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Satya Dash  
*University of Cambridge*

Hiroyuki Sano  
*Dartmouth College*

Justin J. Rochford  
*University of Cambridge*

Robert K. Semple  
*University of Cambridge*

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# A truncation mutation in *TBC1D4* in a family with acanthosis nigricans and postprandial hyperinsulinemia

Satya Dash<sup>a,1</sup>, Hiroyuki Sano<sup>b,1</sup>, Justin J. Rochford<sup>a</sup>, Robert K. Semple<sup>a</sup>, Giles Yeo<sup>a</sup>, Caroline S. S. Hyden<sup>a</sup>, Maria A. Soos<sup>a</sup>, James Clark<sup>c</sup>, Andrew Rodin<sup>c</sup>, Claudia Langenberg<sup>d</sup>, Celine Druet<sup>e</sup>, Katherine A. Fawcett<sup>f</sup>, Y. C. Loraine Tung<sup>a</sup>, Nicolas J. Wareham<sup>d</sup>, Inês Barroso<sup>f</sup>, Gustav E. Lienhard<sup>b</sup>, Stephen O'Rahilly<sup>a,2</sup>, and David B. Savage<sup>a,2</sup>

<sup>a</sup>Departments of Medicine and Clinical Biochemistry, University of Cambridge, Box 289 Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QQ, United Kingdom; <sup>b</sup>Department of Biochemistry, Dartmouth Medical School, 7200 Vail Building, Hanover, NH 03755-3844; <sup>c</sup>Department of Endocrinology, St. Helier's Hospital, Wrythe Lane, Carshalton, Surrey SM5 1AA, United Kingdom; <sup>d</sup>Medical Research Council Epidemiology Unit, University of Cambridge, Box 289 Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QQ, United Kingdom; <sup>e</sup>Institut National de la Santé et de la Recherche Médicale (C.D.), U690, FR-75019 Paris, France; and <sup>f</sup>The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom

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Tre-2, BUB2, CDC16, 1 domain family member 4 (*TBC1D4*) (*AS160*) is a Rab-GTPase activating protein implicated in insulin-stimulated glucose transporter 4 (*GLUT4*) translocation in adipocytes and myotubes. To determine whether loss-of-function mutations in *TBC1D4* might impair *GLUT4* translocation and cause insulin resistance in humans, we screened the coding regions of this gene in 156 severely insulin-resistant patients. A female presenting at age 11 years with acanthosis nigricans and extreme postprandial hyperinsulinemia was heterozygous for a premature stop mutation (R363X) in *TBC1D4*. After demonstrating reduced expression of wild-type *TBC1D4* protein and expression of the truncated protein in lymphocytes from the proband, we further characterized the biological effects of the truncated protein in 3T3L1 adipocytes. Prematurely truncated *TBC1D4* protein tended to increase basal cell membrane *GLUT4* levels ( $P = 0.053$ ) and significantly reduced insulin-stimulated *GLUT4* cell membrane translocation ( $P < 0.05$ ). When coexpressed with wild-type *TBC1D4*, the truncated protein dimerized with full-length *TBC1D4*, suggesting that the heterozygous truncated variant might interfere with its wild-type counterpart in a dominant negative fashion. Two overweight family members with the mutation also manifested normal fasting glucose and insulin levels but disproportionately elevated insulin levels following an oral glucose challenge. This family provides unique genetic evidence of *TBC1D4* involvement in human insulin action.

glucose transport | insulin resistance | *AS160*

Blood glucose levels are very precisely regulated in healthy people despite enormous variation in the timing and magnitude of carbohydrate ingestion. Postingestive insulin-stimulated glucose uptake into skeletal muscle and adipose tissue is an essential element of this tight metabolic regulation. Glucose transporter 4 (*GLUT4*) selectively mediates insulin-stimulated glucose transport into myotubes and adipocytes. The importance of this metabolic step in vivo was demonstrated by generating muscle and adipose tissue-specific *GLUT4* knockout mice, both of which are insulin resistant (1). Human physiological studies suggest that defects in insulin-stimulated glucose transport in muscle constitute the earliest abnormality in insulin-resistant people (2). *GLUT4* is the main insulin-responsive hexose transporter. Insulin rapidly increases the amount of *GLUT4* in the plasma membrane by causing specialized *GLUT4*-containing vesicles to move from an intracellular location to the plasma membrane where the vesicles dock and then fuse with the plasma membrane (3). This process is referred to as *GLUT4* translocation. Whilst impaired *GLUT4* translocation appears to be common to many different insulin resistance-inducing metabolic insults (4), precisely how insulin triggers *GLUT4* vesicle

