Disruption of the striated muscle glycogen-targeting subunit of protein phosphatase 1: influence of the genetic background

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Abstract

A prediabetic phenotype of glucose intolerance, insulin resistance and obesity was observed at ~12 months of age in mice homozygous for a null allele of the major skeletal muscle glycogen-targeting subunit G_M of protein phosphatase 1 (PP1) and derived from a 129/Ola donor strain. In this study, backcrossing of these $G_M^{-/-}$ mice (termed obese $G_M^{-/-}$ mice) onto two different genetic backgrounds gave rise to lean, glucose-tolerant, insulin-sensitive $G_M^{-/-}$ mice (termed lean $G_M^{-/-}$ mice), indicating that at least one variant gene in the 129/Ola background, not present in the C57BL/6 or 129s2/sV background, is required for the development of the prediabetic phenotype of obese $G_M^{-/-}$ mice. Slightly elevated AMP-activated protein kinase $\alpha 2$ activity in the skeletal muscle of lean C57BL/6 $G_M^{-/-}$ mice was also observed to a lesser extent in the obese $G_M^{-/-}$ mice. Normal or slightly raised *in vivo* glucose transport in lean C57BL/6 $G_M^{-/-}$ mice compared with decreased glucose transport in the obese $G_M^{-/-}$ mice supports the tenet that adequate transport of glucose may be a key factor in preventing the development of the prediabetic phenotype. The pH 6·8/pH 8·6 activity ratio of phosphorylase kinase was increased in lean C57BL/6 $G_M^{-/-}$ mice compared with controls indicating that phosphorylase kinase is an *in vivo* substrate of PP1-G_M.

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Introduction

One of the problems in understanding type 2 diabetes is that multiple genes as well as environmental factors contribute to this disorder. In addition, current evidence indicates heterogeneity in the underlying genetic defects (Hansen & Pedersen 2005, O'Rahilly *et al.* 2005). A feature commonly observed in type 2 diabetes is that insulin-stimulated glycogen synthesis is decreased in skeletal muscle (Shulman 2000). In this tissue, insulin stimulates glycogen synthesis via the translocation of the glucose transporter (GLUT4) and activation of glycogen synthase (GS; DeFronzo 1997, Shepherd & Kahn 1999). Phosphorylation of GS by GS kinase-3 (GSK3) at sites 3a, b and c (now known to be Ser640, Ser644 and Ser648) leads to its inactivation, while dephosphorylation of these sites and activation of GS are catalysed by glycogen-bound protein phosphatase 1 (PP1) that is predominantly bound to the glycogen-targeting subunit G_M/R_{GL} (encoded by the gene *PPP1R3A*) in skeletal muscle (Cohen 1999). Glycogen breakdown is catalysed by phosphorylase kinase and phosphorylase and the latter had been shown to be dephosphorylated with inactivation by PP1-G_M *in vivo* (Toole & Cohen 2007). Variations in *PPP1R3A*, the gene-encoding G_M, have been noted in human populations and associated with diabetes in some populations but not others. For example, a five bp deletion mutation leading to decreased stability of G_M mRNA was associated with diabetes in the Pima Indian population (Xia *et al.* 1998). In addition, the same mutation was found at higher frequencies in males diagnosed early with type 2 diabetes in a Scottish population (Doney *et al.* 2003). Individuals with severe insulin resistance within one family were identified as the only family members who were doubly heterozygous with respect to frameshift/premature stop mutations in the *PPP1R3A* and peroxisome proliferator-activated receptor γ genes (Savage *et al.* 2002).

In order to determine the physiological role of G_M, two murine models homozygous for a null allele of G_M have been generated in C57/BL6 backgrounds using donor cells from different substrains, 129/SvJ (Suzuki *et al.* 2001) and 129/Ola (Delibegovic *et al.* 2003). In both models, glycogen levels in skeletal muscle of $G_M^{-/-}$ mice were 10% of the levels in control mice, but blood glucose levels were not significantly different from controls. The proportion of phosphorylase in the active form in skeletal muscle was elevated in both models. However, the proportion of GS in the active form in the insulin-stimulated $G_M^{-/-}$ mice of Delibegovic *et al.* (2003) was less than that in the unstimulated control mice, whereas the proportions of GS in the active form in the insulin-stimulated $G_M^{-/-}$ and control mice of Suzuki *et al.*

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(2001) were not significantly different. Nevertheless, the most striking difference between the two models was that the $G_M^{-/-}$ mice of Suzuki *et al.* (2001) remained lean, glucose tolerant and insulin sensitive up to 12 months of age, whereas the $G_M^{-/-}$ mice of Delibegovic *et al.* (2003) were obese with large abdominal fat deposits, glucose intolerant and insulin resistant at 11–12 months of age, and glucose uptake into skeletal muscle *in vivo* was decreased. This prediabetic phenotype is consistent with the concept that when blood glucose cannot be taken up and converted via GS into glycogen in skeletal muscle, the glucose is redirected (probably via the liver) into fat deposits that increase gradually with age. Development of insulin resistance may arise as a consequence of the increased fatty acids in older $G_M^{-/-}$ mice. In order to investigate the factors accounting for the phenotypic differences between the two $G_M^{-/-}$ mice models, we have examined the genetic background, environmental factors and biochemical parameters.

Materials and methods

Generation of G_M-deficient ($G_M^{-/-}$) mice

All animal procedures were approved by the University of Dundee Ethical Committee and were performed under a UK Home Office Project Licence. Mice heterozygous for a null allele of G_M (Delibegovic *et al.* 2003) were backcrossed to either C57BL/6 or 129s2/sV for at least six generations, before heterozygotes were intercrossed to produce mice homozygous for the null allele ($G_M^{-/-}$), heterozygous, and homozygous for the wild-type allele ($G_M^{+/+}$), which were used in the analyses described herein. All mice were maintained in temperature- and humidity-controlled conditions with a 12 h light:12 h darkness cycle and were allowed access to food and water *ad libitum* unless otherwise stated. Animals were fed either standard chow (nitrogen-free extract (NFE) 62%), a low-carbohydrate diet (NFE 51%) or a diet containing 30% extra glucose (NFE 72%) all from Special Diet Services (Witham, Essex, UK). NFE is a measure of 'usable' carbohydrate content of a diet. Mice were genotyped by Southern blotting of ear DNA digested with *Xba*I, as described previously (Delibegovic *et al.* 2003).

Immunological techniques

Unless indicated otherwise, 20 μg protein lysates were subjected to SDS-PAGE (Novex, Invitrogen) and transferred to nitrocellulose membranes. The membranes were incubated with affinity purified antibody at 1 μg/ml or at dilutions recommended by the suppliers in 50 mM Tris–HCl (pH 7·5), 0·15 M NaCl, 0·1% (v/v) Tween-20 containing 5% (w/v) skimmed milk for 16 h at 4 °C. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence or fluorescently labelled secondary antibodies and analysis using a Li-Cor Odyssey infrared detection system following the manufacturer's guidelines (Li-Cor, Cambridge, UK). The band intensity was quantified using Li-Cor software.

Antibodies to G_M, PP1α and PP1β, have been described previously (Delibegovic *et al.* 2003). Antibodies to human$PP2A_C$α/β (289-FDPAPRRGEPHVTRRTPDY-307) and the PH domain of protein kinase B (PKB) were raised in sheep by Diagnostics Scotland (Penicuik, Midlothian, UK) and affinity purified by the Division of Signal Transduction Therapy, University of Dundee, co-ordinated by Dr Hilary McLauchlan and Dr James Hastie. Peptides were synthesised by Dr G Bloomberg (University of Bristol, UK). Antibodies to phosphorylase kinase purified from rabbit skeletal muscle (Cohen 1983) were similarly raised in sheep and the immunoglobulin G (IgG) was isolated using protein G-Sepharose. Antibodies to rat AMP-activated protein kinase (AMPK) α1 (344-CTSPPDSFLDDHHLTR-358), AMPKα2 (352-CMDDSAMHIPPGLKPH-366) and AMPK phospho-Thr172 were supplied by Prof. D G Hardie (University of Dundee). Antibodies against PKB (phospho-Thr308), PKBα/PKBβ (phospho-Ser473), GSK3α/GSKβ (phospho-Ser 21/9), acetyl-CoA carboxylase (ACC) and ACC phospho-Ser212 were from Cell Technology (Hitchin, UK). Other antibodies employed were: anti-GSK3α/GSK3β (Biosource, Nivelles, Belgium), anti-GLUT4 from clone 1F8 ((James *et al.* 1988), Biogenesis, Poole, UK) and anti-GLUT4 C-terminal 15 amino acids (Abcam, Cambridge, UK). For analysis of GLUT4, the anti-GLUT4 C-terminal antibody (Abcam) was covalently coupled to protein G-Sepharose beads and incubated with skeletal muscle lysates overnight at 4 °C. The 10 000 ***g*** immunopellets were washed three times and examined by sodium dodecyl gel electrophoresis followed by immunoblotting with anti-GLUT4 antibodies. Immunoblot analysis of ACC was performed according to Sakamoto *et al.* (2005), except that lysates were prepared with the addition of 1 μM microcystin.

