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Acute exercise and physiological insulin induce distinct phosphorylation signatures on TBC1D1 and TBC1D4 proteins in human skeletal muscle

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Key points

- Phosphorylation signature patterns on TBC1D1 and TBC1D4 proteins in the insulin–glucose pathway were investigated in human skeletal muscle in response to physiological insulin and exercise.
- In response to postprandial increase in insulin, Akt phosphorylation of T308 and S473 correlated significantly with sites on TBC1D1 (T596) and TBC1D4 (S318, S341, S704).
- Exercise induced phosphorylation of TBC1D1 (S237, T596) that correlated significantly with activity of the $\alpha2/\beta2/\gamma3$ AMPK trimer, whereas TBC1D4 phosphorylation (S341, S704) with exercise correlated with activity of the $\alpha2/\beta2/\gamma1$ AMPK trimer.
- TBC1D1 phosphorylation signatures with exercise/muscle contraction were comparable between human and mouse skeletal muscle, and AMPK regulated phosphorylation of these sites in mouse muscle, whereas contraction and exercise elicited different TBC1D4 phosphorylation patterns in mouse compared with human muscle.
- Our results show differential phosphorylation of TBC1D1 and TBC1D4 in response to physiological stimuli in human skeletal muscle and indicate that Akt and AMPK may be upstream kinases.

Abstract We investigated the phosphorylation signatures of two Rab-GTPase activating proteins TBC1D1 and TBC1D4 in human skeletal muscle in response to physical exercise and physiological insulin levels induced by a carbohydrate rich meal using a paired experimental design. Eight healthy male volunteers exercised in the fasted or fed state and muscle biopsies were taken before and immediately after exercise. We identified TBC1D1/4 phospho-sites that (1) did not respond to exercise or postprandial increase in insulin (TBC1D4: S666), (2) responded to insulin only (TBC1D4: S318), (3) responded to exercise only (TBC1D1: S237, S660, S700; TBC1D4: S588, S751), and (4) responded to both insulin and exercise (TBC1D1: T596; TBC1D4: S341, T642, S704). In the insulin-stimulated leg, Akt phosphorylation of both T308 and S473 correlated significantly with multiple sites on both TBC1D1 (T596) and TBC1D4 (S318, S341, S704). Interestingly, in the exercised leg in the fasted state TBC1D1 phosphorylation (S237, T596) correlated significantly with the activity of the $\alpha2/\beta2/\gamma3$ AMPK trimer, whereas TBC1D4 phosphorylation (S341, S704) correlated with the activity of the $\alpha2/\beta2/\gamma1$ AMPK trimer. Our data show differential phosphorylation of TBC1D1 and TBC1D4 in response to physiological stimuli in human skeletal muscle and support the idea that Akt and AMPK are upstream kinases. TBC1D1 phosphorylation signatures were comparable between *in vitro* contracted mouse skeletal muscle and exercised

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human muscle, and we show that AMPK regulated phosphorylation of these sites in mouse muscle. Contraction and exercise elicited a different phosphorylation pattern of TBC1D4 in mouse compared with human muscle, and although different circumstances in our experimental setup may contribute to this difference, the observation exemplifies that transferring findings between species is problematic.

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Abbreviations ACC, acetyl-CoA carboxylase; Akt, serine/threonine-specific protein kinase; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; AU, arbitrary units; EDL, extensor digitorum longus; GLUT4, glucose transporter 4; IB, immunoblot; IP, immunoprecipitation; KD, kinase dead; KO, knockout; PAS, phospho-Akt substrate; Rab-GAP, Rab-GTPase activating protein; TBC1D1/4, TBC1 domain family member 1/4; WT, wild-type; 14-3-3, highly conserved regulatory eukaryotic proteins binding to phosphorylated sites of target proteins.

Introduction

Skeletal muscles constitute a major sink for whole body glucose disposal as evidenced by glucose uptake measurements during hyperinsulinaemic euglycaemic clamp conditions in non-insulin resistant individuals (DeFronzo *et al.* 1981; Richter *et al.* 1989). GLUT4 is the main glucose transporter in skeletal muscle and adipose tissue located within the endosomal and the trans-Golgi network compartments and to small tubule–vesicular structures (Slot *et al.* 1991; Ploug *et al.* 1998). In response to certain stimuli (e.g. muscle contraction, exercise, insulin, or hypoxia; Klip *et al.* 1987; Douen *et al.* 1989; Cartee *et al.* 1991; Kristiansen *et al.* 1996) these transporters are mobilized to the sarcolemma and/or T-tubules to facilitate glucose uptake. Skeletal muscle thus represents an important tissue for studying the intracellular signalling pathways leading to incorporation of GLUT4 molecules into the cell surface membrane.

