolved by SDS-PAGE and immunoblotted with appropriate antibodies against AMP kinase (AMPK), pThr172-AMPK, and pSer79-acetyl-CoA carboxylase (pSer79-ACC; Cell Signaling, Beverly, MA), ACC (Upstate Biotechnology, Lake Placid, NY), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α; Chemicon International, Temecula, CA), uncoupling protein 3 (UCP3; Affinity Bioreagents, Golden, CO), c-Cbl (BD Biosciences, San Jose, CA), and an antibody cocktail that recognizes five subunits of the mitochondrial respiratory chain (MS601; Mitosciences, Eugene, OR). Quantitation of immunolabeled bands was performed using a VersaDoc imaging system (BioRad).

**Enzyme activity measurements.** The activity of cytochrome c oxidase and b-hydroxyacyl-CoA dehydrogenase were measured in muscle homogenates as described previously (2).

**Statistical analyses.** Data are the means ± SE. An unpaired Student's *t* test was used for comparison of *c-Cbl*<sup>A/-</sup> mice with *c-Cbl*<sup>+/-</sup> littermates and *c-Cbl*<sup>F/F</sup> mice with *c-Cbl*<sup>+/+</sup> controls. Differences at *P* < 0.05 were considered to be statistically significant.

## RESULTS

**c-Cbl ubiquitin ligase-deficient mice have reduced adiposity.** *c-Cbl*<sup>A/-</sup> mice weighed ~5% less than their control *c-Cbl*<sup>+/-</sup> littermates, whereas *Cbl*<sup>F/F</sup> mice displayed similar body mass to wild-type (*c-Cbl*<sup>+/+</sup>) controls (Table 1). Body composition was assessed by dual X-ray absorptiometry scanning. All four mouse strains displayed similar lean mass (Table 1). *Cbl*<sup>F/F</sup> mice showed no difference in whole-body adiposity or in mass of epididymal and brown fat depots compared with wild-type mice (Table 1). The loss of one c-Cbl allele in the *c-Cbl*<sup>+/-</sup> mice had no effect on adiposity compared with wild-type mice. *c-Cbl*<sup>A/-</sup> mice, however, displayed a significant reduction in fat mass compared with *c-Cbl*<sup>+/-</sup> littermates (~25%). Examination of specific adipose depots showed that *c-Cbl*<sup>A/-</sup> mice exhibited reduced epididymal (33%) and brown fat mass (~20%) compared with *c-Cbl*<sup>+/-</sup> controls (Table 1). Intriguingly, this reduction in adiposity observed in the *c-Cbl*<sup>A/-</sup> mice compared with control littermates occurred despite a higher energy intake (5.9 ± 0.46 g/day, *n* = 6, vs. 4.5 ± 0.26, *n* = 7; *P* < 0.003). Consistent with the reduction in adiposity observed in the *c-Cbl*<sup>A/-</sup> mice, we also observed a significant decrease in circulating triglycerides



**FIG. 1.** Glucose tolerance test in overnight-fasted *c-Cbl* heterozygous (+/-), *c-Cbl* RING mutant (A/-), wild-type (+/+), and *c-Cbl* PI 3-kinase-binding mutant (F/F) mice. **A:** Blood glucose levels after an intraperitoneal glucose load (2 g/kg). **B:** Area under the curve as an indicator of glucose clearance in the different *c-Cbl* mouse strains. Data are the means  $\pm$  SE of 11–18 mice. \* $P < 0.01$  vs. *c-Cbl*<sup>+/-</sup> mice. **C:** Circulating insulin levels during the glucose tolerance test. Data are the means  $\pm$  SE of five *c-Cbl*<sup>+/-</sup> and four *c-Cbl*<sup>A/-</sup> mice.

levels ( $0.74 \pm 0.05$  mmol/l,  $n = 7$ , vs.  $0.51 \pm 0.02$ ,  $n = 7$ ;  $P < 0.03$ ), a 20% decrease in circulating fatty acids levels ( $0.86 \pm 0.05$  mmol/l,  $n = 6$ , vs.  $0.71 \pm 0.05$ ,  $n = 6$ ;  $P = 0.06$ ), and a ~55% reduction in circulating leptin in the *c-Cbl*<sup>A/-</sup> mice compared with *c-Cbl*<sup>+/-</sup> mice ( $0.70 \pm 0.05$  ng/ml,  $n = 12$ , vs.  $1.53 \pm 0.1$ ,  $n = 11$ ;  $P < 0.001$ ). These results are similar to those observed in *c-Cbl*<sup>-/-</sup> mice (1) and indicate that a deficiency in *c-Cbl* ubiquitin ligase activity is sufficient to induce a lean phenotype.