movement and subsequent membrane fusion remains incompletely understood. *AKT2* is clearly activated in response to insulin and triggers *GLUT4* membrane translocation (5), but until recently the link between proximal insulin signaling steps [insulin receptor substrate (IRS)–phosphatidylinositol 3 kinase (PI3K)–protein kinase B (*AKT*)] and *GLUT4* translocation was unknown. Tre-2, BUB2, CDC16, 1 domain family member 4 (*TBC1D4*) is a 160-kDa *AKT* substrate with a GTPase-activating protein (GAP) domain and several *AKT* phosphorylation sites (6, 7). Insulin-induced activation of *AKT2* leads to phosphorylation of these sites. Considerable evidence indicates that phosphorylation of *TBC1D4* suppresses its GAP activity, thereby promoting elevation of the GTP-bound form of selected Rabs and *GLUT4* translocation (7–9) (Fig. 1). In the basal state, *TBC1D4* appears to maintain these Rabs in their GDP form and so prevents *GLUT4* translocation. *TBC1D4* knockdown in 3T3L1 adipocytes increases basal cell membrane *GLUT4* levels, indicating that *TBC1D4* Rab-GTPase activity is required for full intracellular retention of *GLUT4* storage vesicles (8, 9). Knockdown of *TBC1D4* also inhibits insulin-stimulated *GLUT4* translocation by about 25% by a currently unknown mechanism (8).

Although rare, monogenic syndromes of severe insulin resistance/type 2 diabetes can provide unique insight into the function of the affected gene/protein in humans as well as offering clues to understanding more common polygenic disease (10). To determine whether missense/nonsense mutations within *TBC1D4* might alter glucose transport and predispose carriers to insulin resistance, we screened all of the coding regions of *TBC1D4* in a cohort of 156 severely insulin-resistant patients. Patients were selected for the study if they had acanthosis nigricans, a cutaneous manifestation of extreme hyperinsulinemia, and/or a fasting insulin level of greater than 150 pmol/L, or if already diabetic at the time of referral, if they required more than 200 units of insulin daily.

## Results

**Identification of a Premature Stop Mutation in *TBC1D4*.** We identified a heterozygous substitution of thymine for cytosine at nucleotide

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<sup>1</sup>S.D. and H.S. contributed equally to this work.

<sup>2</sup>To whom correspondence may be addressed. E-mail: dbs23@medschl.cam.ac.uk or so104@medschl.cam.ac.uk.

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**Table 1. Clinical and biochemical characteristics of the proband and her mother**

	Proband	Reference ranges, mean (95% CI)*	Mother	Reference ranges, mean (95% CI)*
Age (years)	11		41	
Sex	F		F	
BMI (kg/m <sup>2</sup> )	27		37	
BMI SDS	2.7		3.1	
Basal glucose (mg/dL)	97.3	84.1 (83.2; 85.0)	81.1	90.8 (86.7; 95.0)
120 min glucose (mg/dL)	158.6	102.1 (100.1; 104.2)	137.0	103.6 (95.0; 112.3)
Basal insulin (pM)	101	94 (85; 103)	115	80 (70; 92)
120 min insulin (pM)	6237	460 (413; 508)	1720	367 (283; 452)
120 min/basal insulin ratio	62	4.8 (4.7; 4.8) <sup>†</sup>	15	4.7 (3.9; 5.4) <sup>†</sup>
HbA1C	5.3%			
Triglyceride (mg/dL)	88.5		115	<177
Cholesterol (mg/dL)	146.7		169.9	<193
HDL Cholesterol (mg/dL)	61.8		46.3	>38.6

To convert the values for glucose into millimoles per liter multiply by 0.0555. To convert the values for insulin to microinternational units per milliliter divide by 6.945. To convert the values for triglycerides to millimoles per liter multiply by 0.0113. To convert the values for cholesterol and HDL to millimoles per liter multiply by 0.0259.

