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# Effects of AMPK Activation on Insulin Sensitivity and Metabolism in Leptin-Deficient *ob/ob* Mice

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**AMP-activated protein kinase (AMPK) is a heterotrimeric complex, composed of a catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ), which act as a metabolic sensor to regulate glucose and lipid metabolism. A mutation in the  $\gamma_3$  subunit (AMPK $\gamma_3^{\text{R225Q}}$ ) increases basal AMPK phosphorylation, while concomitantly reducing sensitivity to AMP. AMPK $\gamma_3^{\text{R225Q}}$  ( $\gamma_3^{\text{R225Q}}$ ) transgenic mice are protected against dietary-induced triglyceride accumulation and insulin resistance. We determined whether skeletal muscle-specific expression of AMPK $\gamma_3^{\text{R225Q}}$  prevents metabolic abnormalities in leptin-deficient *ob/ob* (*ob/ob*- $\gamma_3^{\text{R225Q}}$ ) mice. Glycogen content was increased, triglyceride content was decreased, and diacylglycerol and ceramide content were unaltered in gastrocnemius muscle from *ob/ob*- $\gamma_3^{\text{R225Q}}$  mice, whereas glucose tolerance was unaltered. Insulin-stimulated glucose uptake in extensor digitorum longus muscle during the euglycemic-hyperinsulinemic clamp was increased in lean  $\gamma_3^{\text{R225Q}}$  mice, but not in *ob/ob*- $\gamma_3^{\text{R225Q}}$  mice. Acetyl-CoA carboxylase phosphorylation was increased in gastrocnemius muscle from  $\gamma_3^{\text{R225Q}}$  mutant mice independent of adiposity. Glycogen and triglyceride content were decreased after leptin treatment (5 days) in *ob/ob* mice, but not in *ob/ob*- $\gamma_3^{\text{R225Q}}$  mice. In conclusion, metabolic improvements arising from muscle-specific expression of AMPK $\gamma_3^{\text{R225Q}}$  are insufficient to ameliorate insulin resistance and obesity in leptin-deficient mice. Central**

**defects due to leptin deficiency may override any metabolic benefit conferred by peripheral overexpression of the AMPK $\gamma_3^{\text{R225Q}}$  mutation.**

AMP-activated protein kinase (AMPK) is a heterotrimeric complex, composed of a catalytic subunit ( $\alpha_1$  or  $\alpha_2$ ) and two regulatory subunits ( $\beta_1$  or  $\beta_2$  and  $\gamma_1$ ,  $\gamma_2$ , or  $\gamma_3$ ), which acts as a metabolic sensor to regulate glucose and lipid metabolism (1). AMPK is activated in response to changes in the intracellular AMP/ATP and ADP/ATP ratios in response to cellular stress or nutrient deprivation, changes in calcium concentration, or alterations in circulating levels of various hormones including leptin, cytokines, or adiponectin (2,3). Several lines of evidence highlight AMPK as an intertissue signal integrator among peripheral tissues and the hypothalamus to control whole-body energy and glucose homeostasis (4–10).

Acute activation of AMPK in peripheral tissues stimulates glucose uptake and lipid oxidation to produce energy, while turning off energy-consuming processes including synthesis of glycogen, lipids, and proteins (2). Pharmacological activation of AMPK in rodents or humans with insulin resistance or type 2 diabetes increases skeletal muscle glucose uptake (5,7,11,12) by an insulin-independent mechanism (13). Although AMPK can form up to 12 unique heterotrimeric complexes based on the expression of

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different  $\alpha/\beta/\gamma$  subunits, only three complexes ( $\alpha_1/\beta_2/\gamma_1$ ,  $\alpha_2/\beta_2/\gamma_1$ , and  $\alpha_2/\beta_2/\gamma_3$ ) are found in human skeletal muscle (14). Expression profiling of the AMPK  $\gamma$ -subunits in human and rodent skeletal muscle highlights a specialized role for the  $\gamma_3$  isoform in glycolytic fibers (15). Expression of a naturally occurring mutant (R225Q) form of the AMPK  $\gamma_3$ -subunit (16) in COS7 cells increases basal AMPK phosphorylation, while concomitantly reducing sensitivity to AMP (17). Moreover, AMPK $\gamma_3^{R225Q}$  ( $\gamma_3^{R225Q}$ ) transgenic mice have increased glycogen content and enhanced mitochondrial biogenesis, and are protected against dietary-induced triglyceride accumulation and insulin resistance in glycolytic skeletal muscle (17,18). This phenotype is copied in humans expressing the rare AMPK $\gamma_3^{R225W}$  mutation, which increases basal AMPK activity and muscle glycogen content, and decreases intramuscular triglyceride levels (19). Thus, lifelong expression of activated forms of AMPK $\gamma_3$  may prevent disturbances in glucose and lipid homeostasis that are characteristic of obese people with insulin resistance or type 2 diabetes (20,21).

The hypothalamus is a master regulator of food intake and energy balance, and coordinates glucose and energy homeostasis in response to the adipose-derived peripheral hormone leptin (22). Hypothalamic AMPK signaling plays an important role in the regulation of food intake (4,8,9). Activation of hypothalamic AMPK by counter-regulatory hormones involved in appetite control as well as by pharmacological AMPK activators increases food intake (4,9). Conversely, leptin inhibits hypothalamic AMPK signaling to reduce food intake and body weight, and increases AMPK signaling in peripheral tissues to promote lipid oxidation (4) and deplete triglyceride stores (23,24). Thus, an appropriate balance between AMPK signaling in central and peripheral tissues appears to be important for glucose and energy homeostasis. Previously, we have provided evidence that muscle-specific  $\gamma_3^{R225Q}$  transgenic mice rendered obese and hyperleptinemic by a high-fat diet are protected against excessive intramuscular triglyceride accumulation and the development of insulin resistance, presumably due to increased AMPK activation and lipid oxidation in skeletal muscle (17). This raises the question of whether AMPK activation can improve defects in insulin action and metabolism arising from severe obesity from either leptin deficiency or impaired leptin signaling. For example, in severely obese diabetic leptin receptor-deficient *db/db* mice, GLUT4 overexpression can improve glucose tolerance (25) as well as skeletal muscle insulin sensitivity (26). Furthermore, AMPK activation by AICAR treatment improves glucose homeostasis in rodents (5,7). Given the important role of leptin on energy balance, we determined whether glucose and energy homeostasis are improved by skeletal muscle overexpression of the mutant AMPK $\gamma_3^{R225Q}$  subunit in leptin-deficient *ob/ob* mice and whether the effects of AMPK are leptin-dependent.

## RESEARCH DESIGN AND METHODS

### Reagents

Reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

### Animals

Wild-type (WT) and skeletal muscle-specific  $\gamma_3^{R225Q}$  transgenic mice were generated as described previously (17). The transgenic  $\gamma_3^{R225Q}$  mutant mice were crossed with heterozygous *ob/+* mice to generate *ob/+*- $\gamma_3^{R225Q}$  mice. These *ob/+*- $\gamma_3^{R225Q}$  mice were bred with *ob/+* mice to generate the four mouse models studied in this report: lean WT, lean  $\gamma_3^{R225Q}$ , *ob/ob*-WT (*ob/ob*), and *ob/ob*- $\gamma_3^{R225Q}$  mice. The *ob/+* mice (on a C57BL/6J background) were purchased from Charles River Germany. *ob/ob* mice are obese and insulin-resistant because of a mutation in the hormone leptin; the leptin receptor is, however, intact in *ob/ob* mice. All the animal experiments were approved by the regional ethical committee on animal research Stockholm North, Sweden. Animals had free access to water and standard rodent chow (Lantmännen, Stockholm, Sweden), and were maintained in a temperature- and light-controlled (12-h light/dark cycle) environment. Animals were cared for in accordance with regulations for the protection of laboratory animals. Female and male mice were studied at 12–16 weeks of age.