Glucose and insulin tolerance testing

Blood glucose levels were assessed using the AccuChek Blood Glucose Monitoring System (Roche Diagnostics). Glucose tolerance tests were performed on mice after a 12–14 h overnight fast. Mice were injected intraperitoneally with 2 mg D-glucose/g, and blood glucose levels were determined immediately before and at 15, 30, 60 and 120 min following injection. Insulin tolerance was assessed by measuring blood glucose levels before and

15, 30, 60 and 120 min after mice had received an i.p. injection of 1 mU/g insulin (Human Actrapid, 100 iU/ml; Novo Nordisk Pharmaceuticals Ltd, Crawley, UK) after a 6-h fast. *In vivo* glucose transport was determined after i.p. injection of 2-deoxy-D-[1,2-^{3}H]-glucose mixed with 20% dextrose (2 g/kg body weight; 10 μCi/mouse) according to Zisman *et al.* (2000).

Measurement of serum parameters

Blood samples were measured using the AccuChek Blood Glucose Monitoring System. Up to 100 μl blood was collected from mice after an overnight fast and serum stored at −80 °C. Serum triglycerides and free fatty acids were measured using the commercially available kits (Biostat, Stockport, UK; Wako Diagnostic Systems, Neuss, Germany). Serum insulin was measured by ELISA assay (Crystal Chem Inc., Downers Grove, IL, USA).

Preparation of tissue lysates and subcellular fractions

After an overnight fast, mice were killed by concussion followed by cervical dislocation, and tissues were rapidly extracted and freeze clamped in liquid nitrogen before being stored at −80 °C. Tissues were ground to a fine powder under liquid nitrogen. Skeletal muscle was obtained from whole hind limbs unless otherwise stated and ground under liquid nitrogen. The pulverised tissues were homogenised in 10 vol/g ice-cold 50 mM Tris–HCl (pH 7·5), 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 5% (v/v) glycerol, 0·1% (v/v) 2-mercaptoethanol, 'complete' protease inhibitor cocktail (Buffer A) plus 0·1% (v/v) Triton X-100 and 1 μM microcystin-LR (Life Technologies), using a Polytron PT-1200 and centrifuged at 16 000 ***g*** for 10 min at 4 °C. The supernatant (lysate) was snap-frozen in liquid nitrogen and stored at −80 °C.

For phosphorylase kinase assays, mice were terminally anaesthetised for 20 min by an i.p. injection of 60 μg/g pentobarbital followed by a further injection of 40 μg/g propranolol to inhibit the effects of adrenaline. Skeletal muscle was harvested 30–90 min after propranolol administration and homogenised in 10 vol/g ice-cold 50 mM Tris–HCl (pH 7·5), 2 mM EDTA, 20 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM pyrophosphate, 0·1% (v/v) Triton X-100, 1 μM microcystin-LR, 0·1% (v/v) 2-mercaptoethanol and 'complete' protease inhibitor cocktail.

For preparation of membrane and cytosolic fractions, frozen ground skeletal muscle was homogenised in 4–6 vol/g buffer A. The homogenates were centrifuged at 1000 ***g*** for 10 min at 4 °C to remove nuclei, and the supernatant was centrifuged at 100 000 ***g*** for 1 h at 4 °C. The 100 000 ***g*** supernatant (termed cytoplasmic fraction) was snap-frozen and stored at −80 °C. The pellet (termed membrane fraction) was washed twice in ice-cold Buffer A with repeated centrifugation at 100 000 ***g*** for 30 min, and finally resuspended in 300 μl ice-cold buffer A containing 1% Triton X-100. This lysate was snap-frozen and stored at −80 °C.

Enzyme assays and glycogen content

Phosphorylase kinase was assayed using the peptide KRKQISVRGLA (residues 10–20 of human muscle phosphorylase *b*) as a substrate. Skeletal muscle lysate (50–100 μg) diluted in 100 μl 50 mM Tris–HCl (pH 7·5), 150 mM NaCl, 0·1% (v/v) 2-mercaptoethanol, 'complete' protease inhibitor cocktail was incubated for 1 h at 4 °C on a shaking platform with 30 μg anti-PhK antibody non-covalently coupled to 10 μl protein G-Sepharose. Following centrifugation for 1 min at 16 000 ***g***, the pellets were washed twice with 0·5 ml 50 mM Tris–HCl (pH 7·5), 500 mM NaCl, 0·1% (v/v) 2-mercaptoethanol and once with 50 mM Tris–HCl (pH 7·5), 0·1% (v/v) 2-mercaptoethanol. The pellet was assayed for PhK activity in 50 μl 50 mM Tris–HCl, 50 mM sodium-2-glycerophosphate pH 8·6 or pH 6·8, 2·5 μM cyclic-AMP-dependent protein kinase inhibitor (TTYADFIASGRTGRRNAIHD), 10 mM magnesium acetate, 100 nM okadaic acid, 0·1% (v/v) 2-mercaptoethanol, 0·1 mM [γ-^{32}P]ATP, 0·5 mg/ml (~400 μM) phosphorylase *b* peptide and 0·04 mM $CaCl_2$. Assays were performed for 20 min at 30 °C with constant agitation and were terminated by spotting 40 μl onto Whatman P81 paper, followed by immersion in 75 mM phosphoric acid. Papers were washed for 4× 15 min in 75 mM phosphoric acid, once briefly in water and then acetone. ^{32}P incorporation was determined by Cerenkov counting on a liquid scintillation counter. One unit (U) of kinase activity is defined as that which catalyses the incorporation of 1 μmole of ^{32}P into substrate peptide per minute.

AMPKα1 and α2 activities were assayed by Ser phosphorylation of the peptide substrate AMARAA-SAAALARRR following isoform-specific immunoadsorption (Sakamoto *et al.* 2005). Glycogen was measured using the anthrone reagent after extraction from skeletal muscle with 1 mol/l NaOH at 100 °C for 60 min (Roe & Dailey 1966).

Statistical analysis

All data are presented as mean ± S.E.M. Statistical significance was tested using Student's *t*-test, except where stated otherwise.

Results

Phenotypic analysis of $G_M^{-/-}$ mice backcrossed more than six times onto C57BL/6 and 129s2/sV backgrounds

The $G_M^{-/-}$ mice that developed obesity, glucose intolerance and insulin resistance in late adult life (Delibegovic *et al.* 2003) and lean, glucose-tolerant, insulin-sensitive $G_M^{-/-}$ mice of Suzuki *et al.* (2001) were created using 129/Ola and 129/SvJ mouse embryonic stem cells respectively, backcrossed to the C57BL/6 strain and examined after two to three backcrosses. Extensive genetic variability among 129 substrains has been reported (Simpson *et al.* 1997). Although129/Ola and 129/SvJ are from the same parental lineage, they were separated by a contamination introduced into the Sv and SvJ lines. However, examination of 25 protein markers did not uncover any variation between 129/Ola-Hsd and 129/SvJ at the protein level (Simpson *et al.* 1997). In order to determine whether the different phenotypes of the $G_M^{-/-}$ mouse models reside in the different genetic backgrounds of the 129 substrains used to create them, the obese, glucose-intolerant, insulin-resistant $G_M^{-/-}$ mice of Delibegovic *et al.* (2003) were further backcrossed onto a C57BL/6 background. After at least six backcrosses, the mice, as expected, had no G_M protein in skeletal muscle detectable by immunoblotting and low PP1β levels (Fig. 1A) as observed previously for $G_M^{-/-}$ mice (Delibegovic *et al.* 2003). However, they showed no significant difference in weights or abdominal fat from $G_M^{+/+}$ mice up to 12 months of age (and hence are termed lean C57BL/6 $G_M^{-/-}$ mice, Fig. 1B) in contrast to the obese $G_M^{-/-}$ mice examined earlier, which showed increased weights of ∼20% at 12 months of age with increased fat deposition. The glucose tolerance and insulin sensitivity in the lean C57BL/6 $G_M^{-/-}$ mice were not statistically different from $G_M^{+/+}$ controls (Fig. 1C and D) in contrast to the obese $G_M^{-/-}$ mice that were glucose intolerant and insulin resistant at 11–12 months of age.

129/Ola mice breed poorly and were not available for backcrossing. We therefore crossed the obese $G_M^{-/-}$ mice onto a related 129s2/sV background. After more than six backcrosses, the 129s2/sV $G_M^{-/-}$ mice showed no increase in body weight or abdominal fat compared with 129s2/sV $G_M^{+/+}$ mice up to 12 months of age (Fig. 1E). Glucose tolerance and insulin sensitivity in mice of more than 12 months of age was similar to that of controls (Fig. 1F and G).

No significant differences in fasting blood glucose levels were observed in lean C57BL/6 $G_M^{-/-}$ mice compared with C57/BL/6 $G_M^{+/+}$ mice (Table 1), obese $G_M^{-/-}$ mice compared with control $G_M^{+/+}$ littermates (Delibegovic *et al.* 2003) or 129s2/sV $G_M^{-/-}$ mice compared with 129s2/sV $G_M^{+/+}$ controls (data not shown). Fasting plasma triglycerides of lean C57Bl/6 $G_M^{-/-}$ mice were also similar to C57BL/6 controls (Table 1), but the triglyceride levels of obese $G_M^{-/-}$ mice showed a 1·6-fold increase over C57BL/6 control levels (C57BL/6 $G_M^{+/+}$ 42·8±3·6 mg/dl versus obese $G_M^{-/-}$ 68·9±12·5 mg/dl; $n=4$) at 2–5 months of age, suggesting that triglyceride increase may contribute to the development of obesity in these mice during the early stages. Serum-free fatty acids and serum insulin levels of lean C57BL/6 $G_M^{-/-}$ mice showed a tendency towards lower and higher levels respectively than controls, but the differences were not statistically significant.