\*Age-, gender-, and BMI-matched control data for the proband were derived from oral glucose tolerance tests performed in 215 nondiabetic Caucasian girls (23) [mean and 95% CI for: age 11.46 years (11.13; 11.79); BMI, 29.1 kg/m<sup>2</sup> (28.3; 29.9)]. Gender- and BMI-matched data for the mother were derived from 31 nondiabetic Caucasian adult women participating in the Isle of Ely study (24) (BMI range 35–40 kg/m<sup>2</sup>).

<sup>†</sup>In control subjects, peak insulin values usually occur at 30 or 60 min. Definitive peak insulin data were not available as plasma insulin was only measured at 30 and 120 min after the oral glucose load in the adolescent controls and at 30, 60, and 120 min in the adult controls; however, in these control samples, the mean ratios for 30 min and 120 min to basal insulin concentrations were 9 and 5, respectively, in the gender- and BMI-matched adolescents; and the mean ratios for 60 and 120 min to basal insulin concentrations were 7 and 5, respectively, in gender- and BMI-matched adults.

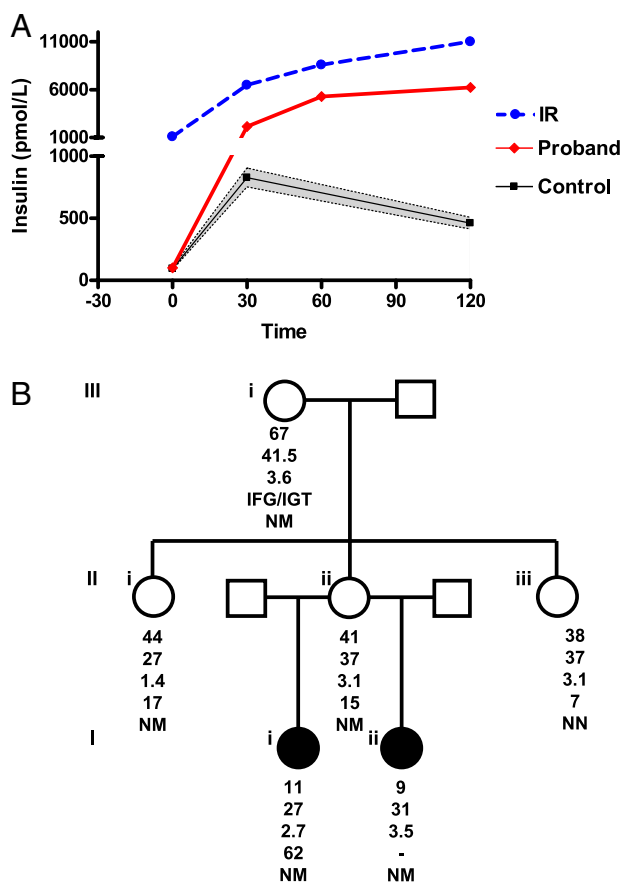
derstanding monogenic  $\beta$ -cell disorders and, more recently, genomewide association studies have identified several common variants thought to be primarily associated with  $\beta$ -cell dysfunction, progress in understanding the genetic basis of insulin resistance remains slow (12, 13). This probably reflects the complexity of the insulin signaling cascade, the fact that insulin sensitivity is determined by insulin action in several different tissues and the number of factors, such as body weight, diet, physical activity, puberty, and old age, which modify insulin sensitivity. All previously described monogenic disorders in the insulin signaling cascade in humans result from proximal defects in the pathway, the vast majority being the result of mutations in the insulin receptor (14). We have also reported 1 kindred with autosomal dominant fasting and postprandial hyperinsulinemia because of a heterozygous *AKT2* mutation (15). Although we and others have screened several genes (including *GLUT4*) implicated in GLUT4 trafficking, the premature stop *TBC1D4* variant (R363X) is the first mutation that significantly impairs GLUT4 translocation.