### Glycogen and Triglyceride Determination

Mice fasted for 4 h were anesthetized with Avertin (2,2,2-tribromoethanol 99% and tertiary amyl alcohol [1:1 w/v], 500 mg/kg body weight) and gastrocnemius muscle and liver were removed, cleaned of fat and blood, and quickly frozen in liquid nitrogen. Glycogen content was determined fluorometrically on HCl extracts as described previously (17). Triglyceride content was determined using a triglyceride/glycerol blanking kit (Roche Diagnostics Scandinavia, Bromma, Sweden) using Seronorm Lipid as a standard (17).

### Intraperitoneal Glucose Tolerance Test

Animals were fasted for 4 h, and baseline glucose levels were measured using a OneTouch Ultra glucose meter (LifeScan, Milpitas, CA). Blood samples were collected from the tip of the tail. Glucose (1 g/kg) was injected intraperitoneally, and blood glucose levels were measured at 15, 30, 60, and 120 min after the injection. Blood was collected at baseline and 15 min after the glucose injection to determine insulin levels. Plasma insulin concentration was determined using an Ultra Sensitive Insulin ELISA Kit (Crystal Chem, Downers Grove, IL).

### Tissue-Specific Glucose Uptake in Conscious Mice

The jugular vein was catheterized 5–7 days prior to the clamp under isoflurane anesthesia. On the day of experiment, animals were fasted for 4 h. Euglycemic-hyperinsulinemic clamps were performed on conscious lean WT and  $\gamma_3^{R225Q}$  mice (10 mU insulin/kg/min) and *ob/ob* and *ob/ob*- $\gamma_3^{R225Q}$  mice (75 mU insulin/kg/min) and insulin-stimulated glucose uptake in glycolytic extensor digitorum longus (EDL)

and gastrocnemius muscles was determined using 2-deoxy-D-[1-<sup>14</sup>C]-glucose (PerkinElmer, Waltham, MA) as a tracer (27). Results are reported in nanograms of glucose per milligram per minute.

#### Lipid Oxidation in Isolated Skeletal Muscle

Palmitate oxidation in isolated EDL muscle was analyzed as described previously (28). EDL muscles were incubated in a Krebs-Henseleit buffer. After recovery, muscles were incubated for 2 h in <sup>3</sup>H-palmitate (PerkinElmer). Palmitate oxidation was determined by analyzing the <sup>3</sup>H-labeled water content using liquid scintillation counting. Results are reported in picomoles per milligram per minute.

#### Mitochondrial Respiration

Mitochondrial respiration in freshly isolated EDL muscle was determined using high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) as described previously (29,30). EDL muscles were dissected out, and fibers were gently separated under a microscope. Following saponin permeabilization in ice-cold relaxing and biopsy preservation solution, tissues were equilibrated in ice-cold mitochondrial respiration medium (MiRO5) and 1–2 mg of tissue was added to the respirometry chamber containing MiRO5. Leak respiration was measured by adding malate and pyruvate in the absence of ADP. Thereafter, ADP was added to measure oxidative phosphorylation. Respiration through complex I (C I) was measured by the addition of glutamate followed by the addition of succinate to measure C I+II respiration. Maximum flux through the electron transfer system (ETS) was measured by the addition of exogenous uncoupler carbonyl cyanide-4-(trifluoromethoxy)-phenyl-hydrazine (ETS I+II). Rotenone was used to inhibit electron transport through C I (ETS II). Absolute oxygen flux is expressed relative to tissue wet weight per second (picomoles of O<sub>2</sub> per milligram per second).

#### In Vivo Leptin Treatment

Mice were acclimatized in individual cages for a period of 2–3 days. They were injected with either saline or leptin (1 mg/kg, reconstituted in saline solution from PeproTech, Rocky Hill, NJ) at 1600 h for a period of 5 days. Food consumption and body weights were recorded daily. On the sixth day, after a 4-h fast, blood glucose level was determined, and the gastrocnemius muscle was dissected and immediately frozen for determination of triglyceride, glycogen, diacylglycerol (DAG), and ceramide content, and Western blot analysis.

#### Body Composition Analysis

Body composition (lean and fat mass) before and after saline and leptin treatment was determined in conscious mice using quantitative magnetic resonance imaging (EchoMRI, Houston, TX).

#### DAG and Ceramide Content

DAG and ceramide content were determined in gastrocnemius muscle from 4-h fasted mice by conversion of

DAG and ceramides to phosphorylation products by externally added DAG kinase from *Escherichia coli* (Enzo Life Sciences, Farmingdale, NY) in the presence of [ $\gamma$ -<sup>32</sup>P] ATP as previously described (31).

#### Circulating Free Fatty Acids

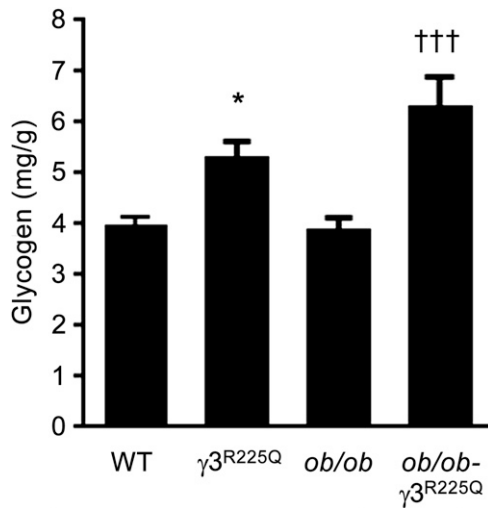
Plasma free fatty acids were determined by a commercially available kit (Wako Chemicals, Dusseldorf, Germany) in *ob/ob* and *ob/ob*- $\gamma_3^{R225Q}$  mice fasted for 4 h.

#### Western Blot Analysis

Gastrocnemius muscle was homogenized in ice-cold homogenization buffer (NaCl 137 mmol/L, KCl 2.7 mmol/L, MgCl<sub>2</sub> 1 mmol/L, Na<sub>4</sub>O<sub>7</sub>P<sub>2</sub> 5 mmol/L, NaF 10 mmol/L, Triton X-100 1%, glycerol 10%, Tris pH 7.8, 20 mmol/L, EDTA 1 mmol/L, phenylmethylsulfonyl fluoride 0.2 mmol/L, Na<sub>3</sub>VO<sub>4</sub> 0.5 mmol/L, and protease inhibitor cocktail  $\times$ 1) (Calbiochem; Merck Millipore, Billerica, MA) using the TissueLyser (Qiagen, Hamburg, Germany). Protein content in the supernatant was determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Proteins were separated on a 4–12% Criterion XT Bis-Tris Precast Gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membrane (100 V, 80 min), then blocked in Tris-buffered saline with 0.02% Tween-20 containing 7.5% nonfat dry milk for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4°C. MitoProfile total oxidative phosphorylation antibody cocktail was from Abcam (Cambridge, U.K.). Abundance of the following complex markers was determined; C I, NADH dehydrogenase (ubiquinone) 1  $\beta$  subcomplex 8 (NDUFB8); C II, succinate dehydrogenase complex, subunit B, iron sulfur (SDHB); C III, ubiquinol cytochrome c reductase core protein 2 (UQCRC2); C IV, cytochrome c oxidase I, mitochondrial (MTCO1); and C V, ATP synthase, H<sup>+</sup> transporting, mitochondrial F1 complex,  $\alpha$  subunit 1 (ATP5A). GLUT4 antibody was from Millipore (Temecula, CA). Phospho-AMPK $\alpha^{Thr172}$ , AMPK $\alpha$ , phospho-acetyl-CoA carboxylase (ACC)  $\alpha^{Ser79}/\beta^{Ser212}$ , and ACC $\alpha/\beta$  antibodies were from Cell Signaling Technology (Danvers, MA). Phospho-ACC $\beta^{Ser219/221}$  and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The AMPK $\gamma^3$  antibody was a gift from Dr. Grahame Hardie (University of Dundee, Dundee, U.K.). Membranes were incubated with appropriate secondary antibody conjugated with horseradish peroxidase (Bio-Rad). The immunoreactive proteins were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantified by calibrated densitometry using Quantity One image analysis software (Bio-Rad). GAPDH was used as a loading control.