Analysis of the effects of environmental factors on lean C57BL/6 $G_M^{-/-}$ mice

Environmental factors such as the composition of the diet and total food intake could also underlie the differences between the two models of $G_M^{-/-}$ mice. However, the food intake of lean C57BL/6 $G_M^{-/-}$ mice was similar to that of C57BL/6 $G_M^{+/+}$ controls (data not shown) and previous studies showed that the food intake of obese $G_M^{-/-}$ mice was similar to the food intake of control mice (Delibegovic *et al.* 2003). Since the $G_M^{-/-}$ mice are defective in the conversion of glucose to glycogen in skeletal muscle, we examined the effects of increasing glucose levels in the diets. After addition of 30% glucose to the diet of lean C57BL/6 $G_M^{-/-}$ and control C57BL/6 $G_M^{+/+}$ mice from weaning to 6 months of age followed by a diet of standard chow, glucose tolerance of the $G_M^{-/-}$ mice was not significantly different from that of the controls at 8 (Fig. 2A) and 12 months of age (Fig. 2B). Insulin sensitivity in the C57BL/6 $G_M^{-/-}$ and control mice was similar at 12 months of age (data not shown). A high-protein, low-carbohydrate diet also did not modulate glucose tolerance or insulin resistance (data not shown).

Biochemical analyses of lean C57BL/6 $G_M^{-/-}$ mice

Genetically determined biochemical variation may account for the different phenotypes of the $G_M^{-/-}$ mice and therefore biochemical analyses were performed on lean C57BL/6 $G_M^{-/-}$ mice. Glycogen levels in skeletal muscle of lean C57BL/6 $G_M^{-/-}$ mice were low (∼30% of controls; Fig. 3A), although not as low as those observed in the obese $G_M^{-/-}$ mice (∼10% of controls; Delibegovic *et al.* 2003). The activity ratios of the PP1-G_M substrates, phosphorylase (−/+AMP) and GS (−/+G6P) were increased and decreased respectively compared with controls, similarly to ratios observed for the obese $G_M^{-/-}$ mice (Delibegovic *et al.* 2003, Toole & Cohen 2007). Phosphorylase kinase (PhK), an *in vitro* substrate of PP1-G_M, was investigated by an immunoadsorption assay. The specificity of this new assay was demonstrated

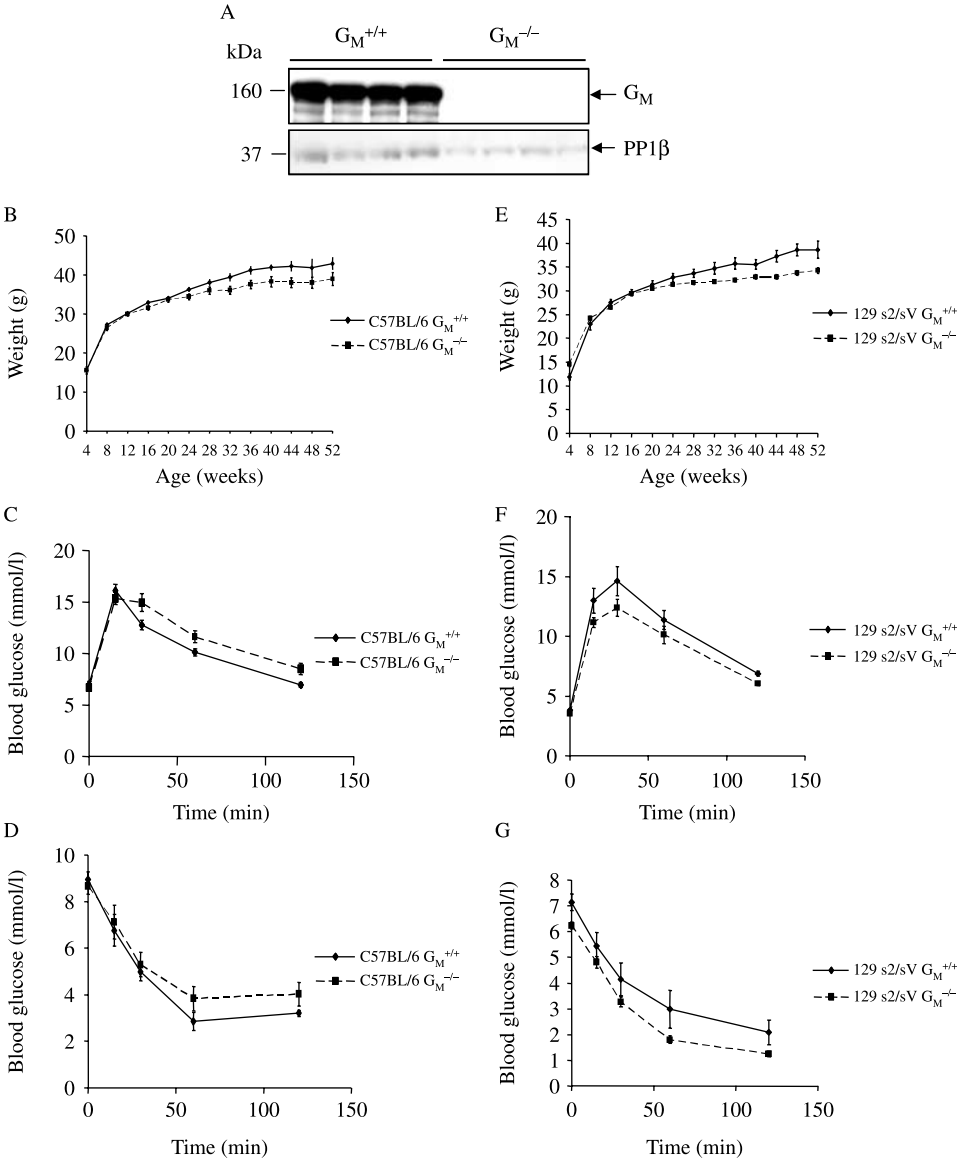

Figure 1 (A) Immunoblot showing G_M protein levels and PP1β levels in skeletal muscle from fasted C57BL/6 $G_M^{+/+}$ and C57BL/6 $G_M^{-/-}$ mice. G_M and PP1β were visualised using anti-G_M and anti-PP1β antibodies. G_M (121 kDa) migrates at ~160 kDa on SDS polyacrylamide gels. (B) Weights of male $G_M^{+/+}$ and $G_M^{-/-}$ mice backcrossed at least six times onto a background of C57BL/6. Results are the mean ± S.E.M. for 12 $G_M^{+/+}$ and 8 $G_M^{-/-}$ mice at each time point. $G_M^{-/-}$ mice do not have increased weight gain compared with $G_M^{+/+}$ mice. (C) Glucose tolerance tests performed on male mice backcrossed at least six times and aged more than 12 months. Results are mean ± S.E.M. for 8 $G_M^{+/+}$ and 11 $G_M^{-/-}$ animals. The blood glucose values for $G_M^{+/+}$ and $G_M^{-/-}$ mice at 0, 15, 60 and 120 min were not significantly different when assessed using Student's *t*-test. (D) Insulin tolerance tests performed on male mice backcrossed at least six times and aged more than 12 months. Results are the mean ± S.E.M. for 7 $G_M^{+/+}$ and 6 $G_M^{-/-}$ animals. The blood glucose values for $G_M^{+/+}$ and $G_M^{-/-}$ mice are not significantly different at any time point. (E) Weights of male $G_M^{+/+}$ and $G_M^{-/-}$ animals backcrossed at least six times onto a background of 129s2/sV. Results are the mean ± S.E.M. for 7 $G_M^{+/+}$ and 16 $G_M^{-/-}$ mice at each time point. $G_M^{-/-}$ mice do not have increased weight gain compared with $G_M^{+/+}$ mice. (F) Glucose tolerance tests performed on male mice backcrossed at least six times onto 129s2/sV and aged more than 12 months. Results are mean ± S.E.M. for 6 $G_M^{+/+}$ and 15 $G_M^{-/-}$ mice. The blood glucose values for $G_M^{+/+}$ and $G_M^{-/-}$ mice at 0, 60 and 120 min are not significantly different. (G) Insulin tolerance tests performed on male mice backcrossed at least six times onto 129s2/sV and aged more than 12 months. Results are mean ± S.E.M. for 6 $G_M^{+/+}$ and 15 $G_M^{-/-}$ mice of each genotype. $G_M^{-/-}$ mice do not have significantly decreased insulin sensitivity.