The signalling pathway in skeletal muscle leading to glucose uptake in response to contraction is not completely understood. It is thought to involve increases in intracellular Ca^{2+} and activation of kinases downstream of LKB1 (i.e. 5′-AMP activated protein kinase (AMPK) and sucrose nonfermenting AMPK-related kinase (SNARK); Friedrichsen *et al.* 2012; Jensen & Richter, 2012). The signalling pathway whereby insulin stimulates glucose uptake has been resolved in greater detail, and is known to involve the insulin receptor – insulin receptor substrate – phosphatidylinositol 3 kinase (IR-IRS-PI3K)–Akt2 axis (Huang & Czech, 2007; Ishikura *et al.* 2008). Akt2 activation is necessary for insulin-stimulated GLUT4 translocation to the plasma membrane in adipocytes (Hill *et al.* 1999) and for insulin-stimulated glucose uptake in skeletal muscle (Cho *et al.* 2001; Bouzakri *et al.* 2006). In the search for novel Akt targets involved in these processes, an Akt substrate of 160 kDa (AS160), known as TBC1D4 (Nagase *et al.* 1998), was identified as a Rab-GTPase activating protein (Rab-GAP) (Sano *et al.* 2003). It is now evident that TBC1D4 has multiple upstream kinases also including AMPK (Kramer *et al.* 2006*a*; Treebak *et al.* 2010) and potentially protein kinase Cζ (Ng *et al.* 2010).

The current thinking is that Rab-GAPs in the signalling pathways to stimulate glucose uptake repress Rab-GTPase function in the basal state by inhibiting the exchange of GDP to GTP leading to intracellular retention of GLUT4 vesicles (Kane *et al.* 2002; Sakamoto & Holman, 2008). In response to stimulation Rab-GAPs are phosphorylated, for instance by Akt or AMPK, allowing for the active exchange of GDP to GTP in the Rab-GTPase and thus stimulating GLUT4 mobilization to the plasma membrane (Hoffman & Elmendorf, 2011). In addition to TBC1D4, the TBC1D4 paralogue TBC1D1 has been identified as a functional Rab-GAP. Similarly to TBC1D4, TBC1D1 has inhibitory effects on GLUT4 translocation in 3T3-L1 adipocytes (Roach *et al.* 2007) and skeletal muscle (An *et al.* 2010). TBC1D4 and TBC1D1 are involved in regulation of both insulin- and contraction-induced glucose uptake in intact mouse skeletal muscle (Kramer *et al.* 2006*b*; An *et al.* 2010), and TBC1D4 has emerged as a nexus for insulin- and contraction-responsive signals, potentially mediating enhanced insulin action in human and rat skeletal muscle after exercise (Arias *et al.* 2007; Funai *et al.* 2009; Treebak *et al.* 2009*b*; Pehmøller *et al.* 2012).

The phosphorylation status of TBC1D1 and TBC1D4 in response to insulin stimulation in human skeletal muscle has been studied only during hyperinsulinaemic euglycaemic clamp conditions and most studies have used the phospho-Akt substrate (PAS) antibody (Karlsson *et al.* 2005*a*,*b*; O'Gorman *et al.* 2006; Frøsig *et al.* 2007; Howlett *et al.* 2007, 2008; Højlund *et al.* 2008; Hoeg *et al.* 2011), whereas other studies (Treebak *et al.* 2009*b*; Vind *et al.* 2011; Pehmøller *et al.* 2012; Vendelbo *et al.* 2012; Consitt *et al.* 2013) have used site- and phospho-specific antibodies. No study has investigated whether physiological

insulin levels (i.e. insulin levels obtained after a meal) would induce changes in phosphorylation status of the two proteins similarly to those observed during sustained elevated insulin levels as applied during the hyperinsulinaemic clamp condition. Moreover, acute exercise in humans is known to induce changes in phosphorylation status of both TBC1D1 and TBC1D4 (Frøsig *et al.* 2010; Jessen *et al.* 2011), but the phosphorylation response to physiological insulin stimulation combined with acute exercise remains to be established. Here we employed the one-legged knee extensor exercise model (Andersen *et al.* 1985) to investigate skeletal muscle site-specific phosphorylation signature patterns of TBC1D1 and TBC1D4 in response to exercise in the fasted or fed condition of healthy human subjects. This design allows for a paired evaluation of the phosphorylation signatures in skeletal muscle at rest and in response to acute exercise alone, physiological insulin stimulation alone, and acute exercise combined with physiological insulin stimulation. We hypothesized the presence of stimuli-dependent phosphorylation signatures on TBC1D1 and TBC1D4. Identification of these signatures in response to physiological stimuli will increase the understanding of how TBC1D1 and TBC1D4 are regulated and how these proteins may impact on metabolism in human skeletal muscle.

Methods

Ethical approval

The human experiments in this study were conducted in accordance with the Helsinki II Declaration and approved by the ethics committee of Capital Region of Denmark (no. KF1277313). Protocols for animal studies were approved by the Danish Animal Experimental Inspectorate and complied with the European Convention for the protection of Vertebrate Animals used for Experiments and Other Scientific Purposes (Council of Europe 123, Strasbourg, France, 1985), UK regulations, and the policies of *The Journal of Physiology*.

Subjects and initial tests

Eight healthy moderately trained male subjects (age 27 ± 2 years, weight 83.5 ± 2.6 kg, body mass index 24.8 ± 0.6 kg m^{-2}) volunteered for this study and gave their written informed consent prior to participation. Subjects initially came to the laboratory on two separate days to complete an incremental cycle ergometer test to determine peak oxygen uptake ($\dot{V}_{O_2peak}$ 52 ± 2 ml O_2 min^{-1} kg^{-1}), and an incremental one-legged knee extensor exercise test to determine the maximal work load for the knee extensors (51 ± 2 W). Half of the subjects exercised with their dominant leg and the other half with their non-dominant leg.