***c-Cbl*<sup>A/-</sup> mice exhibit enhanced whole-body glucose clearance.** To determine whether the reduction in adiposity in *c-Cbl*<sup>A/-</sup> was associated with improved insulin action, we examined whole-body glucose clearance during an intraperitoneal glucose tolerance test (Fig. 1A). *c-Cbl*<sup>A/-</sup> mice displayed substantially enhanced glucose clearance compared with *c-Cbl*<sup>+/-</sup> littermates (Fig. 1B). As observed previously (1), *c-Cbl*<sup>+/-</sup> mice displayed a moderate improvement in glucose clearance compared with *c-Cbl*<sup>+/+</sup> mice. *c-Cbl*<sup>F/F</sup> mice did not exhibit any difference in glucose clearance compared with wild-type controls. The improved glucose tolerance observed in *c-Cbl*<sup>A/-</sup> mice was not attributable to higher circulating insulin levels because *c-Cbl*<sup>A/-</sup> mice exhibited lower circulating insulin levels than *c-Cbl*<sup>+/-</sup> mice in both the fasted state ( $74.9 \pm 5.6$  pmol/l,  $n = 7$ , vs.  $35.5 \pm 4.0$ ,  $n = 7$ ;  $P < 0.0001$ ) and during the glucose tolerance test (Fig. 1C). These results indicate that the higher glucose clearance observed in *c-Cbl*<sup>A/-</sup> mice was attributable to enhanced peripheral insulin sensitivity.

***c-Cbl*<sup>A/-</sup> mice exhibit higher oxygen consumption.** The lean phenotype reported for *c-Cbl*<sup>-/-</sup> mice is linked to an increase in whole-body energy expenditure, potentially through activation of the AMPK pathway in skeletal muscle (1). To establish whether a loss of *c-Cbl* ubiquitin ligase function elicited a similar phenotype, we studied whole-body oxygen consumption by indirect calorimetry. *c-Cbl*<sup>A/-</sup> mice exhibited a 13% increase in whole-body oxygen consumption compared with *c-Cbl*<sup>+/-</sup> littermates (Table 1). This difference in oxygen consumption was present during both the light and dark phase of the day (Fig. 2A).

Brown adipose tissue is recognized as an important tissue for whole-body energy expenditure in rodents (21); however, the brown adipose depots were smaller in *c-Cbl*<sup>A/-</sup> mice, and we observed no difference in the expression of several markers of oxidative capacity (PGC-1 $\alpha$ , UCP3, and mitochondrial respiratory chain proteins) in brown adipose tissue of the *c-Cbl*<sup>A/-</sup> mice compared with *c-Cbl*<sup>+/-</sup> littermates (supplemental Figure), suggesting that the observed increase in energy expenditure is not likely associated with increased thermogenesis in this tissue. The elevated energy expenditure observed in *c-Cbl*<sup>A/-</sup> mice was associated with an apparent increase in substrate oxidation in skeletal muscle. Compared with *c-Cbl*<sup>+/-</sup> littermates, *Cbl*<sup>A/-</sup> mice displayed a significant ( $P < 0.05$ ) increase in the phosphorylation of both AMPK (57%) and its downstream target ACC (22%) in skeletal muscle, a 33% increase in the level of PGC-1 $\alpha$  ( $P = 0.09$ ), and significantly ( $P < 0.05$ ) elevated cytochrome c oxidase and b-hydroxyacyl-CoA dehydrogenase activity (Fig. 3). In contrast, *c-Cbl*<sup>F/F</sup> mice displayed no difference in oxygen consumption or any of the above muscle parameters compared with wild-type controls (Fig. 2B and data not shown).