Table 1 Levels of blood components in C57BL/6 $G_M^{+/+}$ and lean C57BL/6 $G_M^{-/-}$

	C57BL/6 $G_M^{+/+}$	**C57BL/6** $G_M^{-/-}$
Blood component		
Fasting blood glucose (mmol/l)	6·68±0·353 (9)	7·25±0·354 (6)
Fasting serum triglycerides (mg/dl)	67·4±9·4 (7)	68·5±12·0 (5)
Fasting serum NEFA (mmol/l)	1·24±0·4 (7)	0·85±0·09 (5)
Fasting serum insulin (ng/μl)	0·583±0·228 (8)	0·807±0·176 (7)

Blood samples were obtained from the tail veins of mice aged 6–8 months. The values for $G_M^{-/-}$ versus $G_M^{+/+}$ mice were not significantly different using Student's *t*-test for any component. The numbers of mice tested are given in brackets. NEFA denotes non-esterified fatty acids. The fasting serum insulin levels were also not significantly different when assessed using the Mann–Whitney–Wilcoxon *U* test.

by showing that no activity was present in the skeletal muscle of I/ICR PhK-deficient mice (Cohen & Cohen 1973; Fig. 3B, left panel). Increased PhK activity at pH 6·8 in the skeletal muscle lysates of anaesthetised propranolol-treated lean C57BL/6 $G_M^{-/-}$ mice was observed compared with controls (Fig. 3B, right panel). The use of propranolol prevented inadvertent activation of PhK in response to adrenalin, because the latter may take up to 90 min to decline to zero (Toole & Cohen 2007). In contrast to the pH 6·8 activity, the PhK activity at pH 8·6, which is a measure of the total activity, was similar in lean C57BL/6 $G_M^{-/-}$ and control mice. Activation of PhK at pH 6·8 has been linked with phosphorylation of the enzyme (Krebs 1972) and correlated with phosphorylation of a specific serine in a peptide of the β-subunit of PhK (Stewart *et al.* 1981), identified as Ser26 (Kilimann *et al.* 1988). Our data therefore imply that the β-subunit is hyper-phosphorylated on Ser26 and are consistent with PhK being an *in vivo* substrate of PP1-G_M. The pH 6·8/8·6 activity was 0·53 and 0·19 for lean C57BL/6 $G_M^{-/-}$ and control mice respectively.

The insulin-sensitive kinase cascade leading to the activation of GS was functional in the skeletal muscle of lean C57BL/6 $G_M^{-/-}$ mice (Fig. 3C) similarly to obese $G_M^{-/-}$ mice (Delibegovic *et al.* 2003). PKB is activated in response to insulin by phosphorylation on Thr308, which in turn inhibits GSK3α and GSK3β by phosphorylation of Ser21 and Ser 9 respectively. PKB, GSK3α and GSK3β were phosphorylated similarly to controls in the skeletal muscle of lean C57BL/6 $G_M^{-/-}$ mice in response to insulin (Fig. 3C); GSK3α and GSK3β also responded similarly to insulin in the skeletal muscle of obese $G_M^{-/-}$ and control mice (Delibegovic *et al.* 2003).

Since energy derived from glycogen is decreased in $G_M^{-/-}$ mice, we analysed AMPK, which is a sensor of cellular energy (Hardie *et al.* 2006). The activity of the AMPKα1 isoform was similar in the skeletal muscle of C57BL/6 $G_M^{+/+}$, lean C57BL/6 $G_M^{-/-}$ and obese $G_M^{-/-}$ mice, but the activity of the AMPKα2 isoform was more than threefold higher in lean C57BL/6 $G_M^{-/-}$ mice than in controls (Fig. 4A) and twofold higher in obese $G_M^{-/-}$ mice than in controls (data not shown). Consistent with the increase in AMPKα2 activity, phosphorylation of Thr172 in AMPKα2 was increased, while no increase in phosphorylation of AMPKα1 was observed (Fig. 4B). The level of total AMPK (both α1 + α2) was similar in all lines as judged by immunoblotting. A downstream target of AMPK is acetyl-CoA carboxylase (ACC) that can be activated by phosphorylation. The levels of ACC in mouse skeletal muscle were extremely low as judged by immunoblotting, but there was no clear increase in phosphorylation of ACC Ser212 in the total hind limb muscle of lean C57BL/6 $G_M^{-/-}$ mice compared with controls (data not shown) or in the gastrocnemius muscle, which had slightly higher levels of ACC (Fig. 4C).

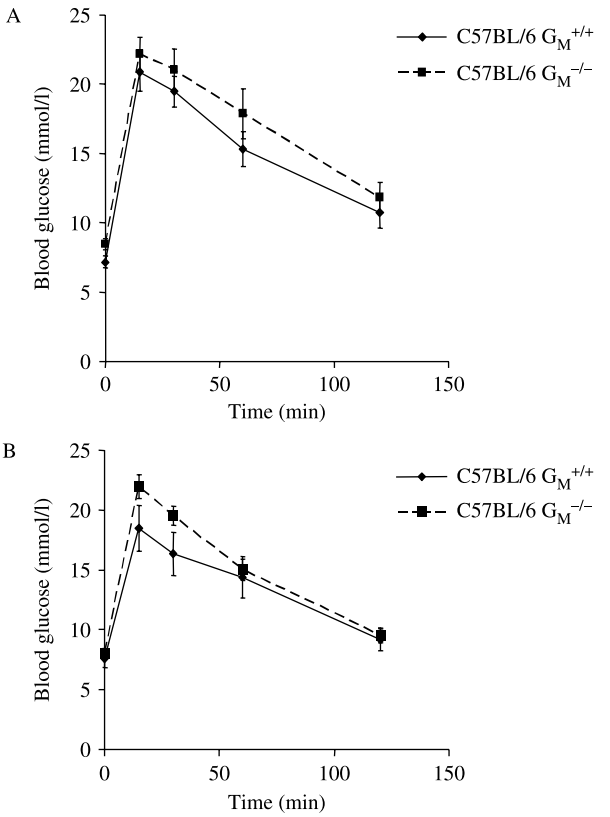

Figure 2 Glucose tolerance tests performed on male C57BL/6 mice born after at least six backcrosses and fed a diet containing 30% extra glucose from weaning to 6 months, then a standard chow diet for either 2 months (A) or 6 months (B). Results are mean±S.E.M. for 11 $G_M^{+/+}$ and 17 $G_M^{-/-}$ animals. The blood glucose values for $G_M^{+/+}$ and $G_M^{-/-}$ mice are not significantly different at any time point in (A) or (B).