Experimental days

Two–three weeks after the initial baseline testing, the subjects came by car or public transportation to the laboratory on two separate occasions. Subjects were instructed to refrain from physical activity 2 days prior to the experiment. On both occasions, subjects came in at 07.00 h after an overnight fast. Upon arrival at the laboratory subjects rested for 30–40 min in the supine position, after which one muscle biopsy was obtained from the vastus lateralis muscle of each leg. Subjects rested for 20 min and then performed a single, 60 min bout of one-legged knee extensor exercise at 80% of peak work load (41 ± 2 W) with two 5 min intervals at 100% of peak work load (51 ± 2 W) after 15 and 35 min. The other leg served as a rested control. On one occasion subjects performed the exercise bout after an overnight fast and on another occasion subjects were given a carbohydrate rich (∼80 E%) meal consisting of cornflakes with skimmed milk corresponding to 5% (∼600–800 kJ) of their daily energy intake. This meal was given after the resting biopsy had been obtained and approximately 15 min before the exercise bout commenced. One participant only took part in the exercise trial in the fed state. Thus $n = 7$ completed the fasted trial and $n = 8$ completed the fed trial. Immediately after termination of the exercise bout muscle biopsies were obtained from vastus lateralis muscles of both legs. Thus, a total of eight (2 × 4) biopsies were obtained from each subject. All biopsies were obtained from different incisions, separated by at least 5 cm. Muscle biopsies were obtained under local anaesthesia (2–4 ml of 2% lidocaine) and were immediately stored at −80°C.

Incubation of intact mouse skeletal muscle *in vitro*

Extensor digitorum longus (EDL) and soleus muscles were quickly removed from anaesthetized (intraperitoneal injection of pentobarbital; 6 mg/100 g body $weight^{-1}$) fed male mice aged 16–20 weeks and incubated in Krebs-Henseleit buffer (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 24.6 mM $NaHCO_3$ with addition of 8 mM mannitol, 2 mM pyruvate, and 0.1% BSA) at 30°C, oxygenated with a gas containing 95% O_2 and 5% CO_2; Multi Myograph system, DMT, Denmark). To assess the effects of contraction on AMPK signalling and TBC1D1 and TBC1D4 phosphorylation, muscles from α2 AMPK KO/KD mice (Mu *et al.* 2001; Viollet *et al.* 2003) and corresponding wild-type (WT) littermates ($n = 8$–12) were incubated for 40 min. Subsequently, contraction was induced by electrical stimulation with a 10 s train (100 Hz, 0.2 ms impulse, ∼30 V) per minute for 10 min. Contralateral muscles were incubated for the same duration under resting condition. After incubation, muscles were harvested, washed in ice-cold Krebs-Henseleit buffer, blotted on filter paper, and quickly

frozen with aluminium tongs pre-cooled in liquid nitrogen and stored at −80°C. We chose to determine TBC1D1 phosphorylation patterns in response to contraction in EDL muscle and TBC1D4 phosphorylation patterns in response to contraction in soleus muscles. This was in part due to the restricted number of animals available for these analyses, but also because of our previous findings showing that TBC1D1 is more abundant in EDL compared with soleus and that TBC1D4 is more abundant in soleus compared with EDL (Pehmøller *et al.* 2009).

Mouse treadmill running protocol

Five days prior to the experimental day, male C57BL/6J WT mice ($n = 24$, 12–14 weeks of age) were familiarised to treadmill running for four consecutive days (10–15 min at 8–18 m min^{-1}). Mice rested 1 day prior to the experiment. On the day of the experiment mice ($n = 12$) were run on a motorized treadmill (Exer 3/6, Columbus Instruments, OH, USA) for 1 h at 18 m min^{-1} with an incline of 5 deg. Control mice ($n = 12$) were rested on a dummy treadmill (no motor) that was placed next to the motorized treadmill so that control mice were exposed to the same environment as the exercised mice. Immediately after exercise mice were killed by cervical dislocation and soleus and EDL muscles were rapidly dissected and quickly frozen in liquid nitrogen. Samples were stored at −80°C until processed as described below.

Blood chemistry

Plasma glucose and lactate levels were determined using an ABL 615 (Radiometer Medical A/S, Denmark). Plasma insulin levels were determined by ELISA (EIA1825, DRG International, Inc., Mountainside, NJ, USA).

Glycogen

Muscle glycogen was measured as glucosyl units after acid hydrolysis. The analysis was performed by fluorometry using freeze-dried, dissected muscle specimens (Passonneau *et al.* 1967).

Muscle sample preparation

A piece of each human muscle sample was freeze-dried and dissected free of visible fat and connective tissue under a microscope. Human and mouse muscle samples were homogenized in ice-cold buffer (50 mM Hepes, 150 mM NaCl, 20 mM $Na_4P_2O_7$, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM Na_3VO_4, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin and 3 mM benzamidine). Homogenates were rotated end-over-end (4°C, 1 h) and were subjected to centrifugation (16,000 *g*, 20 min, 4°C). Supernatants were harvested and protein concentrations were determined (BCA no. 23225, Pierce, Rockford, IL, USA).