#### Statistics

Statistical analysis was performed by unpaired two-tailed Student *t* test or two-way ANOVA, where applicable. The  $\gamma_3^{R225Q}$  mice were compared with WT mice, and the *ob/ob*- $\gamma_3^{R225Q}$  mice were compared with the *ob/ob*, unless



**Figure 1**—Effect of the AMPK $\gamma_3^{R225Q}$  mutation on skeletal muscle glycogen content. Glycogen content was measured in the gastrocnemius muscle from 4-h-fasted mice in WT ( $n = 7$ ),  $\gamma_3^{R225Q}$  ( $n = 6$ ), *ob/ob* ( $n = 11$ ), and *ob/ob- $\gamma_3^{R225Q}$*  ( $n = 5$ ) mice. Results are given as the mean  $\pm$  SEM. \* $P < 0.05$  vs. WT mice; ††† $P < 0.001$  vs. *ob/ob* mice.

otherwise stated. The effect of leptin treatment was compared with saline treatment in the lean and obese mouse models. Results were considered statistically significant at  $P < 0.05$ .

## RESULTS

### Glycogen Content in Skeletal Muscle

Glycogen content was determined in the gastrocnemius muscle from 4-h fasted WT, lean  $\gamma_3^{R225Q}$  transgenic, *ob/ob*, and *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice. As previously reported (17), the AMPK $\gamma_3^{R225Q}$  mutation increased glycogen content in lean mice (Fig. 1). WT and *ob/ob* mice have similar levels of glycogen content in the gastrocnemius muscle. Consistent with the lean  $\gamma_3^{R225Q}$  transgenic

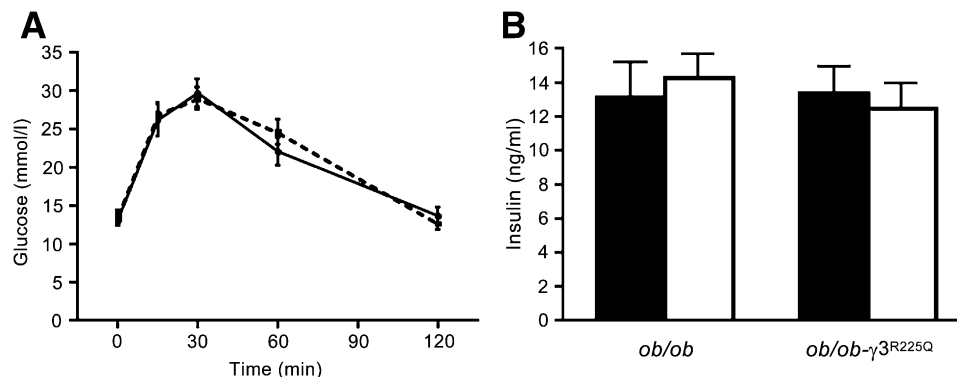
mice, glycogen content in the gastrocnemius muscle was increased in *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice compared with *ob/ob* mice (Fig. 1).

### Glucose Tolerance in *ob/ob- $\gamma_3^{R225Q}$* Transgenic Mice

Glucose tolerance was similar between *ob/ob* and *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice (Fig. 2A), consistent with our previously observation that glucose tolerance is unaltered in lean WT and  $\gamma_3^{R225Q}$  transgenic mice (17). Plasma insulin concentrations determined at baseline and 15 min after the glucose injection were similar between the *ob/ob* and *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice (Fig. 2B).

### In Vivo Insulin-Stimulated Glucose Uptake in Skeletal Muscle

We performed a euglycemic-hyperinsulinemic clamp to assess in vivo glucose uptake in EDL and gastrocnemius muscles. Lean WT and  $\gamma_3^{R225Q}$  transgenic mice were clamped using an insulin infusion of 10 mU/kg/min (baseline plasma insulin levels were  $0.5 \pm 0.1$  and  $0.6 \pm 0.2$  ng/mL, respectively, in WT and  $\gamma_3^{R225Q}$  mice, and levels achieved during the euglycemic-hyperinsulinemic clamp were  $9.4 \pm 0.9$  and  $11.3 \pm 1.2$  ng/mL, respectively, in WT and  $\gamma_3^{R225Q}$  mice ( $n = 6-9$ ). *ob/ob* and *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice were clamped using an insulin infusion of 75 mU/kg/min because of their extreme insulin-resistant state (baseline plasma insulin levels in *ob/ob* and *ob/ob- $\gamma_3^{R225Q}$*  mice were  $15.1 \pm 5.6$  and  $9.3 \pm 1.6$  ng/mL, respectively; levels achieved in *ob/ob* and *ob/ob- $\gamma_3^{R225Q}$*  mice during the euglycemic-hyperinsulinemic clamp were  $281.5 \pm 60.3$  and  $338.1 \pm 17.9$  ng/mL, respectively;  $n = 4$ ). The body weight of lean mice (WT mice  $31.6 \pm 0.8$  g;  $\gamma_3^{R225Q}$  mice  $32.4 \pm 1.1$  g;  $n = 10-12$ ) and *ob/ob* mice (*ob/ob*  $43.6 \pm 1.3$  g; *ob/ob- $\gamma_3^{R225Q}$*   $41.3 \pm 1.7$  g;  $n = 6-9$ ) without or with the  $\gamma_3^{R225Q}$  transgene was unaltered. Four-hour fasted plasma glucose levels were similar between WT and  $\gamma_3^{R225Q}$  mice ( $8.4 \pm 0.4$  and  $9.8 \pm 0.6$  mmol/L, respectively;  $n = 10-12$ ) and *ob/ob* and *ob/ob- $\gamma_3^{R225Q}$*  mice ( $9.3 \pm 0.7$  and  $11.4 \pm 1.8$  mmol/L, respectively;  $n = 6-9$ ).



**Figure 2**—Effect of the AMPK $\gamma_3^{R225Q}$  mutation on glucose tolerance. **A:** Intraperitoneal glucose tolerance tests (1 mg/kg glucose) were performed in 4-h-fasted *ob/ob* mice (solid line,  $n = 9$ ) and *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice (dashed line,  $n = 15$ ). **B:** Plasma insulin levels at baseline (black bars) and 15 min after the glucose injection (white bars) in *ob/ob* and *ob/ob- $\gamma_3^{R225Q}$*  mice ( $n = 9-10$ ). Results are given as the mean  $\pm$  SEM.