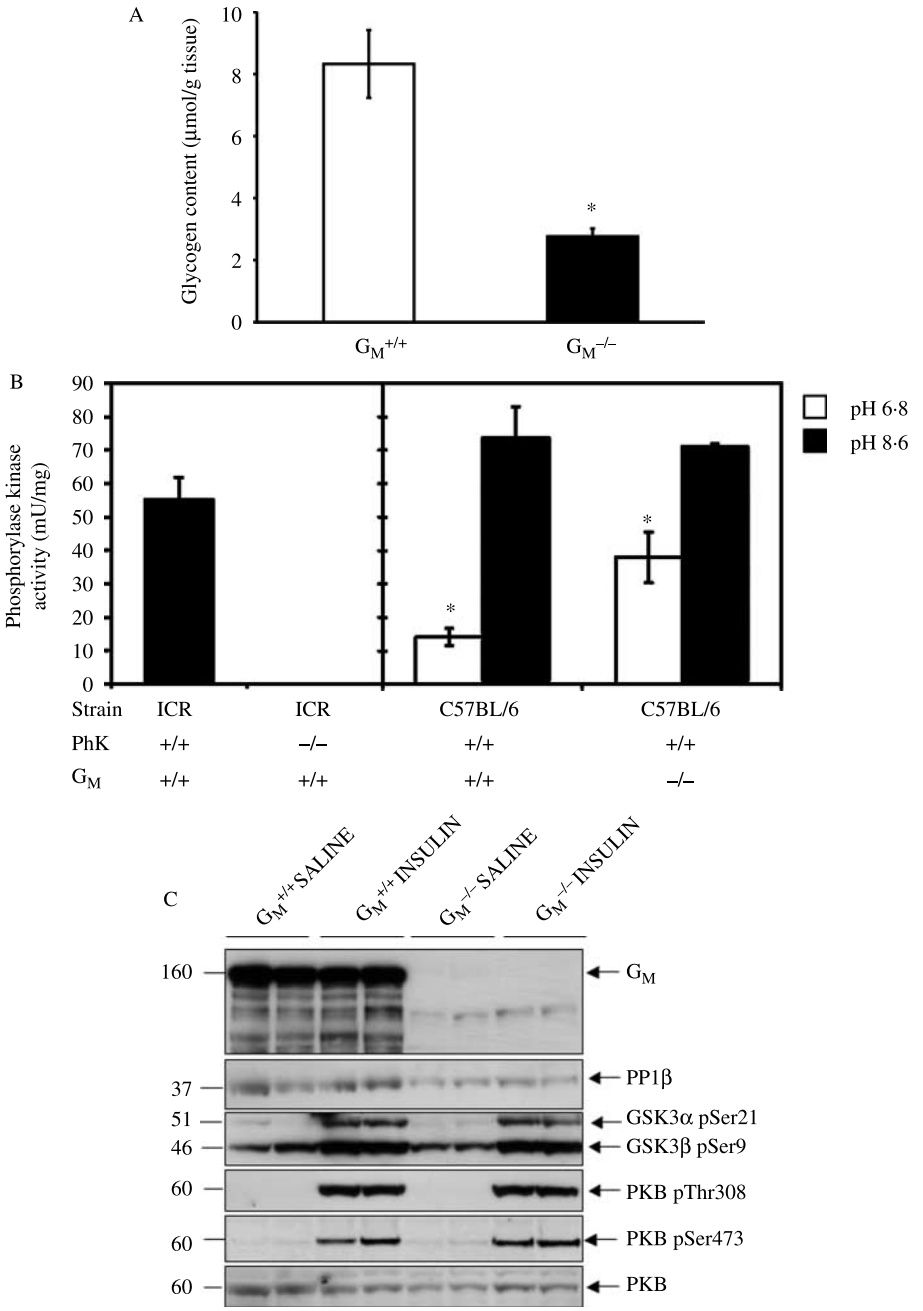

Figure 3 (A) Glycogen content in skeletal muscle of C57BL/6 $G_M^{+/+}$ and lean C57BL/6 $G_M^{-/-}$ mice fasted overnight. Glycogen concentration is expressed in micromoles of glycosyl units per gram of muscle (wet weight). Results are expressed as the mean ± S.E.M. for 6 $G_M^{+/+}$ and 11 $G_M^{-/-}$ animals and the difference between $G_M^{-/-}$ versus $G_M^{+/+}$ is statistically significant. *$P<0{\cdot}001$. (B) Left panel: Phosphorylase kinase activity at pH 8·6 (in the presence of 40 μM Ca^{2+}) in skeletal muscle lysates from wild-type ICR and PhK-deficient ICR/l mice. Results are expressed as mean ± S.E.M. for three $PhK^{+/+}$ and three $PhK^{-/-}$ mice. Right panel: PhK activity in skeletal muscle lysates from C57BL/6 $G_M^{+/+}$ and lean C57BL/6 $G_M^{-/-}$ mice. Assays were performed in the presence of 40 μM Ca^{2-} at pH 6·8 (near physiological pH, open bars) and pH 8·6 (solid bars). Results are expressed as mean ± S.E.M. for three to five animals of each genotype. *The difference in pH 6·8 PhK activities of C57BL/6 $G_M^{+/+}$ and lean C57BL/6 $G_M^{-/-}$ mice was statistically significant, $P<0{\cdot}05$. All assays were performed in triplicate. Control values (1–2 mU/mg) using preimmune IgG in place of anti-PhK antibody were subtracted from the calculated activities. (C) Proteins in the PKB insulin signalling pathway in C57BL/6 $G_M^{+/+}$ and lean C57BL/6 $G_M^{-/-}$ skeletal muscle lysates. Mice were fasted overnight before injection of a bolus of either saline or insulin. Immunoblotting was performed using the indicated antibodies.

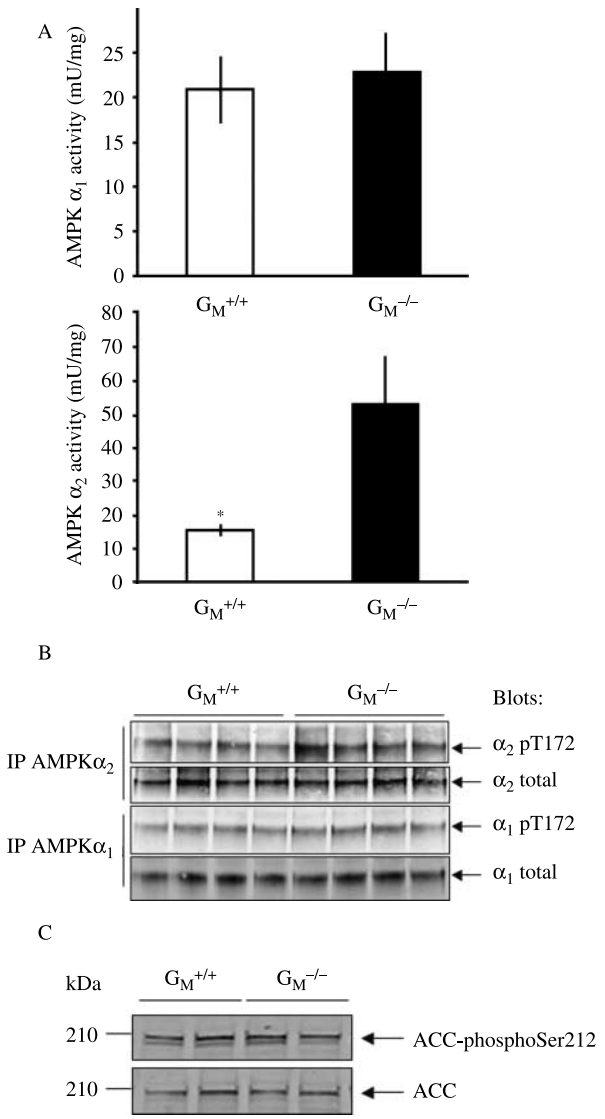

Figure 4 (A) Activities of AMPKα1 and AMPKα2 isoforms in skeletal muscle lysates from five C57BL/6 $G_M^{+/+}$ and seven lean C57BL/6 $G_M^{-/-}$ animals. Activities of AMPKα1 are not significantly different. For AMPKα2 activities, statistical significance (*) for $G_M^{-/-}$ versus $G_M^{+/+}$ was $P<0{\cdot}05$. (B) AMPKα1 or α2 was immunoprecipitated from lysates of $G_M^{+/+}$ or $G_M^{-/-}$ mice and immunoblotted with antibodies against the total proteins and phospho-Thr172. (C) Equal amounts of protein (70 μg) from lysates of the gastrocnemius muscle of C57BL/6 $G_M^{+/+}$ and lean C57BL/6 $G_M^{-/-}$ mice were immunoblotted with the indicated antibodies. ACC phosphorylation was assessed relative to the level of total ACC by quantitative Li-Cor analysis. The ratio ACC phospho-212/total ACC was 1·6 for lean C57BL/6 $G_M^{-/-}$ mice and 1·6 for the control mice.

The uptake of glucose into skeletal muscle *in vivo* was decreased in obese $G_M^{-/-}$ mice compared with controls (Delibegovic *et al.* 2003) but found to be slightly increased in lean C57BL/6 $G_M^{-/-}$ mice compared with controls (Fig. 5). Since AMPK may modulate glucose transport through the insulin-stimulated glucose transporter GLUT4, we analysed GLUT4 and hexokinase II (HKII), which catalyses conversion of the intracellular glucose to glucose-6-phosphate in skeletal muscle. No change in the level of HKII was observed in lean C57BL/6 $G_M^{-/-}$ compared with C57BL/6 $G_M^{+/+}$ mice (Fig. 6A). Of three commercial anti-GLUT4 antibodies, no two antibodies recognised the same 40–50 kDa band in mouse skeletal muscle lysates (prepared in the presence of the detergent Triton X-100 to solubilise GLUT4). Membrane fractions, which would be expected to contain GLUT4, were then prepared and GLUT4 was again released using Triton X-100. Following immunoadsorption of GLUT4 from the lysates (Fig. 6B) or membrane fractions (Fig. 6C) with an antibody raised to the 15 C-terminal amino acids of GLUT4 (Abcam), a 46 kDa band could be recognised by the same antibody and also by a monoclonal antibody 1F8 raised against partially purified GLUT4-containing vesicles (Biogenesis; Fig. 6C). The precise 1F8 antibody epitope is not known but it lies in the cytoplasmic portion of GLUT4 (James *et al.* 1988, Imamura *et al.* 2001). The 46 kDa protein was present in the membrane fraction and not in the cytosolic fraction as might be expected for GLUT4. Although immunoblotting of the immunopellets resulted in some variation between samples, there was no clear difference in the levels of GLUT4 in the membrane fractions from whole hind limb muscles, the quadriceps muscle or the gastrocnemius muscle of lean C57BL/6 $G_M^{-/-}$ mice compared with $G_M^{+/+}$ controls (Fig. 6C).

Discussion

Our studies show that continued backcrossing of the obese, glucose-intolerant, insulin-resistant $G_M^{-/-}$ mice onto two different genetic backgrounds gave rise to lean, glucose-tolerant, insulin-sensitive $G_M^{-/-}$ mice. The loss of the prediabetic phenotype suggests that at least one further variant gene in the 129/Ola background, not present in the C57BL/6 or 129s2/sV background, may be required for the development of the prediabetic $G_M^{-/-}$ phenotype. This situation is similar to the interaction of two variant genes underlying severe insulin resistance in the human population (Savage *et al.* 2002). Environmental changes, including a high-fat diet (Suzuki *et al.* 2001) and high-carbohydrate diet (this study) fed to lean $G_M^{-/-}$ mice, were insufficient to lead to glucose intolerance and insulin resistance, despite the severely decreased ability of lean $G_M^{-/-}$ mice to convert glucose to glycogen in skeletal muscle. However, interestingly, mice completely devoid of glycogen in skeletal muscle due to disruption of the muscle GS gene exhibit either normal or improved glucose tolerance (Pederson *et al.* 2005*a*). It has been suggested that the reason may be because, in contrast to

