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Contraction regulates site-specific phosphorylation of TBC1D1 in skeletal muscle

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TBC1D1 (tre-2/USP6, BUB2, cdc16 domain family member 1) is a Rab-GAP (GTPase-activating protein) that is highly expressed in skeletal muscle, but little is known about TBC1D1 regulation and function. We studied TBC1D1 phosphorylation on three predicted AMPK (AMP-activated protein kinase) phosphorylation sites (Ser^{231}, Ser^{660} and Ser^{700}) and one predicted Akt phosphorylation site (Thr^{590}) in control mice, AMPKα2 inactive transgenic mice (AMPKα2i TG) and Akt2-knockout mice (Akt2 KO). Muscle contraction significantly increased TBC1D1 phosphorylation on Ser^{231} and Ser^{660}, tended to increase Ser^{700} phosphorylation, but had no effect on Thr^{590}. AICAR (5-aminoimidazole-4-carboxyamide ribonucleoside) also increased phosphorylation on Ser^{231}, Ser^{660} and Ser^{700}, but not Thr^{590}, whereas insulin only increased Thr^{590} phosphorylation. Basal and contraction-stimulated TBC1D1 Ser^{231}, Ser^{660} and Ser^{700} phosphorylation were greatly reduced in AMPKα2i TG mice, although contraction still elicited a small increase in phosphorylation. Akt2 KO mice had blunted insulin-stimulated TBC1D1 Thr^{590} phosphorylation. Contraction-stimulated TBC1D1 Ser^{231} and Ser^{660} phosphorylation were normal in high-fat-fed mice. Glucose uptake *in vivo* was significantly decreased in tibialis anterior muscles overexpressing TBC1D1 mutated on four predicted AMPK phosphorylation sites. In conclusion, contraction causes site-specific phosphorylation of TBC1D1 in skeletal muscle, and TBC1D1 phosphorylation on AMPK sites regulates contraction-stimulated glucose uptake. AMPK and Akt regulate TBC1D1 phosphorylation, but there must be additional upstream kinases that mediate TBC1D1 phosphorylation in skeletal muscle.

Key words: Akt, AMP-activated protein kinase (AMPK), contraction, glucose transport, skeletal muscle, TBC1D1.

INTRODUCTION

Skeletal muscle is a major target tissue for insulin-stimulated glucose disposal [1,2], and insulin resistance in this tissue is considered a key factor in the development of Type 2 diabetes. Insulin-stimulated muscle glucose transport is impaired in patients with Type 2 diabetes [3,4], whereas exercise-stimulated glucose transport is normal, or near normal [5–7]. Both exercise and insulin increase glucose transport through the translocation of GLUT4 (glucose transporter 4)-containing vesicles from intracellular depots to the transverse tubules and sarcolemma [8–13]. Although it is well-established that the proximal signalling mechanisms that lead to exercise- and insulin-stimulated GLUT4 translocation are distinct, there is now extensive evidence that there is a convergence of exercise and insulin signalling at TBC1D1 (tre-2/USP6, BUB2, cdc16 domain family member 1) and AS160 (Akt substrate of 160 kDa; also known as TBC1D4). TBC1D1 and AS160 are highly homologous Rab-GAPs (GTPase-activating proteins). There has been considerably more investigation of AS160, and this protein is postulated to function by promoting hydrolysis of GTP to GDP on GLUT4-containing vesicles. In the GDP-bound form, the GLUT4-containing vesicles are bound to specific Rab proteins, inhibiting GLUT4 translocation to the cell surface [14]. Site-specific phosphorylation of AS160 is thought to modulate Rab-GAP activity, allowing release of the transporters to the membranes.

While AS160 has been studied in both skeletal muscle (e.g. [15,16]) and adipocytes [17], much less is known about the regulation and function of TBC1D1 [14,18,19]. TBC1D1 is similar to AS160 in terms of size and structure [20]. However, the tissue distribution of the two proteins is considerably different, with TBC1D1 having little if any expression in adipocytes, and the highest level of expression in skeletal muscle [21,22]. We found that contraction, insulin and the AMPK (AMP-activated protein kinase) activator AICAR (5-aminoimidazole-4-carboxyamide ribonucleoside) increase TBC1D1 phosphorylation on PAS (phospho-Akt substrate) motifs in mouse skeletal muscle, demonstrating that TBC1D1 is an additional point of convergence for insulin and contraction signalling [21]. More recently, others have also shown TBC1D1 PAS phosphorylation in response to exercise and insulin in rat and mouse skeletal muscle [23,24].

In addition to Akt, there is strong evidence that there are other kinases that can phosphorylate TBC1D1, most notably AMPK. For example, we found that phosphorylation of skeletal muscle TBC1D1 with recombinant AMPK *in vitro*, but not recombinant Akt, induced an electrophoretic-mobility shift. Consistent with

Abbreviations used: AICAR, 5-aminoimidazole-4-carboxyamide ribonucleoside; AMPK, AMP-activated protein kinase; AS160, Akt substrate of 160 kDa; EDL, extensor digitorum longus; GAP, GTPase-activating protein; GLUT4, glucose transporter 4; ICR, imprinting control region; KO, knockout; PAS, phospho-Akt substrate; TBC1D1, tre-2/USP6, BUB2, cdc16 domain family member 1; TG, transgenic; WT, wild-type.

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this hypothesis, we identified several novel phosphorylation sites on endogenous TBC1D1 from skeletal muscle using MS [21]. Several of these sites, including Ser^{231}, Thr^{499}, Ser^{660} and Ser^{700}, are consensus or near-consensus sites for AMPK phosphorylation motifs $\{\Phi[X\beta]XX(S/T)XXX\Phi$; Φ = methionine, valine, leucine, isoleucine or phenylalanine, β = arginine, lysine or histidine, and X = any amino acid$\}$ [25] and/or are suggested to be phosphorylated by the AMPK activator AICAR, based on relative peak ion intensities, a semi-quantitative method of analysis [21]. One purpose of the present study was to determine the effects of contraction, insulin and the AMPK activator AICAR on phosphorylation of these TBC1D1 sites.

There is growing evidence that TBC1D1 is involved in the regulation of glucose transport. In 3T3-L1 adipocytes, mutation of multiple Akt sites impairs insulin-stimulated GLUT4 translocation [26]. In skeletal muscle, we found that expression of the obesity-associated R125W mutant significantly decreased insulin-stimulated glucose transport in the absence of changes in TBC1D1 PAS phosphorylation [27]. Surprisingly, expression of TBC1D1 mutated to alanine on four conserved phosphorylation sites had no effect on insulin-stimulated glucose transport, but impaired contraction-stimulated glucose uptake [27]. Whether mutation of the multiple putative AMPK sites on TBC1D1 regulates glucose uptake in skeletal muscle has not been investigated, and this is another major goal of the present study. Our findings from the present study indicate that muscle contraction is a significant regulator of TBC1D1 phosphorylation in skeletal muscle, and that simultaneous mutation of multiple sites impairs contraction-stimulated glucose uptake.

EXPERIMENTAL

Animals

Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines. Female ICR (imprinting control region) mice 8–9 weeks old (25–30 g) were purchased from Charles River Laboratories or Taconic. Female muscle-specific AMPKα2i TG (α2-inactive AMPK transgenic) mice and WT (wild-type) littermates (FVB background; aged 9 weeks) were generated as described previously [28]. Female Akt2 KO (Akt2 knockout) mice and WT littermates (C57BL/6N background; aged 9 weeks) were kindly provided by Dr Morris Birnbaum [29]. All mice were housed in a 12 h:12 h light/dark cycle. The ICR, AMPKα2i TG and Akt KO mice were fed standard laboratory chow and water *ad libitum*. For the high-fat diet studies, 6-week-old C57BL/6NHsd male mice were fed on a high-fat diet (60 % kcal of fat) from Harlan Labs for 9 weeks (TD.06414). Mice were restricted from food overnight (20:00 to 09:00 h) prior to experiments.