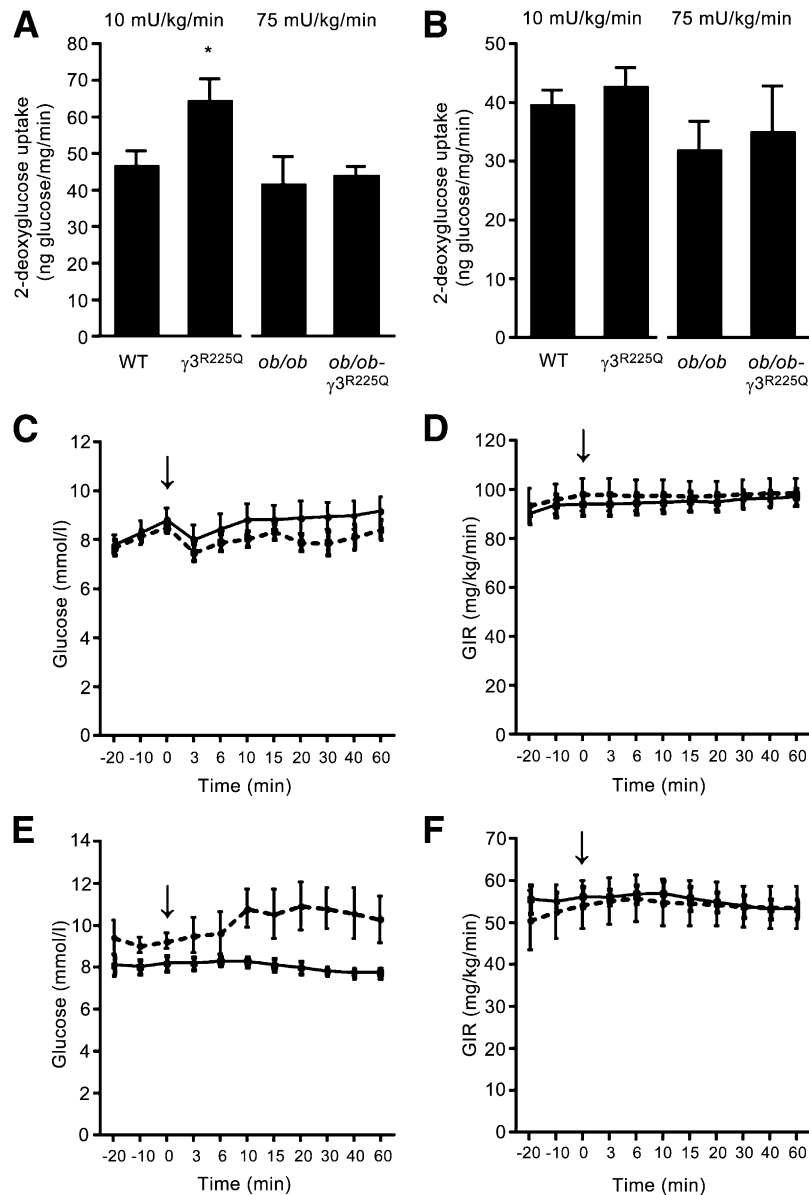
Insulin-stimulated glucose uptake was increased in glycolytic EDL muscle (Fig. 3A), but not in gastrocnemius muscle (Fig. 3B), from lean  $\gamma_3^{R225Q}$  transgenic mice under in vivo conditions. Conversely, insulin-stimulated glucose uptake in EDL and gastrocnemius muscles was unchanged between *ob/ob* and *ob/ob*- $\gamma_3^{R225Q}$  transgenic mice (Fig. 3A and B). Glucose concentration as well as the glucose infusion rate were similar at the steady state of the euglycemic-hyperinsulinemic clamp and after the 2-deoxy-D-[1- $^{14}$ C]-glucose injection in lean (Fig. 3C and D) and *ob/ob* mice (Fig. 3E and F).

### Lipid Oxidation in Isolated EDL Muscle

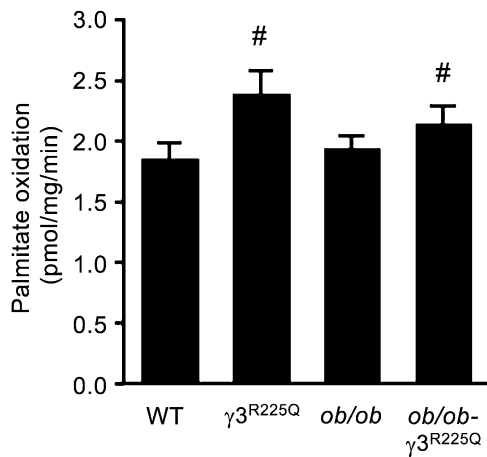
We have previously reported the AMPK $\gamma_3^{R225Q}$  mutation increases oleate oxidation in EDL muscle from high-fat-fed, but not chow-fed mice (17). Basal palmitate oxidation was similar in isolated EDL muscle from WT and *ob/ob* mice (Fig. 4). There was an effect of the  $\gamma_3^{R225Q}$  transgene to increase palmitate oxidation in EDL muscle from WT and *ob/ob* mice (Fig. 4).

### Mitochondrial Respiration in Skeletal Muscle

Mitochondrial respiration in freshly dissected EDL muscles was analyzed using high-resolution respirometry.



**Figure 3**—Glucose uptake in skeletal muscle from lean and obese  $\gamma_3^{R225Q}$  transgenic mice. In vivo insulin-stimulated glucose uptake in EDL (A) and gastrocnemius (B) muscle was assessed in conscious lean ( $n = 9$ – $11$ ) and obese ( $n = 6$ – $9$ ) WT and  $\gamma_3^{R225Q}$  transgenic mice during a euglycemic-hyperglycemic clamp. Plasma glucose concentration and glucose infusion rate (GIR) during the euglycemic-hyperinsulinemic clamp in lean (C and D) and obese (E and F) WT and  $\gamma_3^{R225Q}$  transgenic mice expressing the WT  $\gamma_3$  (solid line) or the  $\gamma_3^{R225Q}$  (dashed line) transgene. At time 0 (indicated by the arrow), 2-deoxy-D-[1- $^{14}$ C]glucose was injected. Results are given as the mean  $\pm$  SEM. \* $P < 0.05$  vs. WT mice.



**Figure 4**—Lipid oxidation in skeletal muscle from lean and obese  $\gamma_3^{R225Q}$  transgenic mice. Basal in vitro palmitate oxidation was measured in EDL muscle from 4-h-fasted lean ( $n = 7$ ) and obese ( $n = 7$ –11) mice. Results are given as the mean  $\pm$  SEM. # $P < 0.05$  indicates the effect of the  $\gamma_3^{R225Q}$  transgene.

The leak respiration, which denotes endogenous uncoupling, was similar between the lean and obese nontransgenic and transgenic mice (Fig. 5A). Mitochondrial respiration at the level of C I, ETS I+II, and ETS II was increased in *ob/ob* versus WT mice (Fig. 5A). The AMPK $\gamma_3^{R225Q}$  mutation did not alter the mitochondrial respiration in skeletal muscle from either WT or *ob/ob* mice. Protein abundance of markers of mitochondrial complexes (NDUFB8, SDHB, UQCRC2, MTCO1, and ATP5A) in gastrocnemius muscle was unaltered between WT and *ob/ob* mice with or without the AMPK $\gamma_3^{R225Q}$  mutation, except for the C II marker UQCRC2, which was increased in *ob/ob* mice compared with WT mice (Fig. 5B–F).

#### Effects of 5 Days In Vivo Leptin Treatment on Food Intake, Body Composition, Muscle Biochemistry, and Signaling

Five-day intraperitoneal leptin treatment reduced food intake in lean and obese nontransgenic and transgenic mice, compared with the respective saline-treated mice (Fig. 6A). Leptin treatment decreased body weight in WT, *ob/ob*, and *ob/ob*- $\gamma_3^{R225Q}$  mice compared with the respective saline-treated mice (Fig. 6B). In contrast, leptin treatment did not significantly decrease body weight in the lean  $\gamma_3^{R225Q}$  transgenic mice (Fig. 6B).

Body composition was assessed using magnetic resonance imaging. Total fat mass was reduced in leptin-treated *ob/ob* and *ob/ob*- $\gamma_3^{R225Q}$  transgenic mice, compared with the respective saline-treated mice (Fig. 6C). Lean mass was unaltered after saline or leptin treatment in the lean and obese nontransgenic and transgenic mice (Fig. 6D). Leptin treatment reduced 4-h fasting blood glucose levels in *ob/ob*- $\gamma_3^{R225Q}$  transgenic mice, with a similar trend in *ob/ob* mice (Fig. 6E).