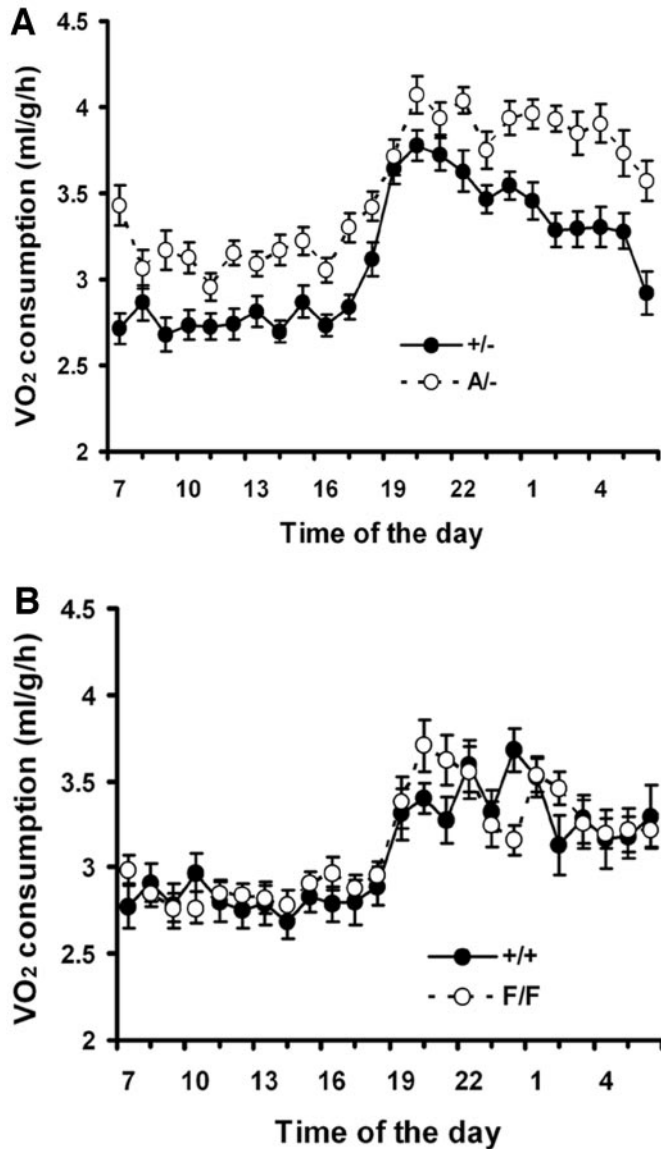


FIG. 2. Whole-body oxygen consumption in  $c-Cbl$  heterozygous ( $+/-$ ) and  $c-Cbl$  RING mutant ( $A/-$ ) mice (A) and wild-type ( $+/+$ ) and  $c-Cbl$  PI 3-kinase-binding mutant ( $F/F$ ) mice (B). Oxygen consumption rate ( $VO_2$ ) was measured over a 24-h period. The light phase corresponds to 0700–1900 and the dark phase to 1900–0700. In A, data are the means  $\pm$  SE of 23–24 mice per group. In B, data are the means  $\pm$  SE of 12–20 mice per group.

**$c-Cbl^{A/-}$  mice are protected against diet-induced obesity and insulin resistance.** We next examined the effect of a high-fat diet on energy homeostasis and insulin action in  $c-Cbl^{A/-}$  mice to determine whether these animals were protected against the development of diet-induced obesity and insulin resistance.  $c-Cbl^{A/-}$  mice and  $c-Cbl^{+/-}$  littermates were provided with a high-fat diet (60% of caloric intake from fat) for 4 weeks.  $c-Cbl^{+/-}$  mice gained significantly more weight than  $c-Cbl^{A/-}$  mice ( $3.5 \pm 0.3$  g,  $n = 8$ , vs.  $1.5 \pm 0.2$ ,  $n = 7$ ;  $P < 0.001$ ) and at the completion of the high-fat feeding regime were an average of 3.5 g heavier than high-fat-fed  $c-Cbl^{A/-}$  mice ( $33.6 \pm 0.6$  g,  $n = 8$ , vs.  $30.1 \pm 0.5$ ,  $n = 7$ ;  $P < 0.001$ ). Consistent with the reduced effect of high-fat feeding on body weight, high-fat-fed  $c-Cbl^{A/-}$  mice exhibited  $\sim 40\%$  less epididymal fat mass than high-fat-fed  $c-Cbl^{+/-}$  littermates ( $0.65 \pm 0.03$  g,  $n = 7$ , vs.  $1.06 \pm 0.1$ ,  $n = 8$ , respectively;  $P < 0.002$ ). Plasma