Plasmid cDNA constructs

The plasmid encoding mouse WT TBC1D1 (long form) was generated in our laboratory [27]. Five mutant TBC1D1 constructs were generated using the QuikChange® II XL mutagenesis kit (Stratagene). This included WT TBC1D1, TBC1D1 mutated on four predicted AMPK phosphorylation sites (Ser^{231}, Thr^{499}, Ser^{660} and Ser^{700}) to alanine (4A mutant) and TBC1D1 mutated on each site individually. Mutations of the TBC1D1 constructs were confirmed for accuracy by utilizing the high-throughput DNA sequencing service at Brigham and Women's Hospital (Boston, MA, U.S.A.). Plasmid DNA was amplified in *Escherichia coli* TOP10 cells (Invitrogen), extracted using an endotoxin-free Plasmid Mega Kit (Qiagen) and suspended in saline at 4 μg/μl.

In vivo gene transfer in mouse skeletal muscle and *in vivo* glucose uptake

Animals were anaesthetized with an intraperitoneal injection of pentobarbital sodium (90–100 mg/kg of body weight). Plasmid DNA (100 μg; empty pCAGGS vector, WT TBC1D1 or mutant TBC1D1 constructs) were directly injected into mouse tibialis anterior muscles followed by electroporation according to our modified protocol [30], originally described by Aihara and Miyazaki [31]. At 7 days after DNA injection, the mice were used to measure contraction-mediated glucose uptake or to test phospho-specific antibodies (see below). Rates of glucose uptake into skeletal muscle were assessed as described previously [32].

In situ muscle contraction

Mice were anaesthetized with an intraperitoneal administration of pentobarbital sodium (90–100 mg/kg of body weight). Peroneal nerves from both legs were surgically exposed. One leg served as a sham-operated control, while the other leg was subjected to electrical stimulation using a Grass S88 pulse generator for 15 min (train rate, 1/s; train duration, 500 ms; pulse rate, 100 Hz; duration, 0.1 ms at 1–8 V). Voltage and electrode position were manually adjusted so that muscles were contracted with a full range of motion.

In vitro muscle incubation

Mice were anaesthetized as described above and EDL (extensor digitorum longus) muscles were dissected. EDL muscles were pre-incubated in Krebs–Ringer bicarbonate buffer with 2 mmol/l pyruvate for 20 min as described previously [33]. After the pre-incubation period, muscles were incubated in the absence or presence of 2 mmol/l AICAR for 40 min and rapidly frozen in liquid nitrogen.

In vivo insulin administration

Mice were anaesthetized as described above followed by intraperitoneal injection of saline (0.9 % NaCl) or a maximal dose of insulin (1 unit). Then, 10 min later, mice were killed by cervical dislocation, and tibialis anterior muscles were rapidly removed and frozen in liquid nitrogen.

Tissue processing and immunoblotting

Frozen muscles were pulverized and homogenized with a Polytron (Brinkman Instruments) in ice-cold buffer as described in [21]. Lysates (20 μg of protein) were separated by SDS/PAGE and transferred on to nitrocellulose membrane (PerkinElmer Life Sciences) for Western blotting. A horseradish-peroxidase-conjugated anti-rabbit antibody (Amersham Biosciences) was used to bind and detect all of the primary antibodies. Antibody-bound proteins were detected by enhanced chemiluminescence reagent (PerkinElmer Life Sciences) and visualized on the FluorChem® imaging system (Alpha Innotech). The FluorChem®-detected protein bands were quantified by densitometry (AlphaEaseFC software 4.0; Alpha Innotech). TBC1D1 phosphorylation was measured using immunopurified phospho-specific antibodies against Ser^{231}, Thr^{590}, Ser^{660} and

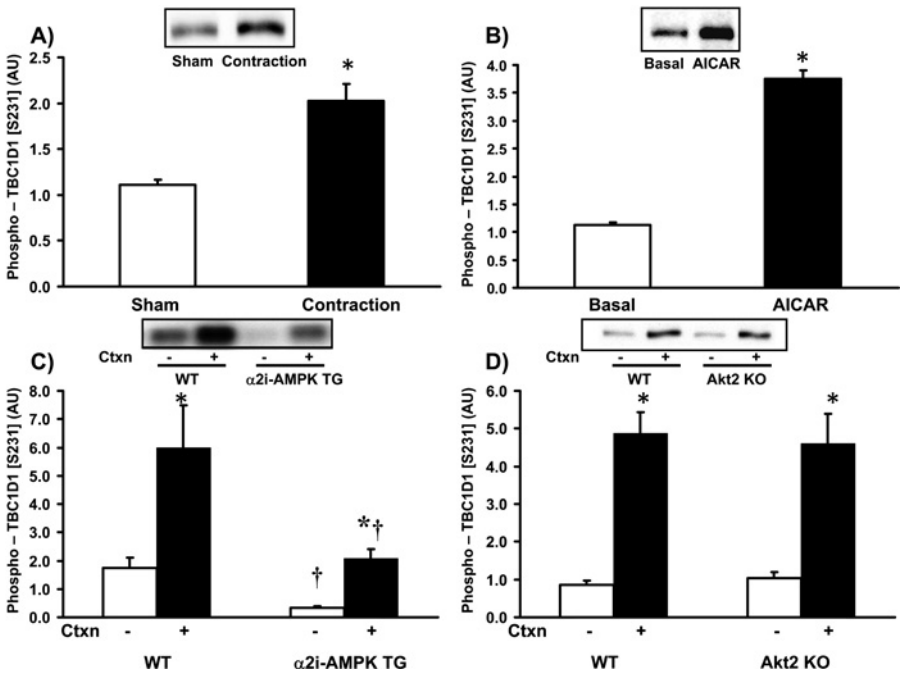

Figure 1 TBC1D1 Ser^{231} phosphorylation is increased by contraction and AICAR

(**A**) Contraction (Ctxn) increased TBC1D1 Ser^{231} phosphorylation in tibialis anterior muscles from ICR mice. Mice were anaesthetized and contraction was generated by electrical stimulation of the peroneal nerve for 15 min, while the contra-lateral side served as a sham-operated control. (**B**) AICAR increased TBC1D1 Ser^{231} phosphorylation. EDL muscles were isolated from ICR mice and incubated with 2 mM AICAR or vehicle for 40 min. (**C**) Phosphorylation of TBC1D1 Ser^{231} was reduced in AMPKα2i TG mice. AMPKα2i TG mice and WT littermates underwent contraction as described in (**A**). (**D**) Basal and contraction-stimulated TBC1D1 Ser^{231} was preserved in Akt2 KO mice. Akt2 KO mice and WT littermates underwent contraction as described in (**A**). Data are means $\pm$ S.E.M.; $n = 5$–7/group. *$P < 0.05$ compared with control; †$P < 0.05$ compared with corresponding WT. AU, arbitrary units.

Ser^{700}, and TBC1D1 protein expression was detected using immunopurified anti-TBC1D1 antibody. TBC1D1 antibodies were developed in collaboration with Cell Signaling Technology. Specificity of the anti-phospho-TBC1D1 Ser^{231}, Thr^{590}, Ser^{660} and Ser^{700} antibodies were validated using tibialis anterior muscles that had been individually transfected with TBC1D1 mutated at each phosphorylation site to alanine, compared with WT TBC1D1-overexpressing muscles and empty-vector-injected control muscles (Supplementary Figure S1A–S1D at http://www.BiochemJ.org/bj/431/bj4310311add.htm).