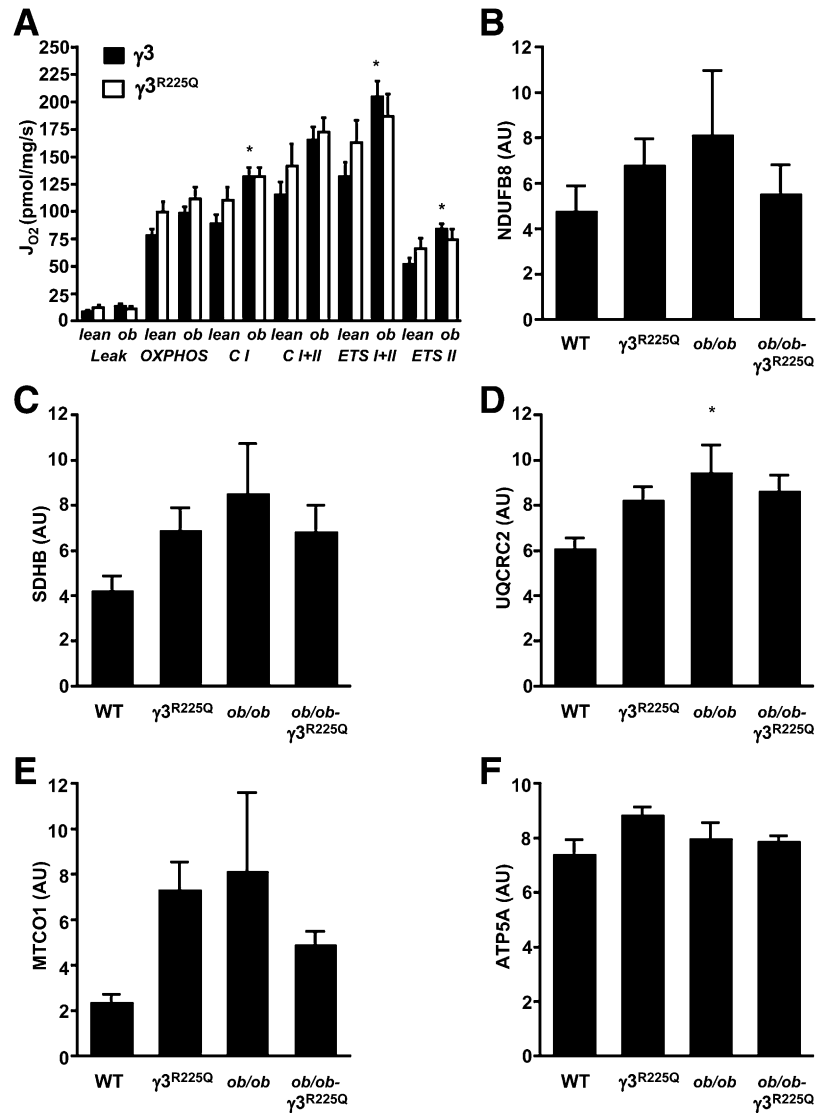
Gastrocnemius muscle was used for biochemical analysis. Glycogen content was increased in the saline-treated lean  $\gamma_3^{R225Q}$  transgenic mice, compared with the WT mice (Fig. 7A). Similarly, glycogen content was increased in *ob/ob*- $\gamma_3^{R225Q}$  transgenic mice, compared with the *ob/ob* mice. Glycogen content was not altered in WT and  $\gamma_3^{R225Q}$  transgenic mice after leptin treatment. However, glycogen content was reduced in *ob/ob* mice compared with saline-treated *ob/ob* mice after leptin treatment, but not in *ob/ob*- $\gamma_3^{R225Q}$  transgenic mice (Fig. 7A). GLUT4 protein abundance was unaltered in gastrocnemius muscle from lean and *ob/ob* mice  $\gamma_3^{R225Q}$  transgenic mice (Fig. 7B).

Triglyceride content in gastrocnemius muscle was unaltered between saline- and leptin-treated WT and  $\gamma_3^{R225Q}$  transgenic mice (Fig. 7C). While triglyceride content was elevated in *ob/ob* versus WT mice, the presence of the AMPK $\gamma_3^{R225Q}$  transgene in the *ob/ob* mice (*ob/ob*- $\gamma_3^{R225Q}$  mice) reduced triglyceride content. Furthermore, leptin treatment reduced triglyceride content in *ob/ob* mice. However, leptin treatment did not further reduce triglyceride content in gastrocnemius muscle from the *ob/ob*- $\gamma_3^{R225Q}$  transgenic mice (Fig. 7C). DAG and ceramide content were assessed in gastrocnemius muscle from saline- and leptin-treated mice. DAG content was increased in *ob/ob* mice compared with WT mice (Fig. 7D). DAG content was unaltered in saline- or leptin-treated  $\gamma_3^{R225Q}$  transgenic mice (Fig. 7D). Ceramide content was unaltered in gastrocnemius muscle from saline- or leptin-treated WT and *ob/ob* nontransgenic and transgenic mice (Fig. 7E). Furthermore, liver triglyceride content was unaltered between *ob/ob* and *ob/ob*- $\gamma_3^{R225Q}$  mice ( $52.6 \pm 5.3$  and  $52.6 \pm 4.3$  mg/g, respectively, for *ob/ob* and *ob/ob*- $\gamma_3^{R225Q}$  mice;  $n = 8$ –11). Circulating free fatty acids were similar between *ob/ob* and *ob/ob*- $\gamma_3^{R225Q}$  mice ( $0.39 \pm 0.07$  and  $0.37 \pm 0.05$  mmol/L, respectively, for *ob/ob* and *ob/ob*- $\gamma_3^{R225Q}$  mice;  $n = 7$ ).

AMPK $\alpha^{Thr172}$  phosphorylation in gastrocnemius muscle from saline-treated lean and obese nontransgenic and transgenic mice was similar (Fig. 8A). Skeletal muscle AMPK $\alpha^{Thr172}$  phosphorylation was increased after the 5-day leptin administration in *ob/ob*- $\gamma_3^{R225Q}$  transgenic mice (Fig. 8A). AMPK $\alpha$  and AMPK $\gamma_3$  subunit protein abundance was increased in lean  $\gamma_3^{R225Q}$  transgenic mice independent of treatment compared with WT mice (Fig. 8B and C), but not in *ob/ob* and *ob/ob*- $\gamma_3^{R225Q}$  transgenic mice. ACC $\alpha^{Ser79}/\beta^{Ser212}$  and ACC $\beta^{Ser219/221}$  phosphorylation was increased in gastrocnemius muscle from lean and *ob/ob*- $\gamma_3^{R225Q}$  transgenic mice (Fig. 8D and E). ACC $\alpha/\beta$  protein abundance was increased in lean  $\gamma_3^{R225Q}$  transgenic mice (Fig. 8F). ACC phosphorylation or abundance was unaltered in leptin-treated lean and obese nontransgenic and transgenic mice compared with the respective saline-treated mice.