Antibodies

The phosphorylation status of TBC1D1/TBC1D4 was measured using a phospho-Akt-substrate (PAS) antibody (no. 9611, Cell Signalling Technology, MA, USA), and site- and phospho-specific antibodies against TBC1D4 (S318, S341, S588, T642, S666, S704 and S751; Geraghty *et al.* 2007; Treebak *et al.* 2010) and TBC1D1 (S237, T596, S660, and S700; Vichaiwong *et al.* 2010), as previously described and validated. All phosphorylation sites refer to the human sequence for TBC1D1 (UniProt entry Q86TI0) and TBC1D4 (UniProt entry O60343), except for S660 and S700 on TBC1D1 which refer to the mouse sequence (UniProt entry Q60949). Supplemental Table S1 provides an overview of the surrounding amino acids of the different phospho-sites. Total TBC1D4 protein was determined using an antibody specific for human TBC1D4 (ab24469, Abcam plc, Cambridge, UK) and total TBC1D1 protein was determined with an antibody specific for human TBC1D1 as described previously (Chen *et al.* 2008). Phosphorylation of T172 on AMPK (no. 2531, Cell Signalling Technology), S221 on acetyl-CoA carboxylase (ACC; no. 07-303, Upstate), Akt on T308 (no. 06-678 Upstate), and on S473 (no. 9271, Cell Signalling Technology) was measured using the site- and phospho-specific antibodies indicated. Total Akt was measured using antibody no. 9272 from Cell Signalling Technology, and AMPK α1 and α2 subunits were detected using antibodies kindly provided by Professor D. Grahame Hardie (University of Dundee, UK).

SDS-PAGE and Western blot analyses

Western blot analyses were performed as previously described (Treebak *et al.* 2007). Membranes probed with the phospho-specific antibodies for TBC1D4/TBC1D1 were stripped in a buffer containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) and re-probed with antibodies recognizing total TBC1D4 and TBC1D1 protein. The signal coming from the phospho-specific antibodies was related to total TBC1D4/TBC1D1 in the sample. Human muscle samples from the two exercise trials (i.e. fasted and fed) were run on the same gel during Western blot analyses, allowing for direct comparisons between the two trials.

AMPK activity assay

AMPK activity was measured on muscle lysates by immunoprecipitation (IP) of the γ3 subunit (isolating the α2/β2/γ3 complex from human and mouse lysate) followed by IP of the α2 (isolating the α2/β2/γ1 from human

lysate, and the $\alpha2/\beta1/\gamma1$ and $\alpha2/\beta2/\gamma1$ from mouse lysate) and lastly the $\alpha1$ subunit (isolating the $\alpha1/\beta2/\gamma1$ from human lysate, and the $\alpha1/\beta1/\gamma1$ and $\alpha1/\beta2/\gamma1$ from mouse lysate) as described previously (Birk & Wojtaszewski, 2006; Treebak *et al.* 2009*a*).

14-3-3 overlays

14-3-3 overlays were carried out as previously described (Pozuelo Rubio *et al.* 2004; Pehmøller *et al.* 2009). Briefly, endogenous TBC1D4 and TBC1D1 protein was immunoprecipitated from 150 μg of skeletal muscle lysates, electrophoretically separated on Invitrogen Nu-PAGE gels, and subsequently transferred to polyvinylidene difluoride membranes (Millipore, Denmark). Membranes were subsequently probed with digoxigenin-labelled 14-3-3 at 4°C overnight followed by incubation with a HRP-conjugated anti-digoxigenin antibody (Roche, UK). Immuno-complexes were visualised and quantified using a Kodak Image Station (2000MM, Kodak, Denmark) and normalised to TBC1D4/TBC1D1 protein.

Statistics

Statistical analyses were performed in SigmaPlot 11.0 (Systat software, Inc., Germany). Unpaired *t* tests and two-, or three-way ANOVAs with repeated measures were used to analyse the data. When sets of data contained more than two factors, data obtained from the samples taken before the exercise bout commenced were analysed to test whether there were differences between the fasted and the fed trial and between the rested and the exercised leg, respectively. In all cases, except for the TBC1D4 pT642 and $\alpha1/\beta2/\gamma1$ AMPK activity data, there were no differences between the trials or legs before the exercise. Thus, within each trial, the delta values (e.g. rested leg post exercise minus rested leg before exercise) were calculated and the resulting set of data was analysed by two-way repeated measures ANOVA. Although presented in the same figure panel (Fig. 7*E*), the TBC1D4 pT642 data from the fasted and the fed trials have been analysed separately by a two-way ANOVA with repeated measures. The $\alpha1/\beta2/\gamma1$ AMPK activity data were analysed with a three-way ANOVA with repeated measures (Fig. 3*C*). In the case of interaction effects, the Tukey test was used *post-hoc* in all analyses. Correlation analyses were performed by calculating Pearson's product moment correlation coefficient. Statistical significance was accepted at $P < 0.05$.