Statistical analysis

Data are presented as means $\pm$ S.E.M. Statistical analyses were performed using an unpaired Student's *t* test or two-way ANOVA. When ANOVA revealed significant differences, a Student–Newman–Keuls post-hoc test was performed. *P* values less than 0.05 were considered statistically significant.

RESULTS

TBC1D1 phospho-antibodies

Our previous study identified multiple phosphorylation sites on endogenous TBC1D1 from skeletal muscle [21]. On the basis of semi-quantitative analysis of our MS results [21], scan-site analysis of the sequences [34] and other published data [20,26], we predicted that Ser^{231}, Ser^{660} and Ser^{700} would be strongly regulated by AMPK, Ser^{499} weakly regulated by AMPK and Thr^{590} regulated by Akt. To investigate site-specific regulation of TBC1D1 phosphorylation, we set out to generate antibodies against these five TBC1D1 residues: Ser^{231}, Ser^{499}, Thr^{590}, Ser^{660} and Ser^{700} (Supplementary Figure S1). Of these, we were not successful in generating the Ser^{499} antibody. Therefore for subsequent experiments we focused on TBC1D1 phosphorylation on Ser^{231}, Thr^{590}, Ser^{660} and Ser^{700}.

Muscle contraction and AICAR increase TBC1D1 Ser^{231} phosphorylation

Since Ser^{231} of TBC1D1 is a consensus match for the AMPK phosphorylation motif, we hypothesized that stimuli that increase AMPK activity would increase TBC1D1 Ser^{231} phosphorylation in skeletal muscle. Using an antibody specific for TBC1D1 phosphorylation at Ser^{231}, we investigated the site-specific regulation of TBC1D1 phosphorylation in response to muscle contraction, AICAR and insulin. Contraction of tibialis anterior muscle *in situ* for 15 min increased Ser^{231} phosphorylation by approx. 2-fold compared with sham-treated muscles (Figure 1A). In addition, incubation of isolated EDL muscles with AICAR for 40 min increased TBC1D1 Ser^{231} phosphorylation by nearly 4-fold compared with basal control muscles (Figure 1B). To determine whether AMPKα2 regulates contraction-induced increases in Ser^{231} phosphorylation, we studied WT and AMPKα2i TG mice [28]. Under basal conditions, Ser^{231} phosphorylation was nearly abolished in tibialis anterior muscles from AMPKα2i TG mice compared with WT controls (Figure 1C). *In situ* contraction increased Ser^{231} phosphorylation in the WT mice, an effect that was markedly impaired in the AMPKα2i TG mice. However, the lack of AMPKα2 activity did not fully inhibit TBC1D1 Ser^{231} phosphorylation. These data demonstrate that AMPKα2 regulates TBC1D1 Ser^{231} phosphorylation under basal conditions, and also contributes to contraction-mediated Ser^{231} phosphorylation. However, there

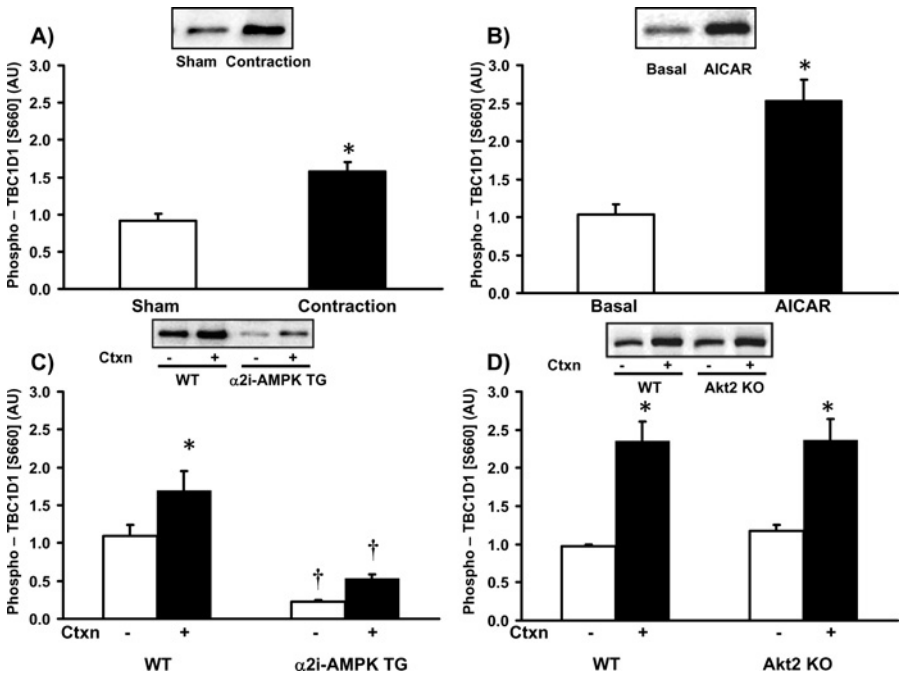

Figure 2 TBC1D1 Ser^{660} phosphorylation is regulated by contraction and AICAR

Experimental details are as described in the legend to Figure 1. (**A**) Contraction (Ctxn) *in situ* for 15 min increased TBC1D1 Ser^{660} phosphorylation in tibialis anterior muscles from ICR mice. (**B**) AICAR increased TBC1D1 Ser^{660} phosphorylation. (**C**) Phosphorylation of TBC1D1 Ser^{660} was reduced in AMPKα2i TG mice. (**D**) Basal and contraction-stimulated TBC1D1 Ser^{660} was preserved in Akt2 KO mice. Data are means $\pm$ S.E.M.; $n = 5$–7/group. $^*P < 0.05$ compared with control; $\dagger P < 0.05$ compared with corresponding WT. AU, arbitrary units.

must be one or more additional upstream kinases that mediate the effects of muscle contraction on TBC1D1 Ser^{231} phosphorylation.

Muscle contraction increases Akt2 phosphorylation and activity in skeletal muscle [33]. To determine whether Akt2 regulates contraction-stimulated TBC1D1 Ser^{231} phosphorylation, WT and Akt2 KO mice were studied in the basal state and after *in situ* muscle contraction. In contrast with experiments using the AMPKα2i mice, knockout of Akt2 had no effect on basal or contraction-stimulated TBC1D1 Ser^{231} phosphorylation (Figure 1D).

To study the effects of insulin on TBC1D1 Ser^{231} phosphorylation, anaesthetized mice were injected with saline or a maximal dose of insulin, and tibialis anterior muscles were dissected 10 min later. Insulin had no effect on Ser^{231} phosphorylation (Supplementary Figure S2A at http://www.BiochemJ.org/bj/431/bj4310311add.htm). Consistent with this finding, there was no change in basal or insulin-stimulated Ser^{231} phosphorylation in Akt2 KO mice (Supplementary Figure S2B). These data demonstrate that insulin and Akt2 do not regulate TBC1D1 Ser^{231} phosphorylation.

Muscle contraction and AICAR increase TBC1D1 Ser^{660} phosphorylation

TBC1D1 Ser^{660} on endogenous TBC1D1 was identified through MS [21], and this phosphorylation site is a consensus match for the AMPK phosphorylation motif, except for the lack of one basic residue at either the -3 or -4 position. Contraction *in situ* significantly increased Ser^{660} phosphorylation in tibialis anterior muscles by approx. 1.5-fold compared with sham-treated muscles (Figure 2A). Incubation of EDL muscles with AICAR increased Ser^{660} phosphorylation by 2.5-fold (Figure 2B). To determine whether AMPKα2 regulates contraction-induced increases in Ser^{660} phosphorylation, we studied WT and AMPKα2i TG mice. Similar to Ser^{231}, TBC1D1 Ser^{660} phosphorylation in the basal state was severely blunted in tibialis anterior muscles from the AMPKα2i TG mice (Figure 2C). *In situ* contraction increased Ser^{660} phosphorylation in the WT mice, and in the AMPKα2i TG mice there was a tendency to increase TBC1D1 Ser^{660} phosphorylation, although this did not reach statistical significance (Figure 2C). Contraction similarly increased Ser^{660} phosphorylation in WT and Akt2 KO mice (Figure 2D). Maximal insulin stimulation *in vivo* did not increase TBC1D1 Ser^{660} phosphorylation (Supplementary Figure S2C), and Akt2 KO mice had normal basal levels of TBC1D1 Ser^{660} phosphorylation (Figure 2D and Supplementary Figure S2D). Taken together, these data suggest that Ser^{231} and Ser^{660} share similar regulatory mechanisms. Both sites exhibit significant increases in phosphorylation in response to contraction and AICAR, and both sites are regulated by AMPKα2, but not by insulin or Akt2.