#### DISCUSSION

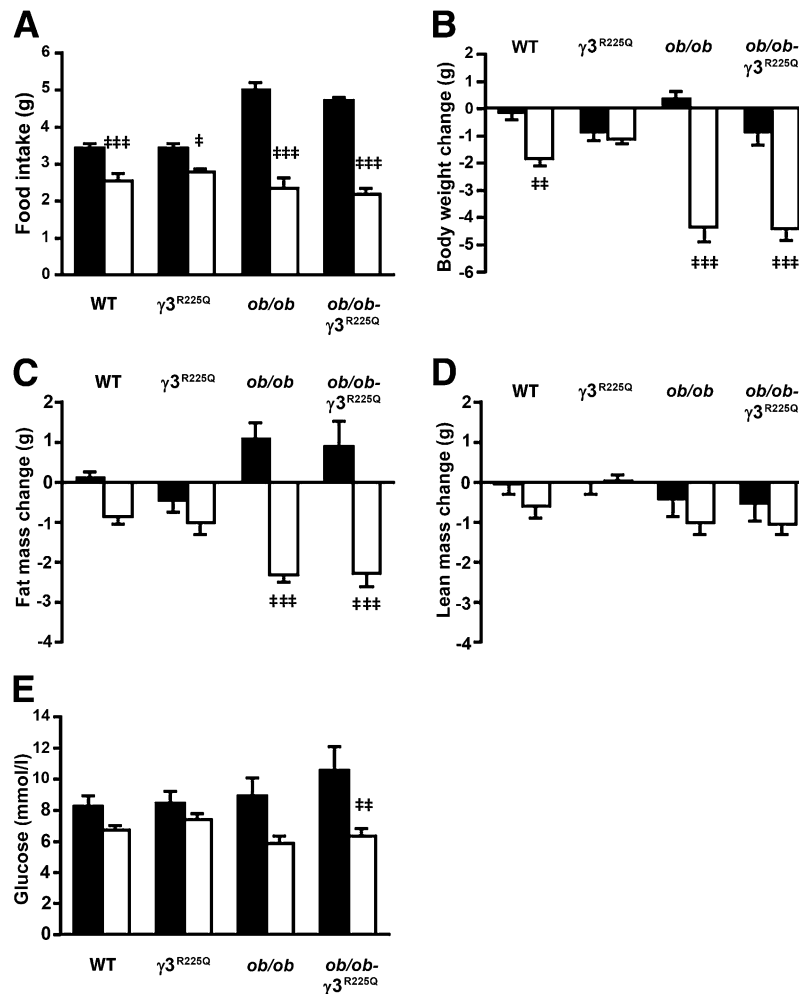
Overexpression of key signaling proteins regulating energy metabolism in skeletal muscle can improve



**Figure 5**—Mitochondrial respiration in skeletal muscle. **A**: Oxidative phosphorylation and ETS capacity were analyzed in glycolytic EDL muscle from lean and obese WT (black bars) or AMPK  $\gamma_3^{R225Q}$  transgenic (white bars) mice using high-resolution respirometry. WT  $\gamma_3$ , black bars,  $n = 8-10$ ;  $\gamma_3^{R225Q}$ , white bars,  $n = 8-9$ . Protein abundance of markers of C I (NDUFB8) (**B**), C II (SDHB) (**C**), C III (UQCRC2) (**D**), C IV (MTCO1) (**E**), and C V (ATP5A) (**F**) were determined in gastrocnemius muscle from WT,  $\gamma_3^{R225Q}$ , *ob/ob*, and *ob/ob- $\gamma_3^{R225Q}$*  mice ( $n = 5-11$ ). Results are given as the mean  $\pm$  SEM; \* $P < 0.05$  vs. WT mice. AU, arbitrary units; ob, obese; J<sub>o2</sub>, absolute oxygen flux.

metabolic disturbances associated with insulin resistance. Improvements in glucose homeostasis have been achieved by overexpression of either GLUT4 or uncoupling protein 3 in skeletal muscle of mice (25,32). AMPK activation by AICAR treatment can also improve glucose homeostasis in rodents (5,7). Here we tested the hypothesis that expression of a single missense mutation (R225Q) in the AMPK $\gamma_3$  isoform in skeletal muscle of *ob/ob- $\gamma_3^{R225Q}$*  mice improves glucose and energy homeostasis. We also determined whether leptin treatment would further normalize metabolic disturbances in obese individuals and whether the effects of AMPK activation on glucose homeostasis are leptin-dependent.

Impairments in whole-body glucose metabolism in type 2 diabetes patients are mainly attributed to defects in skeletal muscle glucose uptake and glycogen synthesis (33). Despite severe insulin resistance, AMPK activation promotes GLUT4 translocation and increases glucose uptake directly in skeletal muscle from insulin-resistant type 2 diabetes patients (11). A role for the AMPK $\gamma$ -subunit in glucose metabolism was first appreciated from studies of Hampshire pigs expressing a naturally occurring dominant mutation (denoted RN-) in the gene encoding the AMPK $\gamma_3$ -isoform (34). This mutation results in an excessive amount of glycogen storage in glycolytic skeletal muscle. In vitro studies in COS7 cells reveal that AMPK $\gamma_3^{R225Q}$  complexes have higher basal AMPK activity



**Figure 6**—Effect of leptin treatment on food intake and body composition. WT ( $n = 7$ – $10$ ),  $\gamma_3^{R225Q}$  ( $n = 7$ – $11$ ), *ob/ob* ( $n = 6$ – $8$ ), and *ob/ob- $\gamma_3^{R225Q}$*  ( $n = 7$ – $8$ ) mice were treated with saline or leptin (1 mg/kg) for 5 days. **A**: Food intake was assessed in saline- and leptin-treated mice. **B**: The effect of saline or leptin on body weight was assessed over a 5-day treatment period. Changes in body composition (fat mass [C] and lean mass [D]) were measured by magnetic resonance imaging after 5 days of saline or leptin treatment. **E**: Four-hour fasting glucose concentration was determined 16 h after the 5-day saline/leptin treatment period. Saline treatment, black bars; leptin treatment, white bars. Results are given as the mean  $\pm$  SEM.  $\dagger P < 0.05$ ,  $\dagger\dagger P < 0.01$ ,  $\dagger\dagger\dagger P < 0.001$  vs. respective saline-treated mice.

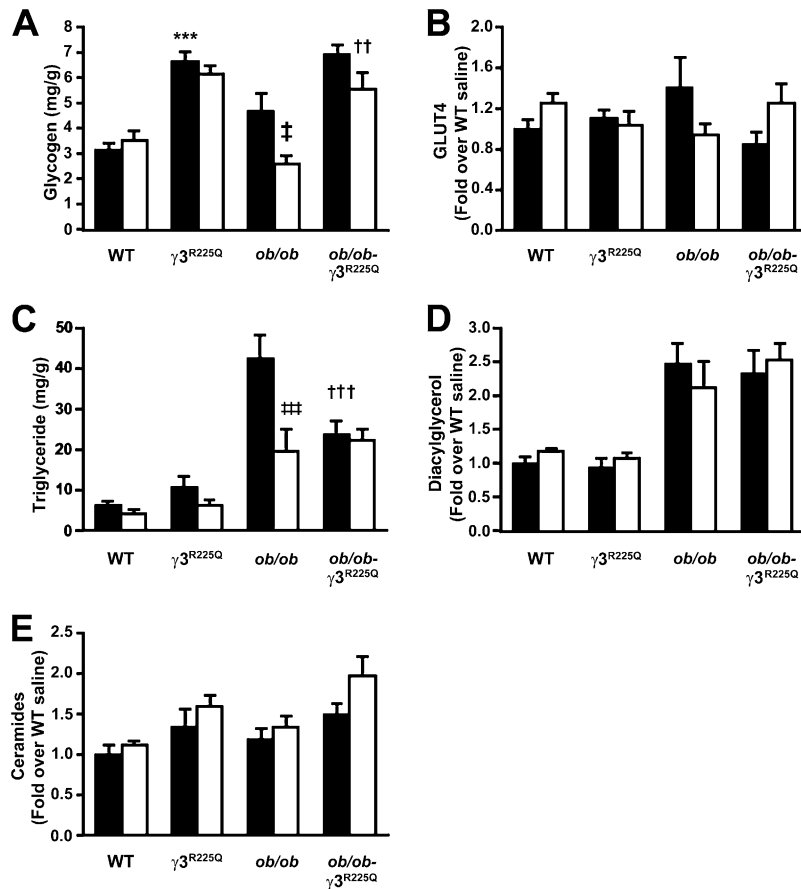
and lack AMP dependence (17). Thus, the R225Q mutation is a gain-of-function mutation that abolishes allosteric regulation by AMP/ATP, which thereby increases basal AMPK activity.