The prematurely truncated *TBC1D4* protein, which lacks the Rab-GTPase domain and AKT phosphorylation sites, slightly increases basal GLUT4 cell membrane levels. Expression of the truncation mutant may displace some wild-type *TBC1D4* from the GLUT4 vesicles. Since the truncated protein lacks a GAP domain, one might expect an increase in GTP-bound Rab substrate proteins and consequently increased basal GLUT4 translocation. The inhibition of GLUT4 at the cell surface in the insulin-stimulated state caused by overexpression of the R363X and 383Tr is qualitatively consistent with the concept that the R363X mutation inhibits GLUT4 translocation in humans. However, it should be noted that quantitatively the degree of inhibition would be expected to be small when the amount of the truncated protein is expressed at about the same level as the wild-type protein, as would be the case in the human heterozygote, since  $\approx$ 30-fold overexpression in 3T3L1 adipocytes decreased the amount of GLUT4 at the cell surface by 34%. Thus, it remains possible that some unknown role of *TBC1D4* in other tissues contributes to the human phenotype. *TBC1D4* mRNA is widely expressed in human tissues with highest expression in skeletal muscle and white adipose tissue (16, 17). Exactly how the

truncated mutant inhibits insulin-stimulated GLUT4 translocation remains unclear. One possibility is that association of the truncated mutant with wild-type *TBC1D4* reduces the suppression of its GAP activity that normally occurs when *TBC1D4* is phosphorylated in response to insulin. As a result, insulin stimulation leads to a smaller increase in GTP-bound Rabs and thus some inhibition of GLUT4 translocation. Alternatively, it is possible that phosphorylated full-length *TBC1D4* also has a positive role in GLUT4 translocation. This notion is consistent with observations made in *TBC1D4* knockdown experiments; knocking down *TBC1D4* in 3T3L1 adipocytes caused a 3-fold increase in GLUT4 at the cell surface in the basal state and a 25% reduction in cell surface GLUT4 levels in the insulin-treated state (8).

We have found that *TBC1D4* dimerises with itself (Fig. 3D). The fact that the truncated protein can associate with full-length *TBC1D4* offers a potential mechanism whereby relatively low-level expression of the variant might impair GLUT4 translocation. This is important for 2 reasons: first, the variant is heterozygous and second, at least in the 3T3L1 adipocytes, we observed considerable readthrough of the premature stop codon. Previous studies suggest that TGA stop codons, which is what the patients in this kindred have, appear to be the leakiest of all stop codons (18, 19), but whether or not this is occurring in vivo and to what extent it might be occurring is difficult to quantify accurately. Nevertheless, immunoblots of lysates from the proband's transformed lymphocytes suggest that total *TBC1D4* expression levels are reduced and that the truncated *TBC1D4* protein is expressed. Alternatively, given the increased basal GLUT4 levels with reduced insulin-induced GLUT4 translocation seen in *TBC1D4* knockdown 3T3L1 adipocytes (8), it is also plausible that the phenotype may simply be explained by reduced *TBC1D4* expression in vivo.

Two aspects of the human R363X phenotype were particularly striking: first, the proband presented with a very modestly elevated fasting insulin level of 101 pmol/L, but a massively elevated postglucose insulin peak of 6,237 pmol/L. We hypothesize that this selective preservation of fasting glucose and insulin levels with marked postprandial hyperinsulinaemia might reflect (i) preserved insulin action in the liver being primarily



**Fig. 4.** Phenotypic characterization of the R363X *TBC1D4* kindred. (A) Plasma insulin levels (pmol/L) before (0 min) and after (30, 60, and 120 min) a 75-g oral glucose challenge. Black squares and error bars represent mean and 95% CI for plasma insulin levels during oral glucose tolerance tests performed in 215 nondiabetic Caucasian girls (23) (control; mean and 95% CI for: age, 11.46 years (11.13; 11.79); BMI, 29.1 kg/m<sup>2</sup> (28.3; 29.9)). Red diamonds denote data derived from the proband (proband) at age 11 years and blue circles, data from a 13-year-old lean female (IR) with type A insulin resistance because of a dominant negative mutation in the insulin receptor (*INSR* A1135E), which causes severe hepatic and peripheral insulin resistance. (B) Proband's (II) family pedigree. The squares represent male family members and the circles female family members; solid symbols denote family members with acanthosis nigricans. Below each symbol, age (years) is given, followed by the BMI (kg/m<sup>2</sup>), the BMI standard deviation score (SDS), the peak-to-basal insulin ratio, and the genotype, with N denoting the normal (wild-type) allele and M the mutant allele. IFG, impaired fasting glucose; IGT, impaired glucose tolerance.