TBC1D1 Ser^{700} phosphorylation

Similar to Ser^{231}, the Ser^{700} phosphorylation site on TBC1D1 is a full consensus match for the AMPK phosphorylation motif [21]. However, in contrast with Ser^{231} and Ser^{660}, muscle contraction *in situ* only tended to increase Ser^{700} phosphorylation (Figure 3A). AICAR treatment of EDL muscles *in vitro* significantly increased Ser^{700} phosphorylation by 2-fold (Figure 3B). In the AMPKα2i TG mice, basal levels of Ser^{700} phosphorylation were significantly decreased compared with WT littermates (Figure 3C). In these experiments, contraction also tended to increase Ser^{700} phosphorylation in WT mice, and significantly increased phosphorylation in the AMPKα2i TG mice, although to a much lower overall level of phosphorylation compared with the WT mice (Figure 3C). Akt2 KO mice had normal levels

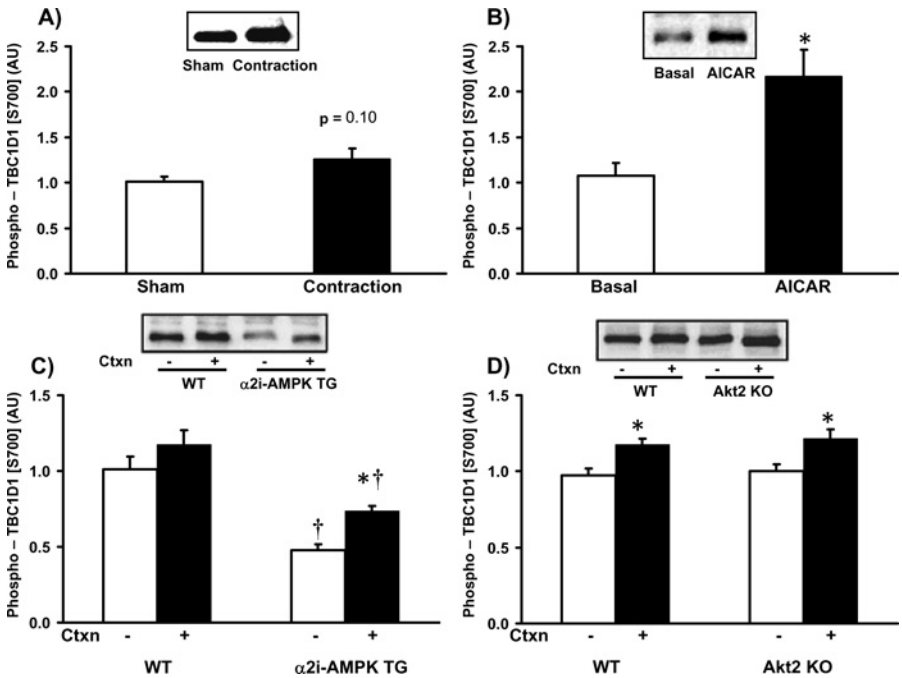

Figure 3 TBC1D1 Ser^{700} phosphorylation is increased by AICAR, but only responds minimally to contraction

Experimental details are as described in the legend to Figure 1. (**A**) Contraction (Ctxn) *in situ* for 15 min tended to increase TBC1D1 Ser^{700} phosphorylation in tibialis anterior muscles from ICR mice. (**B**) AICAR increased TBC1D1 Ser^{700} phosphorylation. (**C**) Phosphorylation of TBC1D1 Ser^{700} was reduced in AMPKα2i TG mice. (**D**) TBC1D1 Ser^{700} phosphorylation was preserved in Akt2 KO mice. Data are means $\pm$ S.E.M.; $n = 5$–7/group. *$P < 0.05$ compared with control; †$P < 0.05$ compared with corresponding WT. AU, arbitrary units.

of TBC1D1 Ser^{700} phosphorylation under basal and contraction conditions (Figure 3D).

Insulin stimulation did not significantly alter Ser^{700} phosphorylation (Supplementary Figure S2E), and there was no change in basal or insulin-stimulated Ser^{700} phosphorylation in Akt2 KO mice (Supplementary Figure S2F). Thus, similar to Ser^{231} and Ser^{660}, AMPKα2, but not Akt2, controls basal levels of TBC1D1 Ser^{700} phosphorylation. TBC1D1 Ser^{700} phosphorylation tends to increase with muscle contraction; however, that increase is much lower compared with increases in phosphorylation at Ser^{231} and Ser^{660}.

TBC1D1 Thr^{590} phosphorylation is regulated by insulin and Akt2

Thr^{590} is a predicted Akt motif that is phosphorylated in response to insulin in 3T3-L1 adipocytes and corresponds to Thr^{649} in AS160 [20,21]. Contraction and AICAR did not increase Thr^{590} phosphorylation in muscle (Supplementary Figures S3A and S3B at http://www.BiochemJ.org/bj/431/bj4310311add.htm). In spite of the lack of Thr^{590} phosphorylation with these two AMPK-activating stimuli, in the AMPKα2i TG mice, basal levels of Thr^{590} phosphorylation were significantly decreased compared with WT littermates (Figure 4A). As expected, insulin stimulation *in vivo* significantly increased TBC1D1 Thr^{590} phosphorylation in muscle (Figure 4C). This effect was mediated by Akt2, as the insulin-stimulated increase in TBC1D1 was abolished in the Akt2 KO mice (Figure 4D). However, there was no change in basal levels of Thr^{590} phosphorylation in the Akt2 KO mice (Figures 4B and 4D). Taken together, these data suggest that, while Akt2 mediates insulin-stimulated TBC1D1 Thr^{590} phosphorylation, AMPKα2 activity is important for maintaining levels of Thr^{590} phosphorylation in the basal state.

Effects of a high-fat diet on TBC1D1 phosphorylation

To determine whether an animal model of insulin resistance alters TBC1D1 phosphorylation with muscle contraction, mice were fed on a high-fat diet (60 % kcal of fat) for 9 weeks. After 9 weeks of high-fat feeding, mice had significantly higher body weights, fasting blood glucose concentrations and fasting insulin concentrations compared with chow-fed mice (Supplementary Table S1 at http://www.BiochemJ.org/bj/431/bj4310311add.htm). High-fat feeding did not change the expression of total TBC1D1 in the tibialis anterior muscles (Figure 5A). High-fat feeding also had no effect on basal levels of TBC1D1 Ser^{231} and Ser^{660} phosphorylation (Figures 5B and 5C). Contraction *in situ* for 15 min significantly increased Ser^{231} and Ser^{660} phosphorylation, and there was no effect of the high-fat feeding (Figures 5B and 5C). There was also no effect of high-fat feeding on TBC1D1 Ser^{700} and Thr^{590} phosphorylation in the basal state or with muscle contraction (Figures 5D and 5E).