Results

Blood chemistry and muscle glycogen

We set out to investigate the specific phosphorylation patterns on TBC1D1 and TBC1D4 in response to an acute bout of one-legged knee extensor exercise, acute physiological 'insulin' stimulation induced by a small carbohydrate rich meal, and the two stimuli combined. Using the one-legged knee extensor exercise model in the fasted and fed condition combined with biopsies from both legs in each trial, muscle samples were obtained representing four different experimental conditions. One leg was rested (non-stimulated), one leg was rested and insulin-stimulated, one leg was exercised, and one leg was exercised and insulin-stimulated. When the subjects were fed a meal just prior to (i.e. 15 min) the exercise bout, plasma glucose levels were significantly elevated compared with the fasted trial (Fig. 1*A*). At baseline, plasma insulin levels were not different between the two trials, but after the meal, plasma insulin levels were >6-fold higher than during the fasted trial (Fig. 1*B*). During the course of the exercise period in the fasted state, plasma insulin levels did not change significantly. However, in the fed state, plasma insulin levels decreased significantly after 30 and 60 min but remained elevated compared with the fasted trial (Fig. 1*B*). Plasma lactate increased to a similar extent in response to the exercise bout in the fasted and fed trials (Fig. 1*C*), and muscle glycogen was similarly reduced in the exercised leg in the two trials (Fig. 1*D*).

Activation of potential upstream TBC1D1/4 kinases

AMPK and Akt are putative upstream kinases of TBC1D1/4 (Kane *et al.* 2002; Treebak *et al.* 2006; Kramer *et al.* 2006*a*; Pehmøller *et al.* 2009; Vichaiwong *et al.* 2010). Furthermore, these kinases are known to be activated by insulin and exercise. Phosphorylation of AMPK on T172 is essential for its activation (Hawley *et al.* 1996) and S221 on ACC is a well-established AMPK target (Ha *et al.* 1994). AMPK and ACC were phosphorylated in response to exercise in the exercised leg only, and the increase was similar in the fasted and fed condition (Fig. 2*A* and *B*). Full activation of Akt requires phosphorylation of T308 and S473 (Alessi *et al.* 1996). It has previously been shown that Akt phosphorylation can be induced by submaximal insulin levels (Karlsson *et al.* 2006) and exercise (Sakamoto *et al.* 2004; Deshmukh *et al.* 2006; Treebak *et al.* 2007) in human skeletal muscle. To our knowledge no-one has investigated whether the two stimuli together have additive effects on Akt phosphorylation in human skeletal muscle. In the fasted state, phosphorylation of T308 did not increase significantly with exercise (Fig. 2*C*). In the fed state, on the other hand, T308 phosphorylation was significantly increased in both the rested and exercised leg. Interestingly, we observed an interaction effect indicating a synergistic effect on T308 when exercising in the fed state (Fig. 2*C*). Contrary to T308, phosphorylation on S473 increased significantly with exercise in the fasted state (Fig. 2*D*).

Furthermore, S473 phosphorylation was increased in the rested leg in the fed state, and there was an additive effect on S473 phosphorylation when the two stimuli were combined (Fig. 2*D*).

Specific AMPK trimers are activated in response to one-legged knee extensor exercise

Phosphorylation of AMPK at T172 correlates well with total AMPK kinase activity in human skeletal muscle during whole body exercise (Wojtaszewski *et al.* 2002; Birk & Wojtaszewski, 2006). However, it is not known whether specific AMPK trimer complexes are activated in response to one-legged knee extensor exercise. Furthermore, in order for us to assess the association between specific upstream AMPK kinase complexes and the phosphorylation of TBC1D1 and TBC1D4, we measured the activity of the three different AMPK trimer complexes found in human skeletal muscle (Wojtaszewski *et al.* 2005). Activity of both AMPK $\alpha2/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma3$ trimers increased in the exercised leg only and there was no statistical difference between the fasted and fed condition (Fig. 3*A* and *B*). The AMPK $\alpha1/\beta2/\gamma1$ complex did not respond to exercise, but the activity was higher in the fed compared with the fasted trial (Fig. 3*C*).

TBC1D1 phosphorylation signatures in human skeletal muscle

Site-specific phosphorylation of TBC1D1 in human skeletal muscle in response to exercise has been studied recently (Frøsig *et al.* 2010; Jessen *et al.* 2011), but no prior study has investigated the effect of physiological insulin stimulation or the interaction between exercise and physiological insulin stimulation. Here we found that exercise, but not physiological insulin, increased phosphorylation of S237, a site regulated by AMPK $\alpha2$ in response to muscle contraction *ex vivo* in mice (Pehmøller *et al.* 2009; Frøsig *et al.* 2010; Fig. 4*A*). On the other hand, T596 phosphorylation was regulated by both exercise (~70%) and physiological insulin (~35%), and the two stimuli combined gave an additive response (Fig. 4*B*). TBC1D1 has two other phosphorylation sites (S660 and S700; residues refer to the mouse sequence of TBC1D1; Jessen *et al.* 2011) that have been predicted to be phosphorylated by AMPK (Taylor *et al.* 2008). This was recently confirmed for S660 (Vichaiwong *et al.* 2010). In addition, S660 has been shown to be phosphorylated in response to exercise in human skeletal muscle (Jessen *et al.* 2011). Here we show that both S660 and S700 are phosphorylated exclusively by exercise and not by physiological insulin in human skeletal muscle (Fig. 4*C* and *D*).