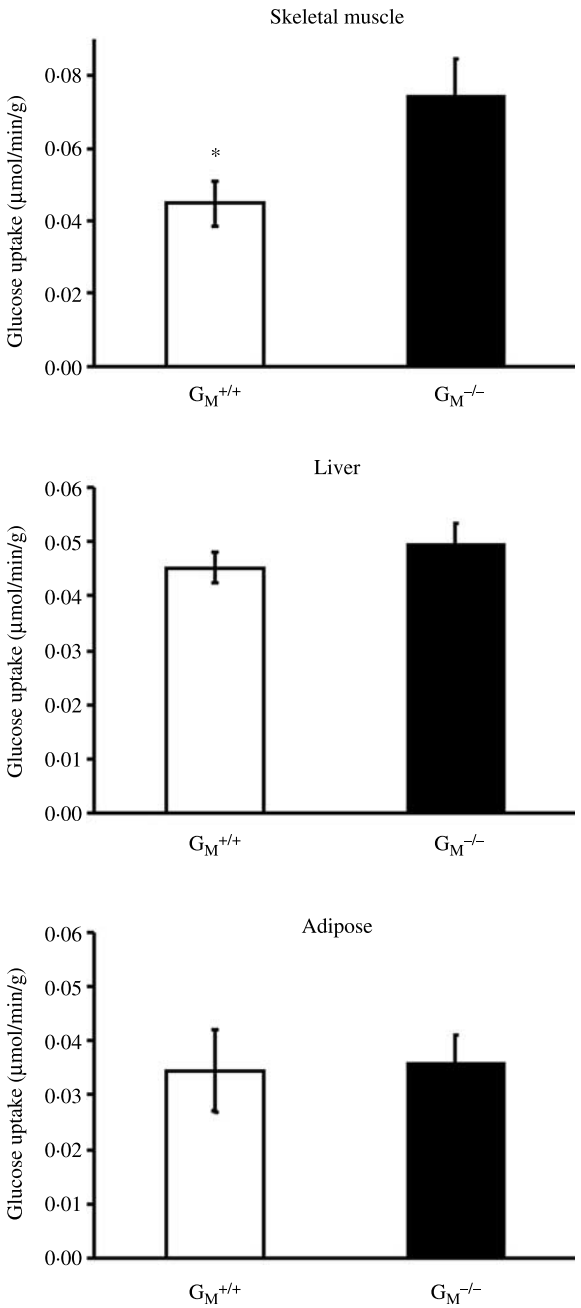

Figure 5 Glucose uptake into the skeletal muscle, liver and adipose tissue of $G_M^{+/+}$ and $G_M^{-/-}$ mice during a glucose tolerance test. A mixture of 2-deoxy-D-[1,2-^{3}H]-glucose tracer and unlabelled glucose was injected into six $G_M^{+/+}$ and eight $G_M^{-/-}$ mice. Data represent the glucose uptake in μmol/min per g tissue and are mean values ± S.E.M. Statistical significance (*) for the $G_M^{-/-}$ versus $G_M^{+/+}$ skeletal muscle glucose uptake was $P<0{\cdot}05$.

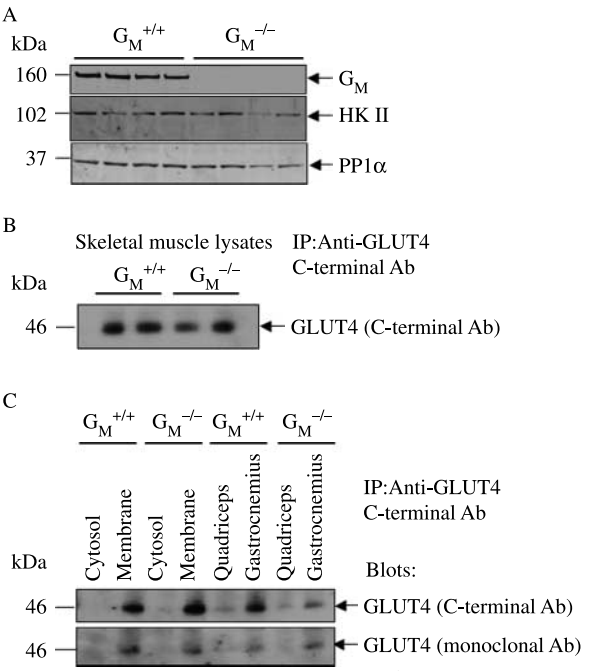

Figure 6 (A) Immunoblots of C57BL/6 $G_M^{+/+}$ and lean C57BL/6 $G_M^{-/-}$ hind limb skeletal muscle lysates probed with antibodies against G_M and hexokinase II (HKII). (B and C) Covalently coupled anti-GLUT4 C-terminal antibody coupled to protein G-Sepharose beads was incubated with hind limb skeletal muscle lysates from C57BL/6 $G_M^{+/+}$ and lean C57BL/6 $G_M^{-/-}$ mice. After centrifugation, the immunopellets were subjected to electrophoresis and examined by immunoblotting with the same anti-GLUT4 antibody. (C) Immunopellets from purified membrane fractions from the whole hind limbs, the quadriceps and gastrocnemius of C57BL/6 $G_M^{+/+}$ and lean C57BL/6 $G_M^{-/-}$ mice were examined similarly (upper blot) and also immunoblotted with a monoclonal anti-GLUT4 antibody (lower blot).

humans who rely on skeletal muscle glycogen for muscle contraction, rodents may be more dependent on liver glycogen stores (Baldwin *et al.* 1973, Pederson *et al.* 2005*b*).

An alternative or additional explanation to the requirement of further variant gene(s) in $G_M^{-/-}$ mice for the development of insulin resistance is that further backcrossing may lead to compensatory changes that ameliorate the prediabetic phenotype. A comparison of the C57BL/6 $G_M^{-/-}$ mice analysed in this manuscript compared with those in previous studies is presented in Table 2. The glycogen level in the skeletal muscle of the lean C57BL/6 $G_M^{-/-}$ mice was higher (30% of controls) than in the obese $G_M^{-/-}$ mice (10% of controls), but this is unlikely to account for the difference between the lean and obese models because the $G_M^{-/-}$ mice of Suzuki *et al.* were lean and had glycogen levels (10% of controls) similar to the obese $G_M^{-/-}$ mice. Other explanations for the obesity include less energy expenditure or a lower metabolic rate. Biochemical analyses revealed that in the skeletal muscle of lean C57BL/6 $G_M^{-/-}$ mice studied in this article the activity of AMPKα2 is increased approximately threefold compared with controls, while the AMPKα1 activities are similar. The low glycogen levels resulting from the

Table 2 Comparison of the lean C57BL/6 $G_M^{-/-}$ mouse model studied in this article with those of Suzuki *et al.* (2001) and Delibegovic *et al.* (2003)

	Comparison of the G_M null mouse models		
Parameter	Paterson *et al.* (this study) (Lean C57BL/6 $G_M^{-/-}$)	Delibegovic *et al.* (2003) (Obese $G_M^{-/-}$)	Suzuki *et al.* (2001) ($G_M^{-/-}$)
Donor strain	129/Ola	129/Ola	129/SvJ
Genetic background	C57BL/6 (6–10 backcrosses)	C57BL/6 (2–3 backcrosses)	C57BL/6 (2–3 backcrosses)
G_M protein in sk. muscle	None	None	None
PP1-G_M activity	≤5% of control[a]	≤5% of control	ND
PP1c (total PP1 activity)	∼50% of control[a]	∼50% of control	∼40% of control
PP1β level	Decreased	Decreased	ND
Sk. muscle glycogen	30% of control	10% of control	10% of control
Phosphorylase total activity	Normal[a]	∼50% of control	Normal
Basal Phosphorylase (−/+AMP) activity ratio	Increased[a]	Increased	Increased
Phosphorylase pSer14	Hyperphosphorylated[a]	ND	ND
Basal PhK (pH 6·8/pH 8·6) activity ratio	Increased	ND	ND
GS total activity	Normal[a]	∼50% of control	∼70% of control
Basal GS (−/+G6P) activity ratio	Decreased[a]	Decreased	Decreased
Insulin-stimulated GS (−/+G6P) activity ratio	Decreased[a]	Decreased	No significant change
GS stimulation by insulin	∼2-fold[a]	∼2-fold	∼2-fold
GS pSer640, pSer644	Hyperphosphorylated[a]	ND	ND[b]
GSK3α pSer21, β pSer9 stimulation by insulin	Normal	Normal	ND
PKB pThr308, pSer473 stimulation by insulin	Normal	ND	ND
AMPKα1 activity	Normal	Normal	ND
AMPKα2 activity	∼3-fold increase	∼2-fold increase	ND
GLUT4 level	Normal	ND	ND
HK level	Normal	ND	ND
PP1-R5/PTG	Activity normal[a]	Activity decreased	R5/PTG level normal
Weight gain in mice over 3 months old	Normal	Increased	Normal
Abdominal fat in mice at ∼12 months old	Normal	Increased	Normal
Fasting blood plasma glucose	Normal	Normal	Normal
Glucose tolerance at up to ∼8 months old	Normal	Normal	Normal
Glucose tolerance at ∼11 months old	Normal	Impaired	Normal
Insulin sensitivity at up to ∼8 months old	Normal	Normal	Normal
Insulin sensitivity at ∼12 months old	Normal	Insulin resistant	Normal
Glucose transport (sk. muscle) at ∼12 months old	Slightly increased (*in vivo*)	∼30% of control (*in vivo*)	Normal (*in vitro*, no age stated)

Data for each model are compared with $G_M^{+/+}$ littermates and classified as normal if no significant differences were detected. Enzyme activities and protein levels are assayed in skeletal muscle.
[a]Toole & Cohen (2007).
[b]Hyperphosphorylation reported but sites not identified;
ND, not determined.

disruption of the G_M gene may be expected to lead to decreased ATP production from glycogen and consequent elevation of AMP, leading to the activation of AMPK, which is a sensor of cellular energy levels and has also been proposed to be a sensor of glycogen stores (Hardie & Sakamoto 2006). One of the downstream actions of AMPK is to phosphorylate and inhibit ACC 2 in skeletal muscle, decreasing the production of malonyl-CoA, an allosteric inhibitor of carnitine palmitoyltransferase 1. Since the latter enzyme is rate limiting for the entry of long-chain fatty acyl-CoA into the mitochondria for oxidation, relief from inhibition leads to an increase in the oxidation of fatty acids (Saha & Ruderman 2003, Kahn *et al.* 2005). Although this mechanism is attractive for allowing fatty acids to be used as an alternative energy source to glycogen in $G_M^{-/-}$ skeletal muscle, we found no evidence for increased phosphorylation of ACC in the lean C57BL/6 $G_M^{-/-}$ mice, which have an approximately threefold increase in AMPKα2 activity compared with controls. Activation of AMPK by approximately tenfold in skeletal muscle of muscle GS knockout (MGSKO) mice compared with controls elicited only an ~1·5-fold increase in the phosphorylation state of ACC in MGSKO mice compared with controls (Pederson *et al.* 2005*a*) and therefore it is possible that an approximately threefold activation of AMPK is insufficient to cause detectable phosphorylation of ACC.