Mutation of TBC1D1 on AMPK consensus sequences decreases contraction-stimulated glucose uptake

To determine whether TBC1D1 phosphorylation at AMPK consensus sites regulates glucose uptake in contracted muscle, we mutated four phosphorylation sites on TBC1D1 (4A mutant) (Ser^{231}, Thr^{499}, Ser^{660} and Ser^{700}) and expressed this mutant TBC1D1 and WT TBC1D1 in tibialis anterior muscle. As shown above, Ser^{231}, Ser^{660} and Ser^{700} are phosphorylated by AMPK, and our previous work predicts that Thr^{499} is an AMPK site [21]. Compared with endogenous levels of TBC1D1 in empty vector-injected control muscles, there was a 5-fold increase in WT and 4A mutant TBC1D1 expression in the tibialis anterior muscles (Figure 6A). Muscle contraction increased glucose

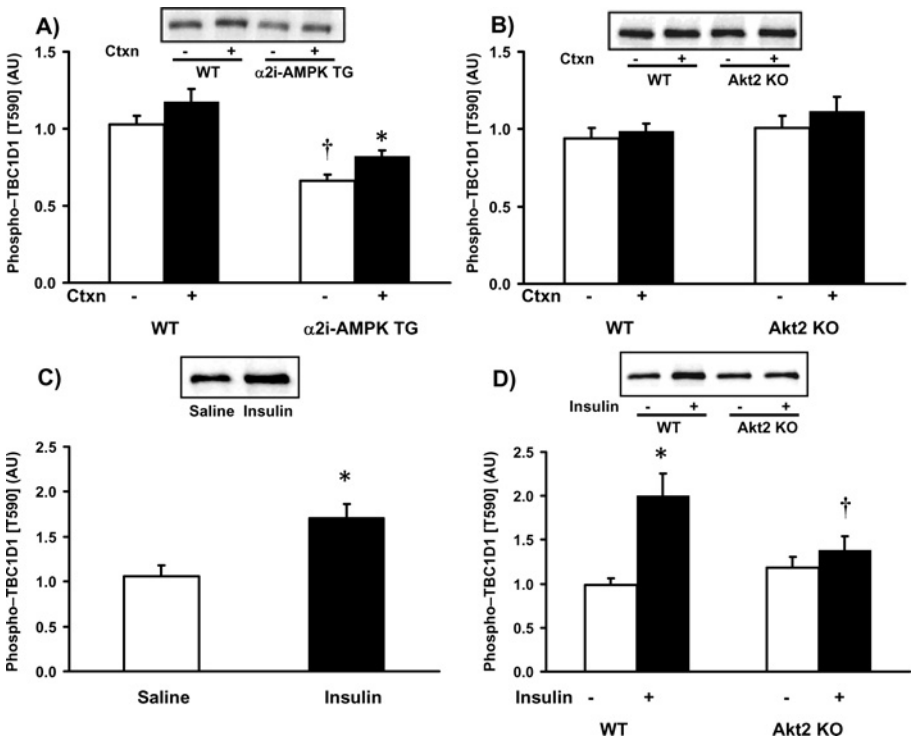

Figure 4 TBC1D1 Thr^{590} phosphorylation is regulated by insulin, but not contraction or AICAR

(**A**) Phosphorylation of TBC1D1 Ser^{590} was significantly reduced in AMPKα2i TG mice at rest. Contraction (Ctxn) *in situ* for 15 min increased phosphorylation in AMPKα2i TG mice, but the increase after contraction in WT mice was not statistically significant. (**B**) The response to contraction was not affected in Akt2 KO mice. (**C**) Maximal insulin injection increased TBC1D1 Ser^{590} phosphorylation in ICR mice. Mice were fasted and injected intraperitoneally with maximal insulin or saline, and tibialis anterior muscles were dissected 10 min later. (**D**) Maximal insulin injection increased TBC1D1 Ser^{590} phosphorylation in WT mice, and the effect was ablated in Akt2 KO mice. Treatment was the same as described in (**C**). Data are means $\pm$ S.E.M.; $n = 5$–7/group. *$P < 0.05$ compared with control; †$P < 0.05$ compared with corresponding WT. AU, arbitrary units.

uptake by 5-fold in control muscles, and overexpression of WT TBC1D1 did not alter contraction-mediated glucose uptake (Figure 6B). However, contraction-stimulated glucose uptake was significantly decreased in muscles overexpressing the 4A TBC1D1 mutant. In contrast with the effects of simultaneous mutation of the four predicted AMPK sites, overexpression of the individual TBC1D1 mutants had no effect on basal or contraction-stimulated glucose uptake (Supplementary Figure S4 at http://www.BiochemJ.org/bj/431/bj4310311add.htm). These findings suggest that phosphorylation of TBC1D1 at multiple AMPK consensus sites is necessary for normal contraction-stimulated glucose uptake in skeletal muscle.

DISCUSSION

Glucose disposal into skeletal muscle is critical for the maintenance of glucose homoeostasis, and insulin- and exercise-stimulated glucose uptake into muscle are the most physiologically relevant stimuli in humans and rodents. It is now well-established that the proximal intracellular signalling molecules regulating exercise-stimulated glucose uptake in skeletal muscle are distinct from that of insulin [14], but the signalling systems converge at AS160 and TBC1D1. AS160 phosphorylation has been investigated in contracting skeletal muscle (e.g. [15,35,36]), and we have studied the role of AS160 in glucose uptake [32,37]. However, much less is known about the regulation and function of TBC1D1.

While AS160 is ubiquitously expressed, the expression of TBC1D1 is highest in skeletal muscle, with very limited expression (e.g. adipocytes) or no expression (e.g. heart) in other tissues throughout the body [21]. This suggests that TBC1D1 has a more specialized function, unique to the properties of skeletal muscle. Therefore we hypothesized that TBC1D1 may have a primary function of mediating signalling inputs derived from muscle contraction. The results of the present study show that muscle contraction increases the phosphorylation of TBC1D1 on multiple sites, including Ser^{231}, Ser^{660}, and to a lesser extent Ser^{700}. On the basis of our initial MS work [21] and the AMPK consensus sequence [25], these three amino acid residues are predicted AMPK phosphorylation sites, which is consistent with our finding that AICAR strongly increased phosphorylation of all three sites. The greater AICAR response compared with the effects of muscle contraction is likely to be due to the more potent effects of AICAR to increase AMPK phosphorylation and activity [15,38]. The lower degree of contraction- and AICAR-stimulated Ser^{700} phosphorylation compared with Ser^{231} and Ser^{660} could be due to a number of factors, including a higher level of Ser^{700} phosphorylation in the basal state. The finding that insulin did not increase phosphorylation of Ser^{231}, Ser^{660} and Ser^{700} is also consistent with AMPK regulation, since insulin does not increase AMPK activity [38]. Thus while both insulin and contraction signalling converge at TBC1D1, the two stimuli exert a distinct pattern of phosphorylation on this Rab-GAP.