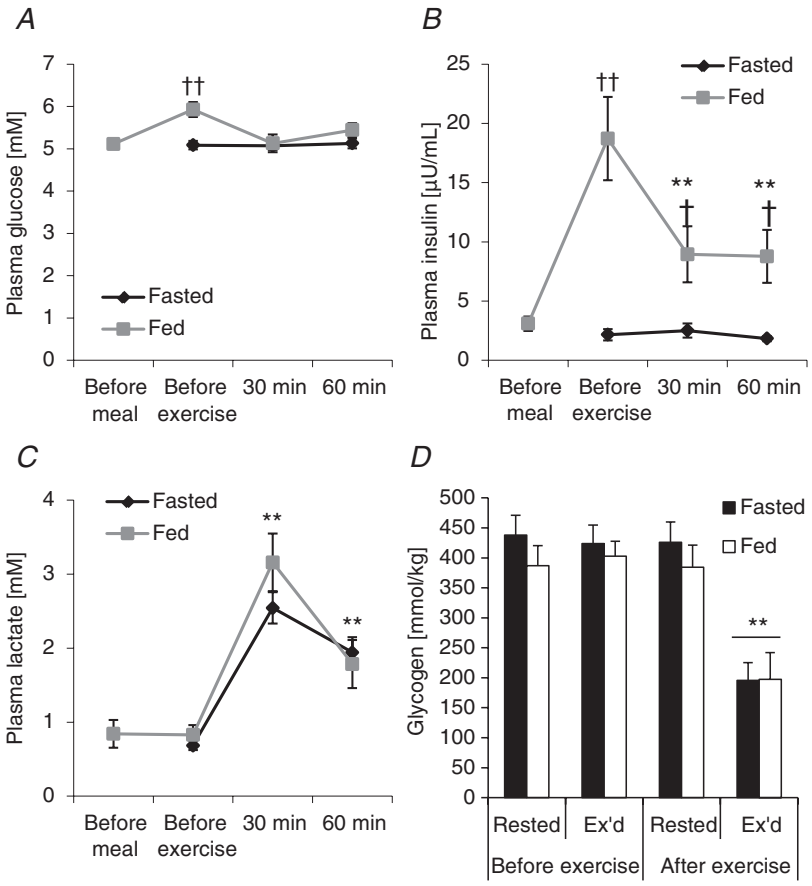

Figure 1. Plasma levels of glucose, lactate and insulin as well as muscle glycogen content
Subjects came to the laboratory in the fasted state and performed the same 1 h one-legged knee extensor exercise bout on two separate occasions in either the fasted (black lines/bars; $n = 7$) or the fed (grey lines/open bars; $n = 8$) state. Blood samples were taken in the rested state prior to the resting biopsies. After the first set of biopsies had been taken, subjects either performed 1 h of one-legged knee extensor exercise (Fasted; see Methods for details), or they consumed a small carbohydrate rich meal and then completed the exercise bout (Fed). In the fed state another blood sample was taken 15 min after the meal and just before the beginning of the exercise bout. Additional blood samples were taken after 30 and 60 min of exercise. *A*, blood glucose concentration (mM); *B*, blood insulin concentration (μU ml^{-1}); *C*, blood lactate concentration (mM); *D*, muscle glycogen (mmol kg^{-1}) was measured from biopsies taken before (Before exercise) and after (After exercise) the exercise bout in the rested (Rested) and exercised (Ex'd) leg. All values are shown as means ± SEM; † and †† indicate effect of the meal ($P < 0.05$ and 0.01, respectively); ** indicates effect of exercise ($P < 0.01$).

Muscle contraction induces phosphorylation of TBC1D1 on specific sites in an AMPK-dependent manner

To investigate the potential upstream kinase regulating the different phosphorylation sites on TBC1D1 in response to exercise in humans we employed a genetic approach. We have previously investigated whether phosphorylation of TBC1D1 in intact skeletal muscle in response to contraction *ex vivo* is AMPK dependent (Pehmøller *et al.* 2009; Frøsig *et al.* 2010; Vichaiwong *et al.* 2010). By incubating and electrically stimulating EDL muscle to contract we confirm that phosphorylation of S237, T596, and S660 are increased with contraction and that this occurs in an AMPK-dependent manner (Fig. 5*A*–*C*). In addition, we show that the increased phosphorylation of S700 in response to contraction is also dependent on AMPK (Fig. 5*D*). Thus, it is