$\gamma_3^{R225Q}$  transgenic mice fed a high-fat diet are protected against the development of skeletal muscle insulin resistance (17). Here we report that in vivo glucose uptake in glycolytic skeletal muscle is increased during a euglycemic-hyperinsulinemic clamp in lean  $\gamma_3^{R225Q}$  transgenic mice. Consistent with our earlier findings in fat-fed  $\gamma_3^{R225Q}$  transgenic mice (17), we found that glycogen content was increased and triglyceride content was decreased in gastrocnemius muscle from *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice. These changes in *ob/ob- $\gamma_3^{R225Q}$*  mice indicate that leptin is not required for AMPK-mediated glycogen synthesis. Moreover, the effect of the AMPK $\gamma_3^{R225Q}$  mutation on glycogen content is

independent of adiposity, consistent with clinical studies in obese humans harboring a similar mutation in the AMPK $\gamma_3$  subunit who have higher skeletal muscle glycogen content compared with WT carriers (19). However, in contrast to our earlier study in fat-fed mice (17), improvements in muscle biochemistry arising from the AMPK $\gamma_3^{R225Q}$  mutation were insufficient to ameliorate whole-body insulin resistance in leptin-deficient mice. Thus, a permissive amount of leptin may be required to fully confer the AMPK $\gamma_3^{R225Q}$ -dependent improvements in skeletal muscle insulin sensitivity.

Several molecular mechanisms account for skeletal muscle glycogen accumulation. Skeletal muscle GLUT4 protein abundance directly influences the rate of insulin-stimulated glucose uptake and metabolism (35). Moreover, GLUT4 overexpression prevents insulin resistance (26) and glucose intolerance (25) in severely obese diabetic leptin



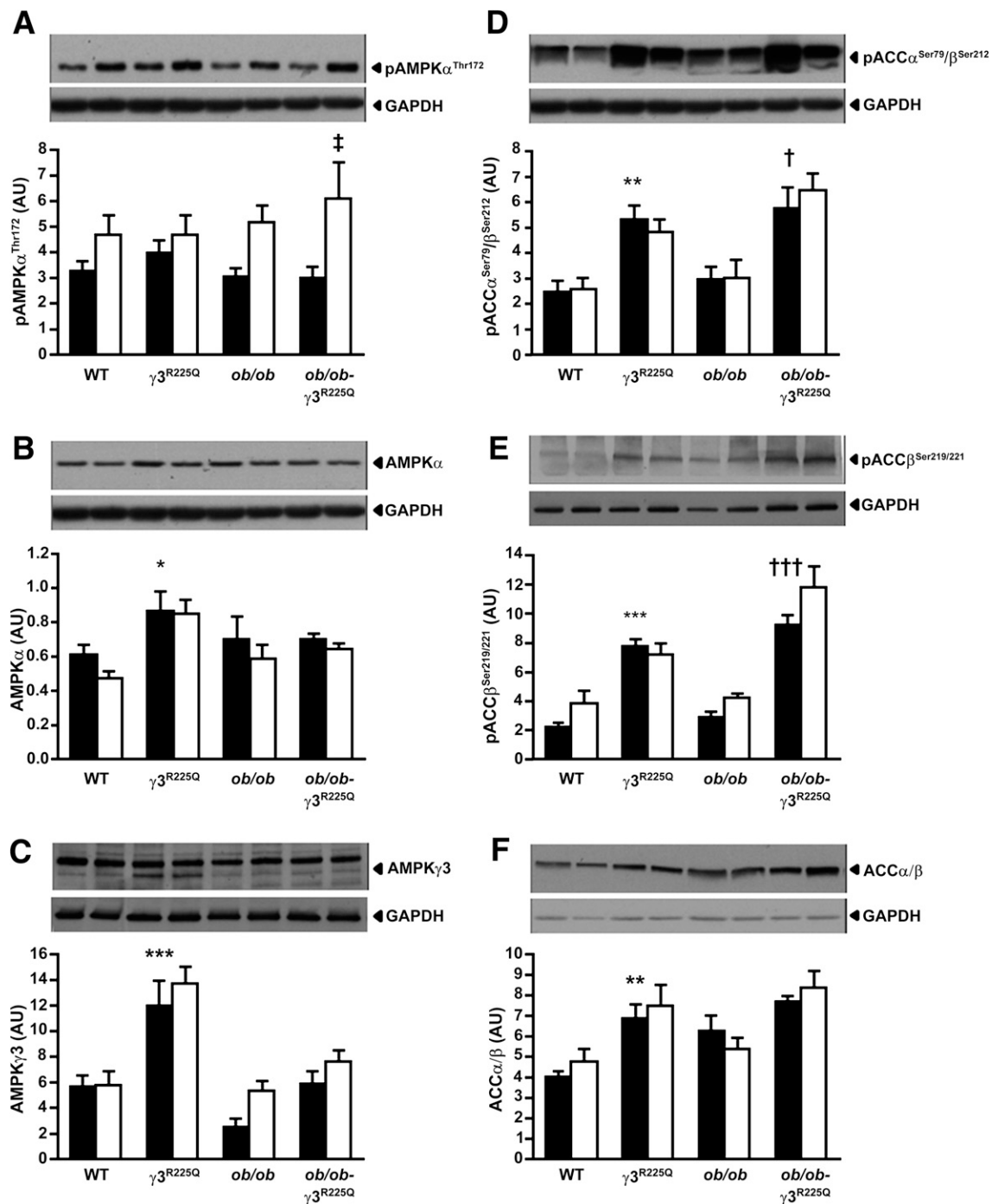


**Figure 7**—Effect of leptin treatment on skeletal muscle glycogen and triglyceride content. Skeletal muscle was dissected from 4-h–fasted WT ( $n = 6$ –11),  $\gamma_3^{R225Q}$  ( $n = 8$ –11), *ob/ob* ( $n = 5$ –7), and *ob/ob- $\gamma_3^{R225Q}$*  ( $n = 9$ –10) mice 16 h after the 5-day saline or leptin (1 mg/kg) treatment period. Glycogen content (A), GLUT4 protein abundance (B), triglyceride content (C), DAG content (D), and ceramide content (E) were measured in the gastrocnemius muscle. Saline treatment, black bars; leptin treatment, white bars. Results are given as the mean  $\pm$  SEM. \*\*\* $P < 0.001$  vs. saline-treated WT mice; †† $P < 0.01$ , ††† $P < 0.001$  vs. saline-treated *ob/ob* mice; † $P < 0.05$ , ††† $P < 0.001$  vs. respective saline-treated mice.

receptor-deficient *db/db* mice, highlighting the importance of skeletal muscle glucose transport in maintaining whole-body glucose homeostasis. Skeletal muscle-specific overexpression of glycogen synthase increases glycogen accumulation by an insulin-independent mechanism not involving glucose transport (36), reinforcing the importance of glucose metabolism. Despite the profound increase in glycogen content in chow-fed lean  $\gamma_3^{R225Q}$  mice, basal and insulin-stimulated glucose uptake in isolated skeletal muscle was similar to WT mice (17). Furthermore, GLUT4 protein abundance was unaltered in gastrocnemius muscle from lean and *ob/ob* nontransgenic and transgenic mice. Thus, the increase in glycogen content in  $\gamma_3^{R225Q}$  transgenic mice is unlikely to arise from constitutive increases in glucose uptake. Consequently,  $\gamma_3^{R225Q}$  mice resemble glycogen synthase transgenic mice, rather than GLUT4 transgenic mice. Glycogen content negatively regulates both AMPK activity (37) and insulin-stimulated glucose uptake (38). However, glucose uptake in glycolytic muscle was increased despite increased glycogen content. Thus, improvements in glucose uptake may require permissive

levels of leptin, since the AMPK $\gamma_3^{R225Q}$  mutation did not increase glucose uptake in leptin-deficient *ob/ob* mice. Alternatively, increased GLUT4 abundance or translocation may be required to achieve improvements in glucose tolerance and insulin sensitivity in individuals with severe obesity due to leptin deficiency.