An important question is to determine the upstream kinase or kinases that mediate the effects of muscle contraction on TBC1D1 phosphorylation. Muscle contraction and exercise

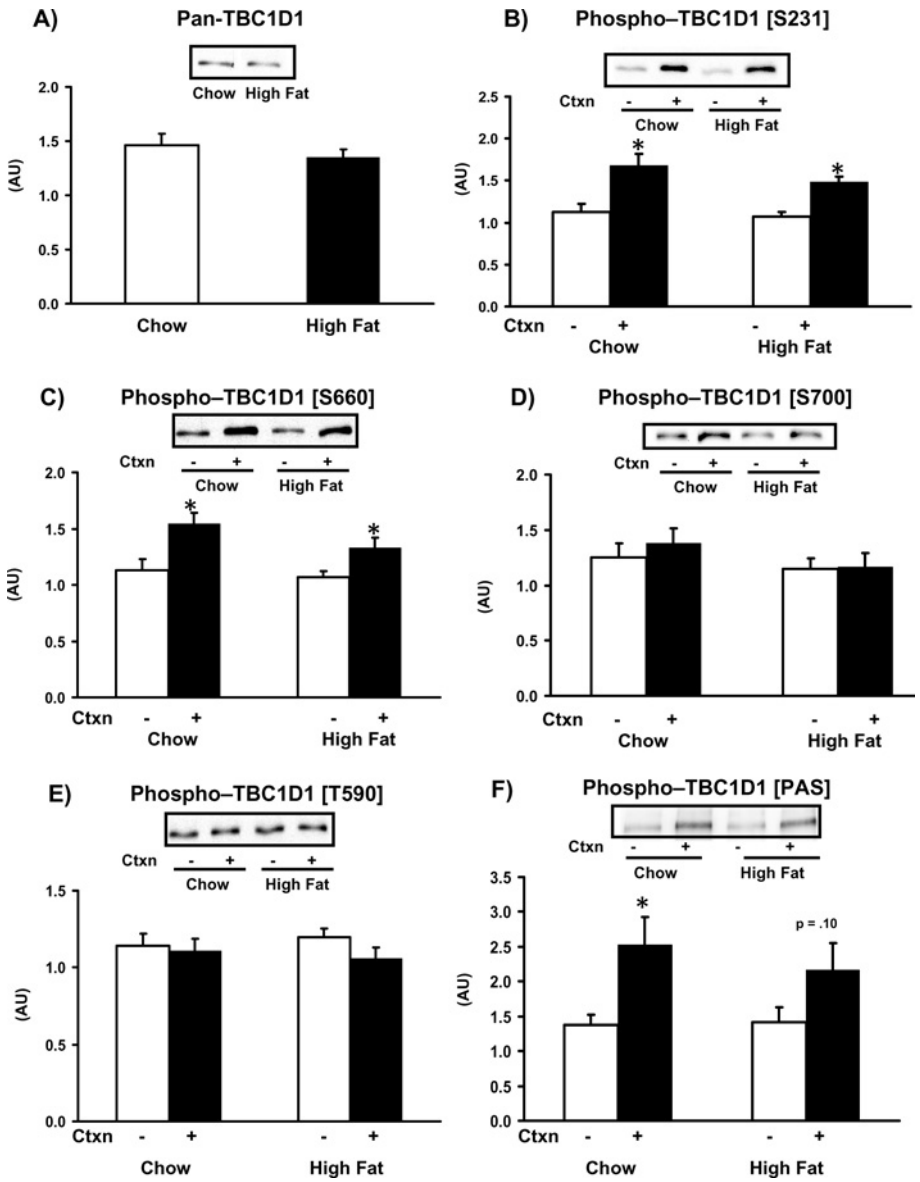

Figure 5 High-fat diet does not affect contraction-stimulated TBC1D1 phosphorylation

(**A**) TBC1D1 expression was not altered in tibialis anterior muscles of mice fed on a high-fat diet (60 % kcal of fat) for 9 weeks. (**B**–**F**) High-fat feeding had no effect on basal or contraction-stimulated levels of (**B**) TBC1D1 Ser^{231} phosphorylation, (**C**) TBC1D1 Ser^{660} phosphorylation, (**D**) TBC1D1 Ser^{700} phosphorylation, (**E**) TBC1D1 Thr^{590} or (**F**) PAS phosphorylation at 150–160 kDa. Data are means ± S.E.M.; $n = 6$/group. $^*P < 0.05$ compared with control. AU, arbitrary units; Ctxn, contraction.

activate numerous signalling responses in skeletal muscle including AMPK [39]. The basal level of phosphorylation of TBC1D1 on Ser^{231}, Ser^{660} and Ser^{700} was significantly reduced in muscles from AMPKα2i TG mice, demonstrating that even low levels of AMPK activity, which are found in the unstimulated or resting condition, are necessary to maintain TBC1D1 phosphorylation in the muscle. Contraction-stimulated TBC1D1 phosphorylation was significantly reduced in the AMPKα2i TG mice, but, interestingly, contraction still resulted in a small increase in phosphorylation of all three sites. Therefore TBC1D1 phosphorylation on Ser^{231}, Ser^{660} and Ser^{700} is not solely dependent on AMPK, implicating regulation by additional upstream kinases. One possibility is Akt2, since under some conditions muscle contraction increases Akt phosphorylation and activity [15,33,35]. However, given that we found both basal and contraction-stimulated TBC1D1 phosphorylation of Ser^{231}, Ser^{660} and Ser^{700} to be normal in the muscle of the Akt2 KO mice, it is unlikely that Akt2 plays a regulatory role. In addition to Akt, exercise and muscle contraction increase the activity of multiple kinases in skeletal muscle including ERK1/2 (extracellular-signal-regulated kinase 1/2), JNK (c-Jun N-terminal kinase), p38 MAPK (mitogen-activated protein kinase), the AMPK-related kinase SNARK (sucrose-non-fermenting protein kinase) and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) [39,40]. Given that AMPKα2-inactive TG mice and AMPKα2 KO mice have normal or near normal increases in contraction-stimulated glucose uptake [28,41,42], both *in vitro* and *in vivo*, the regulation of TBC1D1 phosphorylation by these other kinases may be critical in glucose uptake regulation. Interestingly, compound C has recently been reported to decrease

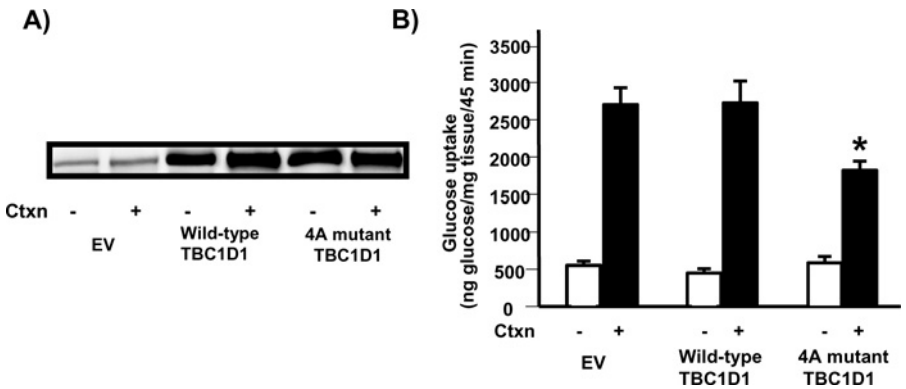

Figure 6 Overexpression of the TBC1D1 4A mutant impairs contraction-stimulated glucose uptake

To determine whether TBC1D1 phosphorylation at AMPK consensus sites regulates glucose uptake in contracted muscle, four phosphorylation sites on TBC1D1 (Ser^{231}, Thr^{499}, Ser^{660} and Ser^{700}) were mutated to alanine (4A) and this mutant and WT TBC1D1 were expressed in tibialis anterior muscles. (**A**) Representative Western blot of total TBC1D1 showing overexpression 7 days after plasmid DNA injections. (**B**) Contraction-stimulated glucose uptake was not affected by overexpression of WT TBC1D1, whereas expression of the TBC1D1 4A mutant impaired contraction-stimulated glucose uptake by 42 %. Data are means ± S.E.M.; $n = 8$–15/group. $^*P < 0.05$ compared with contraction-stimulated empty vector (EV) controls. Ctxn, contraction.

contraction-stimulated TBC1D1 PAS phosphorylation and glucose uptake in rat epitrochlearis muscle [43]. Whether this decrease in PAS phosphorylation was associated with decreases in AMPK activity or muscle contractility was not determined, making it difficult to determine the exact mechanism of the inhibitor compound. Nevertheless, this work is another example where TBC1D1 phosphorylation status is associated with glucose uptake. In future studies it will be important to determine whether these contraction-stimulated proteins or other proteins can behave as TBC1D1 kinases, and whether these kinases could function as redundant signals regulating glucose transport in the absence of AMPK.