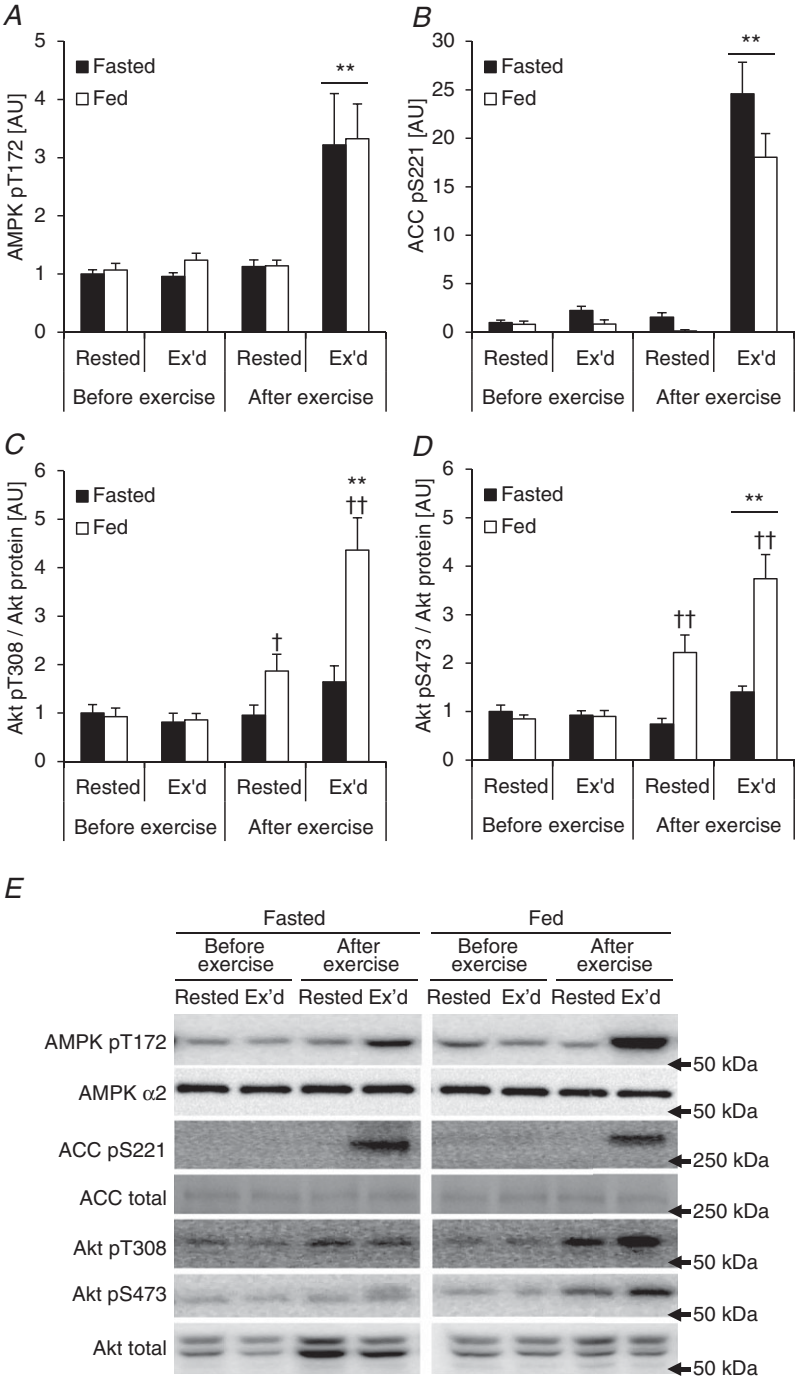

Figure 2. Acute exercise and physiological insulin stimulation affect AMPK and Akt signalling in human skeletal muscle
Phosphorylation of AMPK (T172; *A*), ACC (S221; *B*), Akt (T308; *C*) and Akt (S473; *D*) were measured in biopsies obtained from the subjects before and after exercise in the fasted (black bars; $n = 7$) or in the fed (open bars; $n = 8$) state. *E*, representative Western blot images with molecular weight marker indications. In the Akt pT308 dataset there was a significant interaction effect ($P < 0.05$) indicating a synergistic effect on this phosphorylation site when exercise is performed in the presence of physiological insulin. All values are shown as means ± SEM; † and †† indicate effect of the meal ($P < 0.05$ and 0.01, respectively); * and ** indicate effect of exercise ($P < 0.05$ and 0.01, respectively).

possible that in human skeletal muscle, the kinase responsible for phosphorylation of S237, T596, S660, and S700 during exercise is indeed AMPK. In order to test whether specific AMPK trimer complexes could be responsible for phosphorylating TBC1D1, we measured AMPK activity in EDL muscles from WT and α2 AMPK knockout mice. Both AMPK γ1- and γ3-containing trimers were robustly activated in response to contraction in the WT mice (Fig. 6), and we were therefore not able in this way to identify a specific AMPK trimer as the responsible TBC1D1 kinase.