Although lipids serve as an important fuel source for skeletal muscle, excessive levels may trigger insulin resistance (39). Strategies to reduce excess triglyceride levels in skeletal muscle improve insulin sensitivity (17,40). Intramuscular triglyceride content is reduced and insulin sensitivity is increased in  $\gamma_3^{R225Q}$  transgenic mice fed a high-fat diet concomitant with increased lipid oxidation (17). Likewise, intramuscular triglyceride content was reduced in *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice, presumably due to decreased triglyceride synthesis and a modest increase in lipid oxidation. DAG and ceramide content, intermediates in lipid metabolism, have been linked to the development of insulin resistance in skeletal muscle (41). Here we confirm that DAG content is increased in gastrocnemius muscle from *ob/ob* mice (42), yet it was unaltered



**Figure 8**—AMPK and ACC phosphorylation in skeletal muscle. Mice were treated with saline or leptin (1 mg/kg) for 5 days (WT [ $n = 6-9$ ],  $\gamma_3^{R225Q}$  [ $n = 6-11$ ], *ob/ob* [ $n = 5-6$ ], and *ob/ob*- $\gamma_3^{R225Q}$  [ $n = 6-9$ ] mice). Phospho-AMPK $\alpha$ <sup>Thr172</sup> (pAMPK $\alpha$ <sup>Thr172</sup>) (A), AMPK $\alpha$  (B), AMPK $\gamma_3$  protein abundance (C), phospho-ACC $\alpha$ <sup>Ser79/βSer212</sup> (pACC $\alpha$ <sup>Ser79/βSer212</sup>) (D), phospho-ACC $\beta$ <sup>Ser219/221</sup> (p-ACC $\beta$ <sup>Ser219/221</sup>) (E), and ACC $\alpha/\beta$  protein abundance (F) were determined by immunoblot analysis in the gastrocnemius muscle. Saline treatment, black bars; leptin treatment, white bars. Results are given as the mean  $\pm$  SEM. † $P < 0.05$  vs. respective saline-treated mice; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. WT mice; †† $P < 0.05$ , ††† $P < 0.001$  vs. saline-treated *ob/ob* mice. AU, arbitrary units.

in  $\gamma_3^{R225Q}$  transgenic mice. Ceramide content in gastrocnemius muscle was similar between transgenic and nontransgenic WT and *ob/ob* mice, confirming previous reports (42,43). However, phosphorylation of ACC, a downstream target of AMPK (44), was increased in  $\gamma_3^{R225Q}$  transgenic

mice. Increased ACC phosphorylation leads to inactivation of the enzyme, which thereby increases lipid oxidation and decreases triglyceride levels (45). The increase in ACC phosphorylation suggests that the cellular energy charge driven by the AMPK mutation is altered (17), consistent

with increased lipid oxidation in the  $\gamma_3^{R225Q}$  transgenic mice.

Leptin influences AMPK signaling in central and peripheral tissues. Leptin inhibits AMPK activity in the brain and reduces food intake (4), while enhancing lipid metabolism in skeletal muscle (46). Thus, we explored whether leptin treatment of *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice would lead to a further metabolic improvement imposed by the AMPK $\gamma_3^{R225Q}$  mutation. The AMPK $\gamma_3^{R225Q}$  mutation did not alter the body weight response to leptin treatment in *ob/ob* mice. Thus, the metabolic differences observed between the nontransgenic and *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice are directly related to the mutation, rather than to changes in food intake. Leptin treatment improved the fasting glucose level in *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice, concomitant with a normalization of food intake and a reduction in adiposity. Moreover, we found that skeletal muscle glycogen content was decreased in leptin-treated *ob/ob* mice, consistent with the effects of leptin in decreasing glycogen synthesis in *ob/ob* mice (47). However, leptin treatment did not decrease skeletal muscle glycogen content in  $\gamma_3^{R225Q}$  transgenic lean and *ob/ob* mice, indicating that the AMPK $\gamma_3^{R225Q}$  mutation has a dominant influence on fuel partitioning within skeletal muscle, which may be overcome by hyperleptinemia. Although the concentration of leptin used in this study was sufficient to improve blood glucose and body weight, higher doses trigger a shift in substrate use such as that observed in fat-fed  $\gamma_3^{R225Q}$  mice (17).

AMPK activation is linked to mitochondrial biogenesis, providing a mechanism for the increased lipid oxidation observed in fat-fed  $\gamma_3^{R225Q}$  transgenic mice (17). The increase in mitochondrial respiration in *ob/ob* mice confirms our previous findings that obesity induces molecular adaptations in glycolytic skeletal muscle to enhance mitochondrial respiration (48). Nevertheless, these mice are severely insulin-resistant. Mitochondrial biogenesis is increased in glycolytic skeletal muscle from  $\gamma_3^{R225Q}$  transgenic mice, concomitant with increased expression of the coactivator peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  and transcription factors that drive different mitochondrial proteins expression (18). However, mitochondrial respiration is unaltered between WT and  $\gamma_3^{R225Q}$  transgenic mice (18), as well as *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice. The increase in skeletal muscle mitochondrial markers in  $\gamma_3^{R225Q}$  transgenic mice (18) may account for the increase in lipid oxidation in the  $\gamma_3^{R225Q}$  transgenic mice. However, the regulation of insulin sensitivity is complex and not entirely coupled to increased skeletal muscle mitochondrial content. Nevertheless, cultured myotubes from probands carrying a homologous mutation (AMPK $\gamma_3^{R225W}$ ) reinforce the profound effect of this mutation on glucose uptake and metabolism, mitochondrial content, and oxidative capacity, and raise the clinical implications of mutations in the AMPK $\gamma_3$  subunit (49).

Defective leptin action leads to metabolic abnormalities associated with obesity. The effects of leptin are partly

mediated via the AMPK pathway in central and peripheral sites. Here we show that the expression of a mutant form of the AMPK $\gamma_3$  subunit in glycolytic skeletal muscle increases glycogen content and decreases intramuscular triglyceride levels. However, DAG and ceramide content were unaltered. The triglyceride depletion in *ob/ob- $\gamma_3^{R225Q}$*  transgenic mice does not appear to improve glucose utilization and insulin sensitivity in *ob/ob- $\gamma_3^{R225Q}$*  mice. Thus, the lack of central leptin signaling may override the favorable metabolic milieu conferred by peripheral overexpression of the AMPK $\gamma_3^{R225Q}$  mutation to improve glucose and energy homeostasis. Further studies in hypothalamic-specific AMPK transgenic *ob/ob* mice may clarify the central role of this protein kinase in the control of glucose and energy homeostasis in leptin deficiency. Given our findings (Supplementary Table 1), targeting both peripheral and central AMPK actions may be required to improve glucose homeostasis.

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**Author Contributions.** R.Z.T. researched the data, wrote the manuscript, and approved the final version of the manuscript. P.M.G.-R., A.V.C., and M.B. researched the data, reviewed and edited the manuscript, and approved the final version of the manuscript. R.J.O.S., L.Q.J., M.H.H., A.S.D., and E.V. researched the data and approved the final version of the manuscript. J.R.Z. wrote the manuscript and approved the final version of the manuscript. J.R.Z. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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