Consistent with the fact that TBC1D1 Thr^{590} is a full Akt consensus motif, we found that insulin increased TBC1D1 Thr^{590} phosphorylation in skeletal muscle, and this effect of insulin was dependent on Akt2. TBC1D1 Thr^{590} has also been suggested to be a partial AMPK consensus motif [26], but, in our present study, contraction and AICAR had no effect on Thr^{590} phosphorylation. In contrast, it has been reported that contraction increased TBC1D1 Thr^{590} phosphorylation, and that this increase was abolished in AMPKα2 kinase-dead mice [24]. The reason for this discrepancy is not obvious, but could be due to differences in stimulation protocols or differences in antibody specificity. The function of Thr^{590} is not known, but there are several lines of evidence to suggest that it may not function in the regulation of glucose uptake in adult skeletal muscle. While it is established that insulin increases TBC1D1 Thr^{590} phosphorylation in muscle (the present study and [24]), phosphorylation of this site has no effect on 14-3-3 binding, and this binding is thought to be essential for the regulation of glucose uptake [24]. Furthermore, we found that mutation of Thr^{590} to alanine, along with three other conserved TBC1D1 phosphorylation sites, had no effect on insulin-stimulated glucose uptake in muscle [27]. In addition, in the present study the contraction protocol used stimulated glucose uptake without concomitant phosphorylation of Thr^{590}, providing evidence that this site is also not essential for contraction-stimulated glucose uptake. It is also interesting that TBC1D1 Thr^{590} phosphorylation does fully correlate with contraction- and AICAR-stimulated PAS phosphorylation. It is possible that a site other than TBC1D1 Thr^{590}, such as Ser^{501}, which is an established Akt site [26], may be more critical in regulating insulin-stimulated glucose uptake in skeletal muscle.

Our previous study has shown that mutation of four highly conserved TBC1D1 phosphorylation sites that represent both Akt or AMPK consensus motifs (Ser^{231}, Thr^{499}, Thr^{590} and Ser^{621}) impaired contraction-stimulated glucose uptake, but had no effect on insulin-stimulated glucose uptake. However, the decrease in contraction-stimulated glucose uptake was 22 %, suggesting that other sites on TBC1D1 may also function in contraction-regulated glucose uptake. In the present study we mutated the phosphorylation sites that we had shown were responsive to contraction (Ser^{231}, Ser^{660} and Ser^{700}), as well as Thr^{499}, an AMPK consensus site [21]. In mutating all four of these sites simultaneously, we found an even greater decrease in contraction-stimulated glucose transport (42 %) compared with our previous study (22 %) [27]. Interestingly, we found that expression of single point mutants had no effect on contraction-stimulated glucose uptake in skeletal muscle. Taken together, these data raise the possibility that multiple phosphorylation sites may work co-operatively to control TBC1D1 Rab-GAP activity and, subsequently, glucose uptake.

In conclusion, signals emanating from muscle contraction and insulin both phosphorylate TBC1D1, but the pattern of phosphorylation is distinct for the two stimuli. AMPKα2 and Akt2 phosphorylate TBC1D1, but there must be additional upstream kinases that phosphorylate TBC1D1 in skeletal muscle. We found that contraction-stimulated TBC1D1 phosphorylation was not affected by diet-induced insulin resistance, consistent with most of the literature showing that high-fat-fed animals have normal increases in exercise- or contraction-stimulated glucose uptake [44,45]. Our data establish TBC1D1 as an important regulator of contraction-stimulated glucose uptake in skeletal muscle. Given that TBC1D1 expression is highly specific to skeletal muscle, pharmacological inhibition of TBC1D1 Rab-GAP activity may be a desirable means to enhance glucose uptake and lower blood glucose concentrations, without off-target effects in other tissues.

AUTHOR CONTRIBUTION

Kanokwan Vichaiwong, Suneet Purohit and Michael Hirshman designed and performed the experiments, and contributed to writing the manuscript. Taro Toyoda and Ding An contributed to discussion and performed the experiments. Niels Jessen contributed to discussion and edited the manuscript. Laurie Goodyear designed the experiments, contributed to discussion, and wrote and edited the manuscript.

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online data

SUPPLEMENTARY ONLINE DATA

Contraction regulates site-specific phosphorylation of TBC1D1 in skeletal muscle

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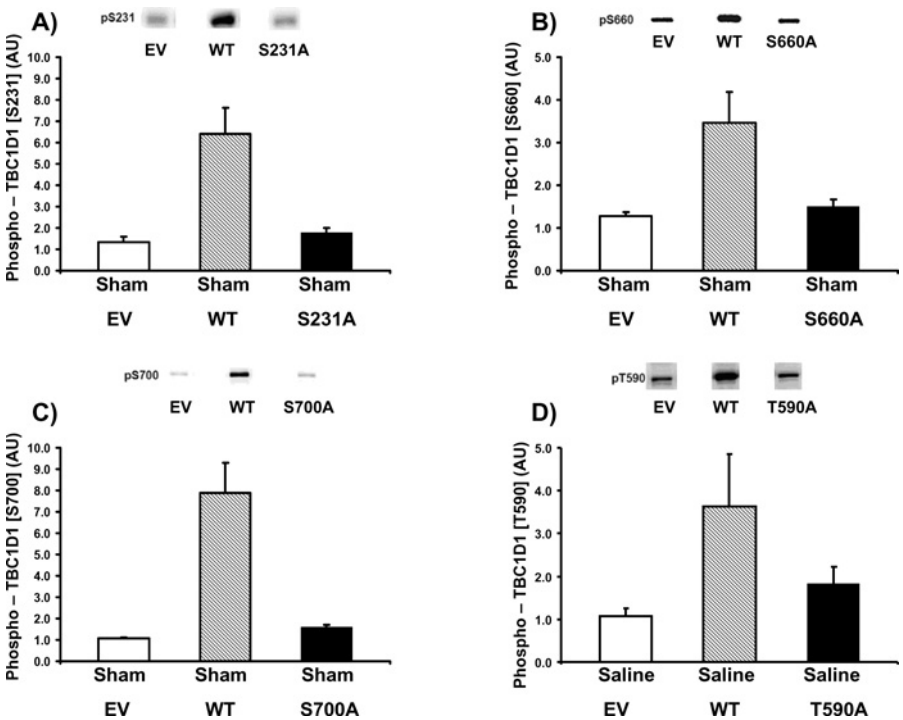

Figure S1 Specificity of anti-phospho-TBC1D1 Ser^{231}, Thr^{590}, Ser^{660} and Ser^{700} antibodies

Point mutations of the TBC1D1 phosphorylation sites Ser^{231} (S231A), Thr^{590} (T590A), Ser^{660} (S660A) and Ser^{700} (S700A) to alanine were generated separately. Empty vector (EV) control, TBC1D1 WT and the four TBC1D1 single alanine mutants were injected into tibialis anterior muscles followed by *in vivo* electroporation. Muscles were collected 1 week later. Immunoblotting was with anti-phospho-TBC1D1 (**A**) Ser^{231}, (**B**) Ser^{660}, (**C**) Ser^{700} and (**D**) Thr^{590} antibodies. Data are means ± S.E.M.; $n = 3$–6/group. Au, arbitrary units.