responsible for maintaining fasting glucose levels, (ii) increased basal (fasting) membrane GLUT4 levels, and (iii) impaired insulin-stimulated GLUT4 translocation. In vivo, the tendency to increase basal GLUT4 membrane expression does not appear to be sufficient to cause hypoglycemia as none of the carriers manifest this problem, but supraphysiological doses of insulin are required to compensate for the postprandial defect in insulin-stimulated GLUT4 translocation. Within a cohort of over 200 probands with severe insulin resistance, we have only identified 3 other probands with similarly disproportionate postglucose hyperinsulinemia. We have yet to identify causative genetic variants in these patients.

Second, the phenotype observed in this kindred appears to be very subtle in lean carriers (in keeping with the apparently subtle in vitro effects of the truncated mutant) but particularly sensitive to secondary modifiers such as puberty and obesity. Puberty is a well-recognized but ill-understood cause of insulin resistance. Intriguingly, it also appears to cause predominantly peripheral as

opposed to hepatic insulin resistance (20). Although the kindred described herein is small, the proband's phenotype was most apparent during puberty and improved significantly following the completion of puberty and weight loss. Following puberty and significant weight loss the proband's acanthosis nigricans resolved and her insulin levels fell considerably although she was still overweight and had a high peak-to-basal insulin ratio. Her aunt normalized her fasting and postglucose insulin levels after reducing her BMI to 24 kg/m<sup>2</sup>, whereas her mother, who remained obese, maintained elevated peak insulin levels. The fact that GLUT4 levels are known to be specifically reduced in adipose tissue in obese states might explain the predisposition of this phenotype to be exacerbated by obesity (21).

A limitation of this study is the absence of direct in vivo measurements of GLUT4 expression and of glucose transport in adipose tissue and skeletal muscle. Family members declined our invitation to undergo hyperinsulinemic euglycemic clamps which, whilst still not providing direct measurements of in vivo glucose transport, might at least have indicated impaired peripheral glucose disposal.

In summary, we have described a family in which the proband, who presented with acanthosis nigricans and extreme postprandial hyperinsulinemia, was found to have inherited a heterozygous premature stop mutation in *TBC1D4*. In vitro, the mutant protein binds wild-type *TBC1D4* and inhibits GLUT4 translocation. Thus, it is plausible to suggest that this family may represent the first inherited disorder of GLUT4 translocation in humans. Of clinical importance is the fact that reliance on biochemical assessments of insulin resistance that incorporate only fasting insulin would not have suggested a diagnosis of a state of severe insulin resistance in the proband, when she presented with acanthosis nigricans, and could have led to subsequent misdirected investigation. This family demonstrates that clinical syndromes of severe isolated postprandial hyperinsulinemia, sufficient to produce the classical dermatological marker of severe insulin resistance, exist in the human population and that their recognition requires measurement of circulating insulin levels in the postprandial and fasting states.

## Methods

**Genetic Studies.** Each participant, or a parent in the case of children under 16 years, provided written informed consent; minors provided oral consent. All clinical studies were conducted in accordance with the principles of the Declaration of Helsinki and were approved by the National Health Service Research Ethics Committee.

Genomic DNA was isolated from leukocytes derived from whole blood from 156 probands with severe insulin resistance and the coding regions and splice junctions of *TBC1D4* (AS160) were amplified by PCR and sequenced. EBV-transformed lymphocytes were lysed in 50 mmol/L Hepes (pH 7.4), 150 mmol/L NaCl, 10 mmol/L ethylenediamine tetraacetate, 0.5 mmol/L phenylmethylsulfonyl fluoride, 2.5 mmol/L leupeptin, 1 μg/mL leupeptin, antipain, pepstatin, and 1% Triton X-100. Protein concentration was determined using the Bradford dye-binding procedure (Bio-Rad Laboratories). Cell lysates were subjected to 8% reducing SDS/PAGE, proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore), and membranes were probed with a rabbit polyclonal antibody raised against the C terminus of *TBC1D4* and a horseradish peroxidase-conjugated secondary antibody (Abcam). Antibody binding was detected by enhanced chemiluminescence (Amersham ECL Western blotting system, GE Healthcare).