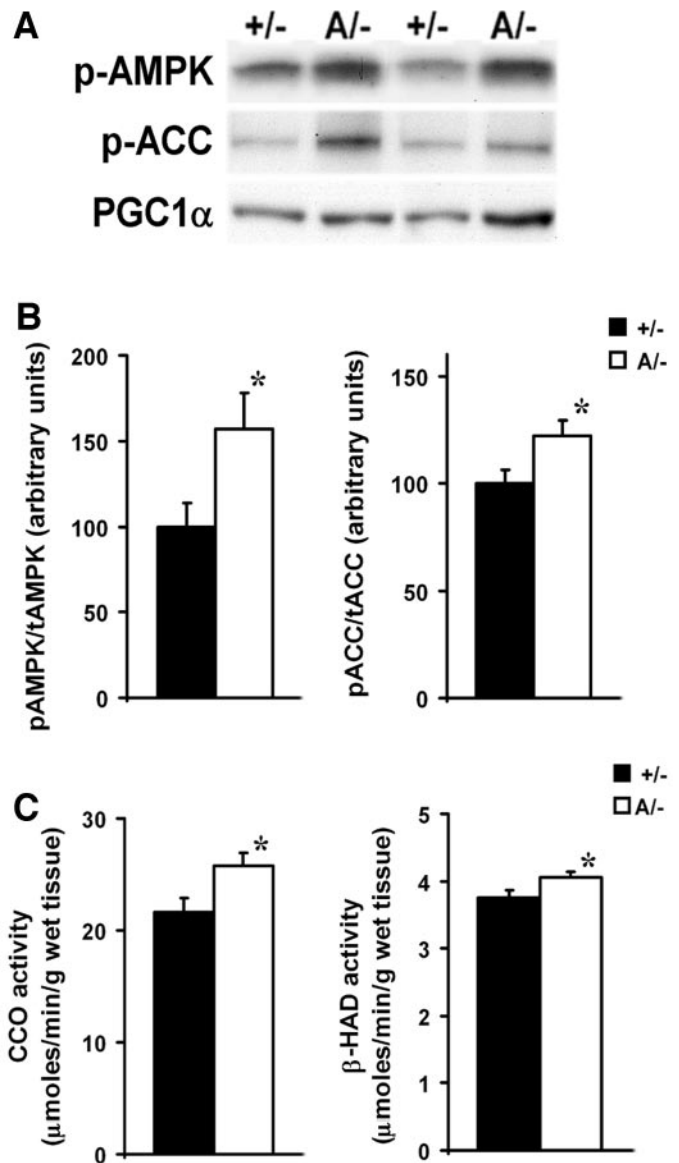
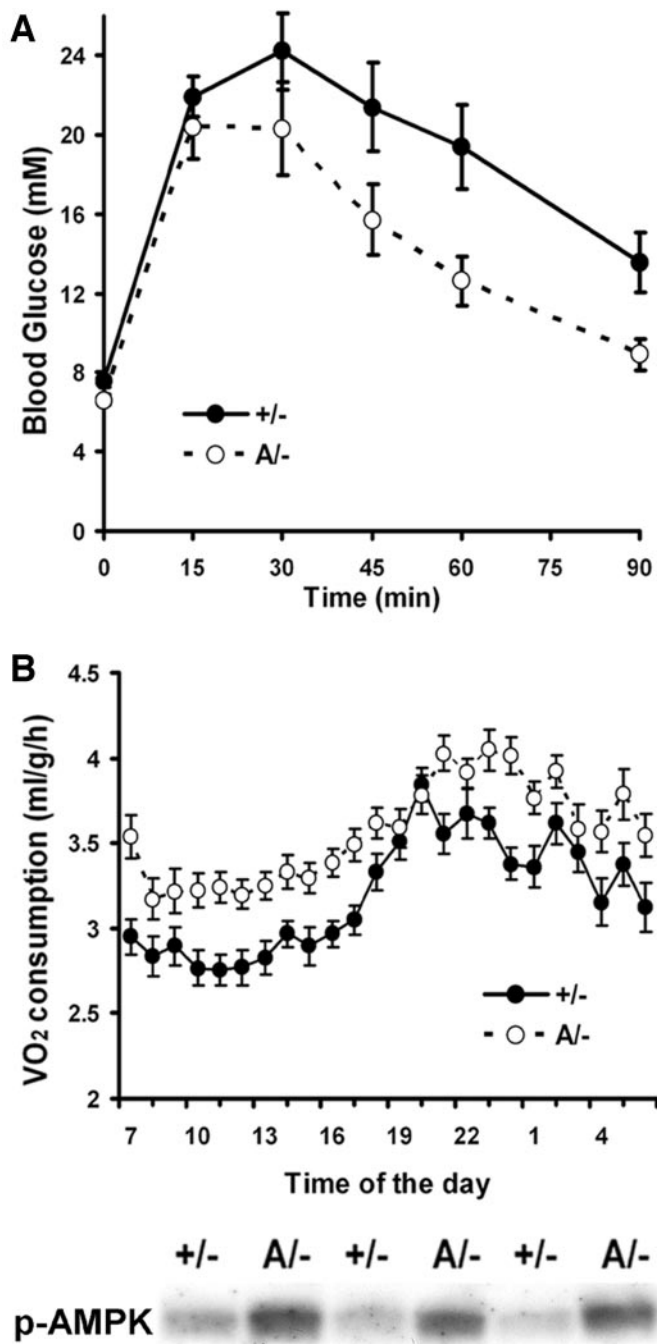