AMPK also increases the expression of the insulin-stimulated glucose transporter GLUT4, which is highly regulated by a number of factors (Zorzano *et al.* 2005). Nevertheless, following immunoadsorption and centrifugation, immunoblotting of the pellets yielded no clear differences in level of GLUT4 in lysates or membrane fractions of skeletal muscle from fasted lean C57BL/6 $G_M^{-/-}$ mice compared with $G_M^{+/+}$ controls. The approximately threefold rise in AMPK activity therefore also appears to be insufficient to elicit a detectable change in the expression level of GLUT4 in the basal state.

Normally, in the basal state, GLUT4 is excluded from the plasma membrane and resides in the endosomal system and specialised intracellular vesicles termed GLUT4 storage vesicles (Shepherd & Kahn 1999, Welsh *et al.* 2005). The addition of insulin results in translocation of GLUT4 to the plasma membrane and 10- to 20-fold increases in GLUT4 levels at the plasma membrane (sarcolemma and T-tubule) are observed after insulin stimulation. Alterations in GLUT4 localisation that enhance glucose uptake are observed not only in response to insulin but also during exercise and muscle contraction, although the mechanisms and pathways are not fully defined (Jessen & Goodyear 2005). AMPK has been implicated in the translocation of GLUT4 in response to exercise and contraction (Merrill *et al.* 1997, Hayashi *et al.* 1998, Mu *et al.* 2001, Jessen & Goodyear 2005, Sakamoto *et al.* 2005). Although we cannot completely eliminate an altered GLUT4 localisation, an initial evaluation of the GLUT4 molecules at the sarcolemma by electron microscopy (kindly performed by Dr John Lucocq and John James) gave no evidence for increased localisation of GLUT4 in this region in starved lean C57BL/6 $G_M^{-/-}$ mice compared with $G_M^{+/+}$ control mice. Furthermore, augmented expression and/or altered localisation of GLUT4 by AMPK are unlikely to underlie the differences between lean C57BL/6 $G_M^{-/-}$ mice and obese $G_M^{-/-}$ mice because AMPK is activated in both lines, although to a slightly lower extent (approximately twofold) in obese $G_M^{-/-}$ mice compared with the approximately threefold in lean C57BL/6 $G_M^{+/+}$ mice.

Insulin stimulation of GLUT4 translocation to the membrane is believed to involve the phosphoinositide 3-kinase (PI3K) pathway (Shepherd & Kahn 1999, Watson *et al.* 2004). The production of PtdIns(3,4,5)P3 in the plasma membrane by PI3K leads to the activation of PKB in muscle cells (Ueki *et al.* 1998, Wang *et al.* 1999) and also stimulates atypical PKC isoforms (PKCλ and PKCζ) in adipocytes (Farese 2002). Both of these pathways have been implicated in GLUT4 translocation (Watson *et al.* 2004, Ishiki & Klip 2005). However, PKB does not appear to be phosphorylated in the basal state in lean $G_M^{-/-}$ mice or control mice and in response to insulin it is similarly phosphorylated in $G_M^{-/-}$ and control mice. Insulin levels are not significantly raised in lean $G_M^{-/-}$ mice. Thus, there is no evidence for increased insulin signalling and alterations in the flux of the PI3K pathway in lean C57Bl/6 $G_M^{-/-}$ mice compared with controls.

Interestingly, mice with a muscle-specific deletion of GLUT4 are glucose intolerant, insulin resistant and mildly diabetic (Zisman *et al.* 2000). Recent studies have shown that, rather surprisingly, glycogen levels are raised by 31–83% in different skeletal muscles of these mice in the fasted state, despite a 75% decrease in glucose transport (Kim *et al.* 2005). The underlying mechanism resides in part in a twofold increase in HKII, which leads to an increase in glucose-6-phosphate, an allosteric activator of GS. In addition, the levels of the glycogen-targeting subunit R_{GL}/G_M and the less abundant glycogen-targeting subunit R5/protein targeting to glycogen (PTG) (Doherty *et al.* 1996, Printen *et al.* 1997) are increased 3·2- to 4·2-fold and PP1 activity is elevated. This increase in glycogen-targeted PP1 decreases phosphorylase activity and further enhances GS activity, leading to a rise in glycogen levels (Kim *et al.* 2005). However, in lean C57BL/6 $G_M^{-/-}$ mice studied in this article, there was no change in the HK levels compared with $G_M^{+/+}$ controls (Fig. 6) nor was the R5/PTG activity increased (Toole & Cohen 2007).

Compensatory changes involving the upregulation of enzymes may often occur in mutant mice during

embryonic development in successive generations. However, we found no evidence for augmented expression of AMPK, ACC, GLUT4, HKII, PKB and R5/PTG in lean C57BL/6 $G_M^{-/-}$ mice compared with $G_M^{+/+}$ controls, although such alterations might be expected to ameliorate the phenotype of the lean C57BL/6 $G_M^{-/-}$ mice. Overall, the elevation of AMPKα2 activity in lean C57BL/6 $G_M^{-/-}$ mice is rather small and appears to be insufficient to appreciably alter the downstream targets of AMPK.

The differences between lean C57BL/6 $G_M^{-/-}$ mice and obese $G_M^{-/-}$ mice do not appear to reside in the differences in AMPK activity, which are marginal. A more likely explanation, as discussed above, is the presence of one or more genes in the 129/Ola background interacting with the $G_M^{-/-}$ alleles. Although we cannot exclude an additional alteration closely linked to the G_M gene, such a mutation would have to be very closely linked to the original G_M disruption, but probably not in the adjacent genes since it was eliminated by six to ten backcrosses. In addition, the mutation would have to give rise to glucose intolerance, insulin resistance and obesity, since $G_M^{+/+}$ littermates of the obese $G_M^{-/-}$ mice did not develop the prediabetic phenotype. Genetic interaction of G_M with a gene in the 129/Ola background would therefore appear more likely. Elevated triglycerides were observed in the blood plasma of 2–5 month obese $G_M^{-/-}$ mice suggesting that a plausible mechanism underlying the development of glucose intolerance in these mice is that the higher lipid levels may lead to obesity and substantially decrease the transport of glucose into the skeletal muscle. Decreased glucose transport may underlie the insulin resistance phenotype, but it should be noted that obesity and insulin resistance are not always linked (Uysal *et al.* 2000). The influence of the genetic background on the phenotype of gene knockout models has been noted in other instances. For example, depending on the genetic background, insulin receptor substrate 2 knockout mice may die due to a combination of impaired insulin action and insulin deficiency on a C57BL/6×129/Sv background (Withers *et al.* 1998) or develop diabetes with no insulin deficiency on a C57BL/6×CBA background (Kubota *et al.* 2000).

In vitro analyses predicted that phosphorylase, PhK and GS are substrates of PP1-G_M. Biochemical analyses of $G_M^{-/-}$ mice have shown that in the fasted state phosphorylase is hyperphosphorylated on Ser14 with increase of activity, while GS is hyperphosphorylated on Ser640 and Ser644 with decrease in activity compared with controls, confirming that these enzymes are *in vivo* substrates of PP1-G_M (Table 2). From *in vitro* studies, it was observed that PhK is a better substrate for PP1 than it is for PP2A, PP2B/calcineurin or the Mg2+-dependent protein phosphatases (PPM1/PP2C) (Ingebritsen & Cohen 1983) and it can be inactivated by PP1-G_M *in vitro* (Hubbard & Cohen 1989). In addition, both PhK and PP1-G_M are bound to glycogen. The pH 6·8/pH 8·6 activity ratios presented here indicate that PhK is hyperphosphorylated on its regulatory β-subunit in the basal state, demonstrating for the fist time that PhK is an *in vivo* substrate of PP1-G_M. The only other enzyme that we found to be hyperphosphorylated with alteration of activity was AMPKα2, but there is no *in vitro* data linking PP1-G_M with dephosphorylation of AMPK. The low level of activation and phosphorylation of AMPKα2 in $G_M^{-/-}$ mice also support the concept that this alteration is likely to arise as a secondary event, possibly as a consequence of lower cellular energy in the presence of low levels of glycogen.

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References

Baldwin KM, Reitman JS, Terjung RL, Winder WW & Holloszy JO 1973 Substrate depletion in different types of muscle and in liver during prolonged running. *American Journal of Physiology* **225** 1045–1050.

Cohen P 1983 Phosphorylase kinase from rabbit skeletal muscle. *Methods in Enzymology* **99** 243–250.

Cohen P 1999 The Croonian Lecture 1998. Identification of a protein kinase cascade of major importance in insulin signal transduction. *Philosophical Transactions of the Royal Society of London. Series B* **354** 485–495.

Cohen PTW & Cohen P 1973 Skeletal muscle phosphorylase kinase deficiency. Detection of a protein lacking any activity in ICR/IAn mice. *FEBS Letters* **29** 113–116.