To demonstrate expression of the truncated protein, we obtained a custom-made affinity-purified rabbit antibody raised against the N terminus of *TBC1D4* (21st Century Biochemicals). EBV lysates were immunoprecipitated with this N-terminal *TBC1D4* antibody using the Exactacruz F kit from Santa Cruz Biotechnology (catalog no. sc-45043) as per manufacturer's instructions. Protein A agarose beads (Sigma, catalog no. P3476-5ML) were used to bind the N-terminal anti-*TBC1D4* antibody. Bead-antibody-protein complexes were washed 4 times in lysis buffer and then finally in PBS. The immunoprecipitated protein was eluted in 50 μL of 2× nonreducing electrophoresis buffer by gentle pipetting at room temperature. Nonreducing buffer was used because the mobility of the reduced truncated protein is approximately the same as that of the antibody heavy chain. The supernatant was run on an

8% SDS gel and immunoblotted with the N-terminal TBC1D4 antibody as described above except that a HRP-conjugated secondary antibody from the Exactacruz kit was used for detection. The truncated R363X protein is detected at just above the 50-kDa marker when dissolved in reduced electrophoresis buffer. To determine its molecular weight in a nonreducing buffer, protein lysates from 3T3L1 adipocytes overexpressing a myc-tagged 363Tr were dissolved in 2× nonreducing buffer and immunoblotted with an antibody raised against the myc epitope. This confirmed that the truncated protein was detectable just above the 75-kD marker (Fig. S1).

**Cellular Studies of Mutant TBC1D4 Function.** Human wild-type *TBC1D4* (AS160) in the P3XFLAG CMV10 vector (6) was used to generate a Flag-tagged mutant construct (R363X) using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocols. To evaluate the biological effects of the truncated mutant, a second construct was created in this vector with no coding DNA beyond the stop codon identified in the proband (designated 363Tr). All constructs were fully sequenced.

The relative amount of GLUT4 at the cell surface in 3T3L1 adipocytes was assayed by transfecting cells through electroporation with a plasmid for expression of HA-GLUT4-GFP and measuring the HA-GLUT4-GFP at the cell surface by quantitative single-cell immunofluorescence, as previously described (7). In this method, HA-GLUT4-GFP at the cell surface is labeled with anti-HA and Cy3-conjugated secondary antibody; the fluorescence intensities for Cy3 and GFP in individual cells are quantitated, and the relative amount of HA-GLUT4-GFP at the cell surface is expressed as the ratio of Cy3 to GFP, to correct for different levels of expression of the HA-GLUT4-GFP protein.

The association of TBC1D4 with itself and with the 363Tr form was examined by cotransfecting human embryonic kidney 293E cells with plasmids for expression of N-terminal T7-tagged human TBC1D4 and either N-terminal

Flag-tagged TBC1D4 or the 363Tr with Lipofectamine 2000. The cells were treated with insulin (1  $\mu$ M for 10 min) or left unstimulated, then lysed in 40 mM Hepes, 150 mM NaCl, pH 7.4 containing 1.5% nonionic detergent octaethylenglycol dodecyl ether and protease and phosphatase inhibitors. The lysate was cleared by centrifugation at 12,000  $\times$  g for 15 min and the portions of the supernatant were immunoprecipitated with anti-Flag agarose or irrelevant mouse Ig and protein G-agarose. SDS samples of the immunoprecipitates were immunoblotted for the T7 and FlaQ epitopes.

**Glucose Tolerance Tests and Biochemical Assays.** Whole body insulin sensitivity was assessed with a 2-hour, 75-g oral glucose tolerance test. After insertion of an antecubital i.v. line, blood samples were collected at -30, 0, 10, 30, 60, 90, and 120 min for determination of plasma glucose, insulin, c-peptide, and free fatty acids. Insulin, leptin, and adiponectin were measured using customized autoDELFIa immunoassays as previously described (22).

**Statistical Analysis.** GLUT4 translocation data are expressed as means  $\pm$  SE. Differences between vectors were compared with use of the unpaired Student's *t* test. All reported *P* values are from 2-sided tests, and *P* values of less than 0.05 were considered to indicate statistical significance.

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