FIG. 3. Metabolic parameters in skeletal muscle of  $c-Cbl^{+/-}$  and  $c-Cbl^{A/-}$  mice. A: Quadriceps muscle homogenates (15  $\mu$ g total protein) were immunoblotted with antibodies specific for either pThr172-AMPK (pAMPK), pSer79-ACC (pACC), or PGC-1 $\alpha$ . Representative immunoblots are shown for two animals per group. B: Histograms show the means  $\pm$  SE for the ratio of phosphorylated AMPK (pAMPK) to total protein for AMPK (tAMPK) and ACC ( $n = 9-11$ ).  $*P < 0.05$  vs.  $c-Cbl^{+/-}$  mice. C: Cytochrome c oxidase (CCO) and  $\beta$ -hydroxyacyl-CoA dehydrogenase ( $\beta$ -HAD) enzymatic activities assayed in muscle homogenates from  $c-Cbl^{+/-}$  and  $c-Cbl^{A/-}$  mice. Histograms show the means  $\pm$  SE ( $n = 12$ ).  $*P < 0.05$  vs.  $c-Cbl^{+/-}$  mice.

leptin levels were also 40% lower in high-fat-fed  $c-Cbl^{A/-}$  mice compared with high-fat-fed  $c-Cbl^{+/-}$  mice ( $4.0 \pm 0.4$  ng/ml,  $n = 8$ , vs.  $7.0 \pm 0.5$ ,  $n = 7$ ;  $P < 0.001$ ).

Similar to the chow-fed mice,  $c-Cbl^{A/-}$  mice fed a high-fat diet exhibited  $\sim 45\%$  lower circulating insulin levels compared with high-fat-fed  $c-Cbl^{+/-}$  mice ( $37.9 \pm 2.9$  pmol/l,  $n = 7$ , vs.  $67.1 \pm 7.4$ ,  $n = 8$ ;  $P < 0.005$ ). Animals were subjected to an intraperitoneal glucose tolerance test and, as can be seen in Fig. 4A, high-fat-fed  $c-Cbl^{A/-}$  mice were able to clear a glucose load faster than high-fat-fed  $c-Cbl^{+/-}$  mice. Indeed, in contrast to high-fat-fed  $c-Cbl^{+/-}$  mice, blood glucose levels in high-fat-fed  $c-Cbl^{A/-}$  mice were close to basal levels by 90 min after glucose admin-