DeFronzo RA 1997 Pathogenesis of type 2 diabetes: metabolic and molecular implications for identifying diabetes. *Diabetes Reviews* **5** 177–269.

Delibegovic M, Armstrong CA, Dobbie L, Watt PW, Smith AJH & Cohen PTW 2003 Disruption of the striated muscle glycogen targeting subunit, PPP1R3A, of protein phosphatase 1 leads to increased weight gain, fat deposition and development of insulin resistance. *Diabetes* **52** 596–604.

Doherty MJ, Young PR & Cohen PTW 1996 Amino acid sequence of a novel protein phosphatase 1 binding protein (R5) which is related to the liver and muscle specific glycogen binding subunits of protein phosphatase 1. *FEBS Letters* **399** 339–343.

Doney ASF, Fischer B, Cecil JE, Cohen PTW, Boyle DI, Leese G, Morris AD & Pamer CNA 2003 Male predonderance in early diagnosed type 2 diabetes is associated with the ARE insertion/deletion polymorphism in the PPP1R3A locus. *BMC Genetics* **4** 11.

Farese RV 2002 Function and dysfunction of aPKC isoforms for glucose transport in insulin-sensitive and insulin-resistant states. *American Journal of Physiology. Endocrinology and Metabolism* **283** E1–E11.

Hansen L & Pedersen O 2005 Genetics of type 2 diabetes mellitus: status and perspectives. *Diabetes, Obesity and Metabolism* **7** 122–135.

Hardie DG & Sakamoto K 2006 AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology* **21** 48–60.

Hardie DG, Hawley SA & Scott JW 2006 AMP-activated protin kinase – development of the energy sensor concept. *Journal of Physiology* **574** 7–15.

Hayashi T, Hirschman MF, Kurth EJ, Winder WW & Goodyear LJ 1998 Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* **47** 1369–1373.

Hubbard MJ & Cohen P 1989 Regulation of protein phosphatase-1G from rabbit skeletal muscle. 2. Catalytic subunit translocation is a mechanism for reversible inhibition of activity toward glycogen-bound substrates. *European Journal of Biochemistry* **186** 711–716.

Imamura T, Huang J, Dalle S, Ugi S, Usui I, Luttrell LM, Miller WE, Lefkowitz RJ & Olefsky JM 2001 β-Arrestin-mediated recruitment of the Src family kinase Yes mediates endothelin-1-stimulated glucose transport. *Journal of Biological Chemistry* **276** 43663–43667.

Ingebritsen TS & Cohen P 1983 Protein phosphatases: properties and role in cellular regulation. *Science* **221** 331–338.

Ishiki M & Klip A 2005 Minireview: recent developments in the regulation of glucose transporter-4 traffic: new signals, locations and partners. *Endocrinology* **146** 5071–5078.

James DE, Brown R, Navarro J & Pilch PF 1988 Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. *Nature* **333** 183–185.

Jessen N & Goodyear LJ 2005 Contraction signalling to glucose transport in skeletal muscle. *Journal of Applied Physiology* **99** 330–337.

Kahn BB, Alquier T, Carling D & Hardie DG 2005 AMP-activated protein kinase: ancient energy guage provides clues to modern understanding of metabolism. *Cell Metabolism* **1** 15–25.

Kilimann MW, Zander NF, Kuhn CC, Crabb JW, Meyer HE & Heilmeyer LM, Jr. 1988 The α and β subunits of phosphorylase kinase are homologous: cDNA cloning and primary structure of the beta subunit. *PNAS* **85** 9381–9385.

Kim Y-B, Peroni OD, Aschenbach WG, Minokoshi Y, Kotani K, Zisman A, Kahn CR, Goodyear LJ & Kahn BB 2005 Muscle-specific deletion of the Glut4 glucose transporter alters multiple regulatory steps in glycogen metabolism. *Molecular and Cellular Biology* **25** 9713–9723.

Krebs EG 1972 In *Current Topics in Cellular Regulation*, vol 5, pp 99–133. Eds BL Horecker & ER Stadtman. New York: Academic Press.

Kubota N, Tobe K, Terauchi Y, Eto K, Yamauchi T, Suzuki R, Tsubamoto Y, Komeda K, Nakano R, Miki H *et al.* 2000 Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory beta-cell hyperplasia. *Diabetes* **49** 1880–1889.

Merrill GF, Kurth EJ, Hardie DG & Winder WW 1997 AICA riboside increases AMP-activated protein kinase, fatty acid oxidation and glucose uptake in rat muscle. *American Journal of Physiology* **273** E1107–E1112.

Mu J, Brozinick JT, Jr, Valladares O, Bucan M & Birnbaum MJ 2001 A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Molecular Cell* **7** 1085–1094.

O'Rahilly S, Barroso I & Wareham NJ 2005 Genetic factors in type 2 diabetes: the end of the beginning? *Science* **307** 370–373.

Pederson BA, Schroeder JM, Parker GE, Smith MW, DePaoli-Roach AA & Roach PJ 2005*a* Glucose metabolism in mice lacking muscle glycogen synthase. *Diabetes* **54** 3466–3473.

Pederson BA, Cope CR, Schroeder JM, Smith MW, Irimia JM, Thurberg BL, DePaoli-Roach AA & Roach PJ 2005*b* Exercise capacity of mice genetically lacking muscle glycogen synthase: in mice, muscle glycogen is not essential for exercise. *Journal of Biological Chemistry* **280** 17260–17265.

Printen JA, Brady MJ & Saltiel AR 1997 PTG, a protein phosphatase 1-binding protein with a role in glycogen metabolism. *Science* **275** 1475–1478.

Roe JH & Dailey RE 1966 Determination of glycogen with the anthrone reagent. *Analytical Biochemistry* **15** 245–250.

Saha AK & Ruderman NB 2003 Malonyl-CoA and AMP-activated protein kinase: an expanding partnership. *Molecular and Cellular Biochemistry* **253** 65–70.

Sakamoto K, McCarthy A, Smith D, Green KA, Hardie DG, Ashworth A & Alessi DR 2005 Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *EMBO Journal* **24** 1810–1820.

Savage DB, Agostini M, Barroso I, Gurnell M, Luan JA, Meirhaeghe A, Harding A, Ihrke G, Ratanayagam O, Soos M *et al.* 2002 Digenic inheritance of severe insulin resistance in a human pedigree. *Nature Genetics* **31** 379–384.

Shepherd PR & Kahn BB 1999 Glucose transporters and insulin action. *New England Journal of Medicine* **341** 248–257.

Shulman GI 2000 Cellular mechanisms of insulin resistance. *Journal of Clinical Investigation* **106** 171–176.

Simpson EM, Linder CC, Sargent EE, Davisson MT, Mobraaten LE & Sharp JJ 1997 Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nature Genetics* **16** 19–27.

Stewart A, Hemmings BA, Cohen P, Goris J & Merlevede W 1981 The ATP-Mg dependent protein phosphatase and protein phosphatase-1 have identical substrate specificities. *European Journal of Biochemistry* **115** 197–205.

Suzuki Y, Lanner C, Kim J-H, Vilardo PG, Zhang H, Yang J, Cooper LD, Steele M, Kennedy A, Bock CB *et al.* 2001 Insulin control of glycogen metabolism in knockout mice lacking the muscle-specific protein phosphatase PP1G/RGL. *Molecular and Cellular Biology* **21** 2683–2694.

Toole BJ & Cohen PTW 2007 The skeletal muscle-specific glycogen-targeted protein phosphatase 1 plays a major role in the regulation of glycogen metabolism by adrenaline *in vivo*. *Cellular Signalling* **19** 1044–1055.

Ueki K, Yamamoto-Honda R, Kaburagi Y, Yamauchi T, Tobe K, Burgering BM, Coffer PJ, Komuro I, Akanuma Y, Yazaki Y *et al.* 1998 Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. *Journal of Biological Chemistry* **273** 5315–5322.

Uysal KT, Scheja L, Wiesbrock SM, Bonner-Weir S & Hotamisligil GS 2000 Improved glucose and lipid metabolism in genetically obese mice lacking aP2. *Endocrinology* **141** 3388–3396.

Wang Q, Somwar R, Bilan PJ, Liu Z, Jin J, Woodgett JR & Klip A 1999 Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Molecular and Cellular Biology* **19** 4008–4018.

Watson RT, Kanzaki M & Pessin JE 2004 Regulated membrane trafficking of the insulin responsive glucose transporter 4 in adipocytes. *Endocrine Reviews* **25** 177–204.

Welsh GI, Hers I, Berwick DC, Dell G, Wherlock M, Birkin R, Leney S & Tavaré JM 2005 Role of protein kinase B in insulin-regulated glucose uptake. *Biochemical Society Transactions* **33** 346–349.

Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI *et al.* 1998 Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* **391** 900–904.

Xia J, Scherer SW, Cohen PTW, Majer M, Xi T, Norman RA, Knowler WC, Bogardus C & Prochazka M 1998 A common variant in PPP1R3 associated with insulin resistance and type-2 diabetes. *Diabetes* **47** 1519–1524.

Zisman A, Peroni OD, Abel ED, Michael MD, Mauvais-Jarvis F, Lowell BB, Wojtaszewski JFP, Hirshman MF, Virkamaki A, Goodyear LJ *et al.* 2000 Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nature Medicine* **6** 924–928.

Zorzano A, Palacin M & Guma A 2005 Mechanisms regulating GLUT4 glucose transporter expression and glucose transport in skeletal muscle. *Acta Physiologica Scandinavica* **183** 43–58.

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