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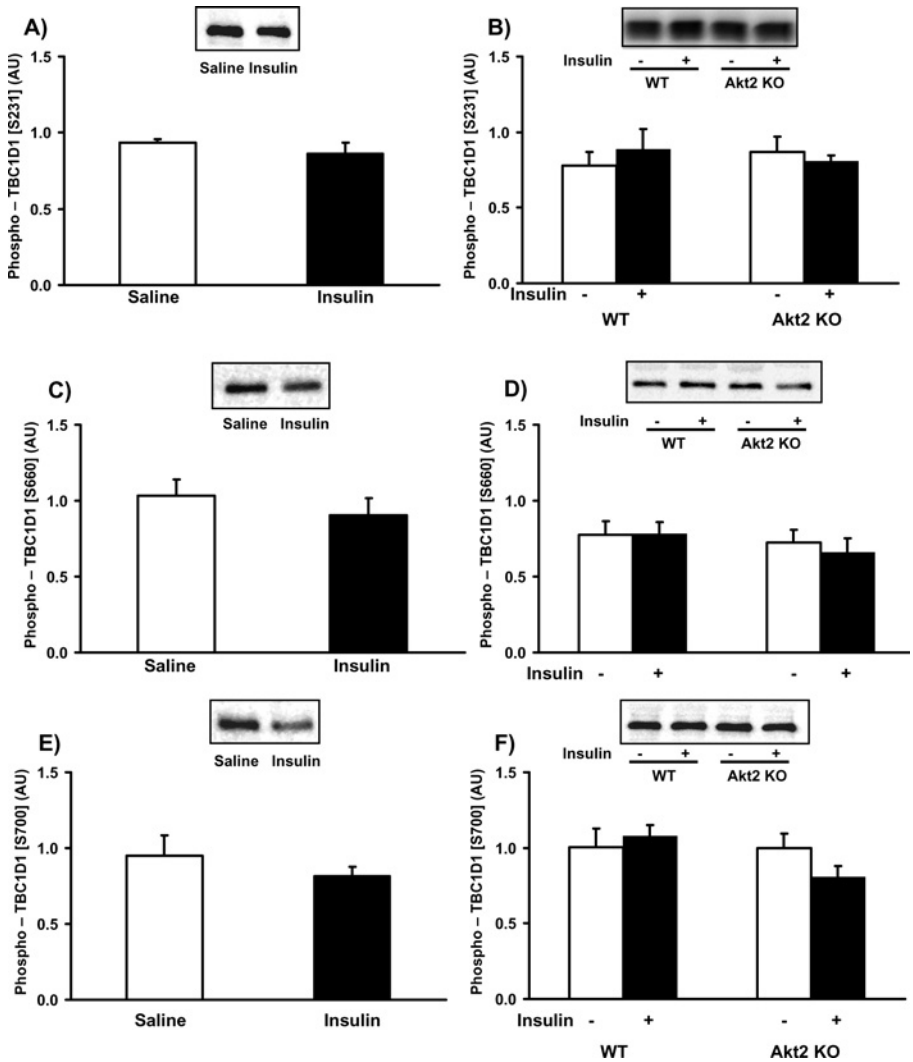

Figure S2 TBC1D1 Ser^{231}, Ser^{660} and Ser^{700} phosphorylation are not increased by insulin

(**A**) Maximal insulin injection did not affect TBC1D1 Ser^{231} phosphorylation in ICR mice. Mice were fasted and injected intraperitoneally with maximal insulin, and tibialis anterior muscles were dissected 10 min later. (**B**) Akt2 KO had normal levels of basal and insulin-stimulated TBC1D1 Ser^{231} phosphorylation. (**C**) Maximal insulin injection did not affect TBC1D1 Ser^{660} phosphorylation in ICR mice. (**D**) Akt2 KO had normal levels of basal and insulin-stimulated TBC1D1 Ser^{660} phosphorylation. (**E**) Maximal insulin injection did not affect TBC1D1 Ser^{700} phosphorylation in ICR mice. (**F**) Akt2 KO had normal levels of basal and insulin-stimulated TBC1D1 Ser^{700} phosphorylation. Data are means ± S.E.M.; $n = 5$–7/group. Au, arbitrary units.

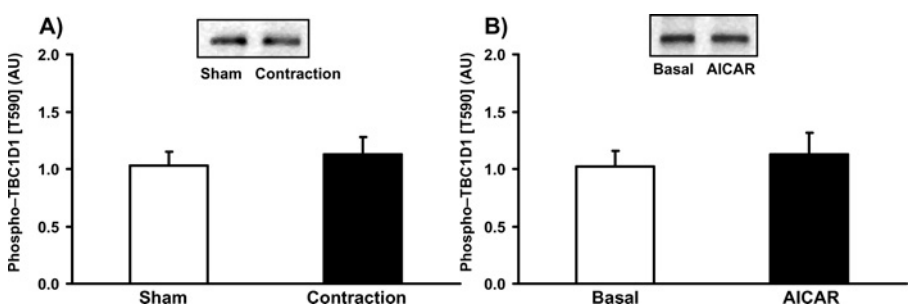

Figure S3 TBC1D1 Ser^{590} was not increased by contraction or AICAR

(**A**) Contraction *in situ* for 15 min did not increase TBC1D1 Ser^{590} phosphorylation in tibialis anterior muscles from ICR mice. (**B**) AICAR incubation did not increase TBC1D1 Ser^{590} phosphorylation in EDL muscles from ICR mice. Data are means ± S.E.M.; $n = 5$–7/group. Au, arbitrary units.

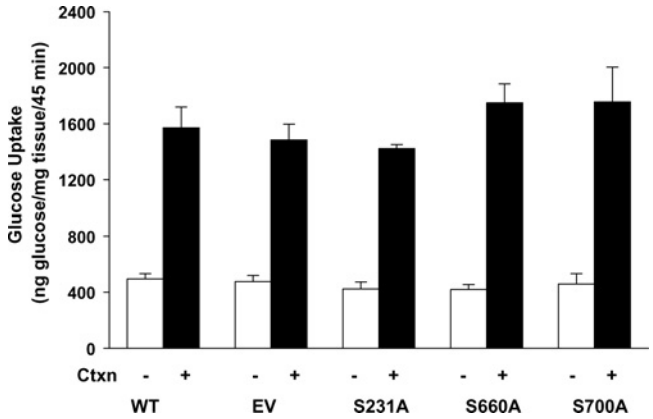

Figure S4 Single point mutations of TBC1D1 do not alter glucose uptake

A separate cohort of mice, including different control empty vector (EV) and WT TBC1D1-injected mice were used to characterize the effects of single mutations on glucose uptake. Individual point mutations of the TBC1D1 phosphorylation sites Ser^{231} (S231A), Thr^{590} (T590A), Ser^{660} (S660A) and Ser^{700} (S700A) to alanine were generated. EV control, TBC1D1 WT and the four TBC1D1 single alanine mutants were injected into tibialis anterior muscles followed by *in vivo* electroporation and 7 days later basal and contraction-stimulated glucose uptake were measured *in vivo*. Basal and contraction-stimulated glucose uptake were not affected by overexpression of WT TBC1D1 or single mutant TBC1D1. Data are means ± S.E.M.; $n = 4$–12/group.

Table S1 Characteristics of high-fat-fed animals

Mice were fed a high-fat diet (60% kcal of fat) for 9 weeks. Mice fed the high-fat diet had significantly higher body weights, fasting blood glucose concentrations and fasting insulin concentrations compared with chow-fed mice. Data are means ± S.E.M.; $n = 6$/group. $^*P < 0.05$ compared with the chow diet.

Characteristic	Chow ($n = 6$)	High fat ($n = 6$)
Body weight (g)	25.2 ± 1.2	33.4 ± 1.2*
Glucose levels (mg/dl)	142.0 ± 17.4	185.2 ± 8.5*
Insulin levels (ng/ml)	0.68 ± 0.12	2.42 ± 0.16*

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