**FIG. 4.** Effect of a high-fat diet on glucose clearance, oxygen consumption, and muscle AMPK phosphorylation in *c-Cbl*<sup>+/-</sup> ( $+/-$ ) and *c-Cbl*<sup>A/-</sup> ( $A/-$ ) mice. **A:** Blood glucose levels after an intraperitoneal glucose load (2 g/kg). **B:** Oxygen consumption rate ( $VO_2$ ) was measured over a 24-h period. The light phase corresponds to 0700–1900 and the dark phase corresponds to 1900–0700. A representative blot of AMPK phosphorylation in skeletal muscle from three mice per group is shown. Data are the means  $\pm$  SE of eight *c-Cbl*<sup>+/-</sup> and seven *c-Cbl*<sup>A/-</sup> mice.

istration. These data indicate that *c-Cbl*<sup>A/-</sup> mice were protected against diet-induced obesity and insulin resistance.

To determine whether the difference in whole-body oxygen consumption remained when animals were fed a high-fat diet, we again conducted indirect calorimetry. High-fat-fed *c-Cbl*<sup>A/-</sup> mice exhibited 10% higher oxygen consumption compared with high-fat-fed *c-Cbl*<sup>+/-</sup> littermates ( $3.6 \pm 0.1 \text{ ml} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ ,  $n = 7$ , vs.  $3.2 \pm 0.04$ ,  $n =$

8;  $P < 0.01$ ), which is consistent with the difference observed in chow-fed animals. The higher oxygen consumption rate in *c-Cbl*<sup>A/-</sup> mice was observed during both the light and dark phase of the day and was again associated with an increased phosphorylation of AMPK in skeletal muscle (2.2-fold,  $n = 7-8$ ,  $P < 0.03$ ) (Fig. 4B) with no significant change observed in total AMPK levels (data not shown).

## DISCUSSION

In this study, we have shown that the majority of the phenotypic characteristics exhibited by *c-Cbl*-deficient mice are present in mice in which ubiquitin ligase activity has been ablated. *c-Cbl*<sup>A/-</sup> mice were leaner, despite having increased food intake; displayed lower circulating levels of insulin, leptin, and triglycerides; and exhibited greater whole-body glucose clearance and oxygen consumption compared with *c-Cbl*<sup>+/-</sup> littermates. This phenotype was maintained even when the animals were challenged with a high-fat diet for 4 weeks (Fig. 4), indicating that similar to *c-Cbl*-deficient mice (2), *Cbl*<sup>A/-</sup> mice are protected against diet-induced obesity and insulin resistance. Mice expressing a form of *c-Cbl* lacking the PI 3-kinase binding motif showed a phenotype that was practically identical to the wild-type mice, indicating that this function of *c-Cbl* does not play an important role in the metabolic phenotype observed in *c-Cbl*-deficient mice.

The balance between energy intake, energy storage, and energy expenditure is both complex and intriguing, and it has become apparent that the level of whole-body adiposity has a major impact on the metabolic status of animals. Mice that lack functional adipose tissue (lipodystrophic mice) are almost uniformly insulin resistant, whereas mice that exhibit reduced adiposity (i.e., lean phenotype) typically display improved glucose tolerance and increased insulin sensitivity (22). In simple terms, less adipose tissue results from an imbalance in the energy equation due to either a reduction in energy availability (intake or absorption), an increase in energy expenditure, or a combination of both. The reality is, however, that there are numerous pathways and multiple tissues involved in the regulation of energy storage, and therefore determining the underlying mechanisms responsible for a lean phenotype presents a significant challenge.

If we consider animal models with a demonstrated increase in energy expenditure, which includes *c-Cbl*<sup>-/-</sup> and *c-Cbl*<sup>A/-</sup> mice, there are three general categories. First, there are mice whose lean phenotype is associated with central nervous system-mediated increases in energy expenditure, often due to hyperactivity, such as the melanin-concentrating hormone (MCH) knockout mouse (23) and, interestingly, PGC-1 $\alpha$  knockout mice (24). The second group of mice are those in which there has been a disruption of genes involved in lipid storage, such as ACC2 (25), acyl-CoA diacylglycerol acyl-transferase (DGAT) (26), acylation-stimulating protein (ASP) (27), stearoyl-CoA desaturase-1 (SCD-1) (28), and interleukin-1 receptor antagonist (IL-1Ra) (29). Intriguingly, in these mice, defects in the pathways of lipid synthesis and storage lead to elevated energy expenditure via a repartitioning of substrates away from adipose tissue with a concomitant upregulation of lipid oxidative pathways and UCPs in other tissues (27,28,30,31). The final group of mice are those in which the lean phenotype of the animals appears to result primarily from increased energy expenditure,

driven by an upregulation of pathways of substrate oxidation (e.g.,  $\beta$ -oxidation enzymes) or energy dissipation (e.g., UCPs) in one or more peripheral tissues, such as muscle, brown and white adipose tissue, and liver. These transgenic and knockout animals have been produced by targeting a variety of genes such as UCP1 and UCP3 (32,33); enzymes involved in signaling pathways, including protein-tyrosine phosphatase 1B (PTP-1B) (34) and S6-kinase (35); and regulators of transcriptional (PGC-1 $\beta$ , transcriptional intermediary factor-2 [TIF2], receptor interacting protein 140 [RIP140]) and translational (eIF4E-binding protein 1 [4E-BP1]) processes (36–39).

Which of these categories *c-Cbl*<sup>-/-</sup> and *c-Cbl*<sup>A/-</sup> mice fall into is unknown, although it is most likely the third category because we have demonstrated an increased activation of the AMPK axis in skeletal muscle of *c-Cbl*<sup>A/-</sup> mice in the current study and in *c-Cbl*-deficient mice (1). AMPK is an energy sensor that is known to integrate nutritional and hormonal signals in peripheral tissue (40). In skeletal muscle, activation of AMPK results in elevated glucose uptake, via enhanced GLUT4 translocation (41), and increased fatty acid oxidation (42). Therefore, a plausible explanation for the phenotype in *c-Cbl*<sup>A/-</sup> and *c-Cbl*<sup>-/-</sup> mice is that the loss of *c-Cbl* ubiquitin ligase activity promotes AMPK activation in skeletal muscle, which increases substrate oxidation and results in reduced circulating and whole-body fat levels. As a consequence of the reduced adiposity and the increased muscle AMPK activation, *c-Cbl*<sup>A/-</sup> and *c-Cbl*<sup>-/-</sup> mice also exhibit improved whole-body glucose clearance and peripheral insulin sensitivity. At this stage, it is unclear whether the increased activation of AMPK in skeletal muscle is caused by reduced ubiquitylation of specific targets in this tissue, or whether other tissues such as liver, brain, or adipose tissue are also playing a part in the phenotype observed in *Cbl*<sup>A/-</sup> and *c-Cbl*<sup>-/-</sup> mice. For example, adipose tissue is known to produce several factors (adipokines) that are able to affect substrate metabolism in peripheral tissues (43), and it is possible that through its ubiquitin ligase function, *c-Cbl* regulates some aspect of adipose biology, which, when removed in *c-Cbl*<sup>-/-</sup> or *c-Cbl*<sup>A/-</sup> mice, alters the secretory profile of adipose tissue, leading to increased AMPK activation in muscle and elevated whole-body energy expenditure. The study of tissue-specific *c-Cbl*-deficient mice will be crucial in clarifying which tissue(s) primarily mediates the effect of *c-Cbl* on metabolic processes.

In summary, we provide evidence that *c-Cbl* regulates whole-body energy homeostasis largely through its ubiquitin ligase function located in the RING finger domain. These findings represent significant progress in understanding the mechanism by which *c-Cbl* affects whole-body energy homeostasis, indicating that *c-Cbl* specifically regulates the ubiquitylation of a target(s) that plays an intimate role in body weight regulation, glucose homeostasis, and energy expenditure. Further investigation is required to identify these mediators of *c-Cbl* action, which are potential targets for the development of new therapies against obesity and diabetes.

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