Using the PAS antibody, we have previously reported that TBC1D4 was phosphorylated during contraction in EDL muscle and that this increased phosphorylation was ablated in the α2 AMPK KO and KD mice (Treebak *et al.* 2006). We and others have subsequently shown that the main PAS signal in EDL muscle derives from TBC1D1 (Taylor *et al.* 2008; Pehmøller *et al.* 2009; Ducommun *et al.* 2012), and data from HEK293 and 3T3-L1 adipocytes suggest that the PAS antibody primarily detects T596 on TBC1D1. Here we reanalysed the samples from our previous α2 AMPK KD/WT contraction experiment (Treebak *et al.* 2006) and evaluated both T596 and PAS phosphorylation. T596 phosphorylation was measured on the individual lysates ($n = 8$–10), whereas PAS phosphorylation was measured on immunoprecipitated TBC1D1 from pools of lysates ($n = 4$–5). In accordance with data from the α2 AMPK KO/WT samples (Fig. 5*B*), contraction increased phosphorylation of T596 in muscle from WT mice, but not in the α2 AMPK KD muscles (Fig. 5*E* and *H*). Furthermore, contraction increased PAS phosphorylation in TBC1D1 immunoprecipitates, but only in WT mice (Fig. 5*F* and top panel in 5*I*). We also performed Western blot analyses to examine the supernatants from the TBC1D1 immunoprecipitates and found that the PAS signal around 150–160 kDa was completely absent (Fig. 5*I*). Finally, we immunoprecipitated TBC1D4 using the same samples as described in the above and analysed these immunocomplexes for PAS phosphorylation. We were unable to detect any phosphorylation of TBC1D4 in these samples using the PAS antibody (Fig. 5*I*, fourth panel from the top). Thus, in EDL muscle, contraction-induced PAS phosphorylation is AMPK dependent and seems to involve only TBC1D1 and not TBC1D4.

TBC1D4 phosphorylation signatures in human skeletal muscle

TBC1D4 contains nine confirmed phosphorylation sites (i.e. S318, S341, T568, S570, S588, T642, S666, S704, and

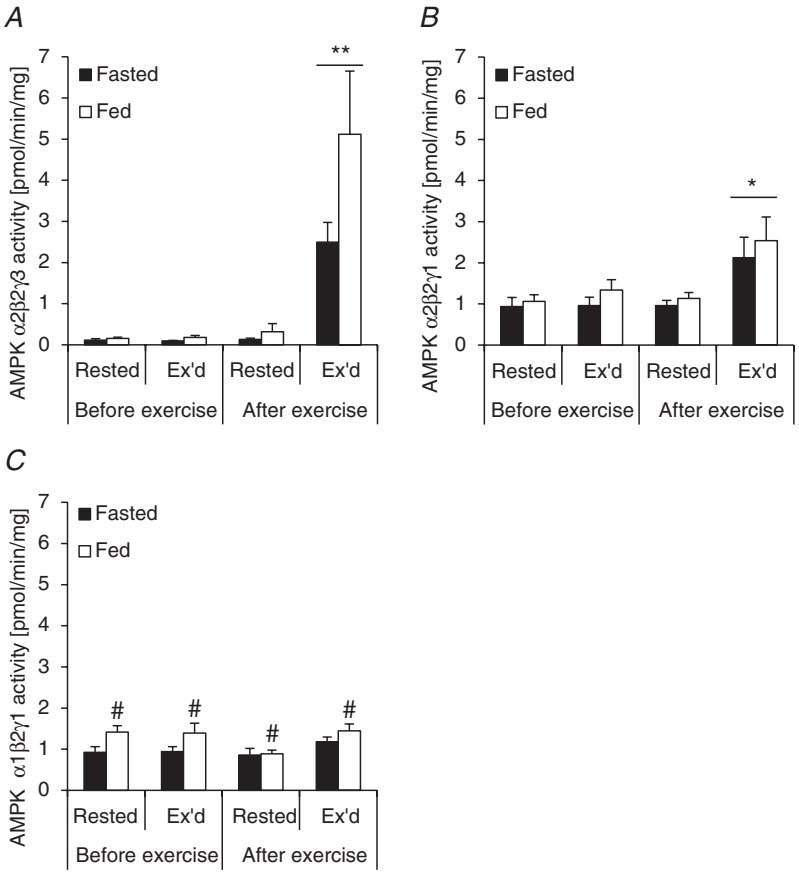

Figure 3. Acute exercise increases AMPK trimer-specific activity in human skeletal muscle
By sequential immunoprecipitation of specific AMPK subunits, AMPK trimer-specific activity (*A*, $\alpha2/\beta2/\gamma3$; *B*, $\alpha2/\beta2/\gamma1$; *C*, $\alpha1/\beta2/\gamma1$) was measured in human skeletal muscle samples obtained from the fasted (black bars; $n = 7$) and the fed (open bars; $n = 8$) exercise trials. All values are shown as means ± SEM; # indicates effect of trial ($P < 0.05$); * and ** indicate effect of exercise ($P < 0.05$ and 0.01, respectively).

S751; Geraghty *et al.* 2007; Treebak *et al.* 2010), and we investigated the phosphorylation signatures of TBC1D4 induced by exercise and physiological insulin, separately or combined on seven of these sites. We have not been able to obtain good quality Western blots with antibodies against T568 and S570. Overall, TBC1D4 contains sites that only respond to insulin (S318), that only respond to exercise (S588 and S751), that respond to both insulin and exercise (S341, T642, and S704), and that do not respond to either stimuli (S666). We have previously shown that phosphorylation of TBC1D4 in human skeletal muscle in response to both exercise and insulin can be detected by the PAS antibody (Treebak *et al.* 2007; Højlund *et al.* 2008) and there is strong evidence that the PAS antibody

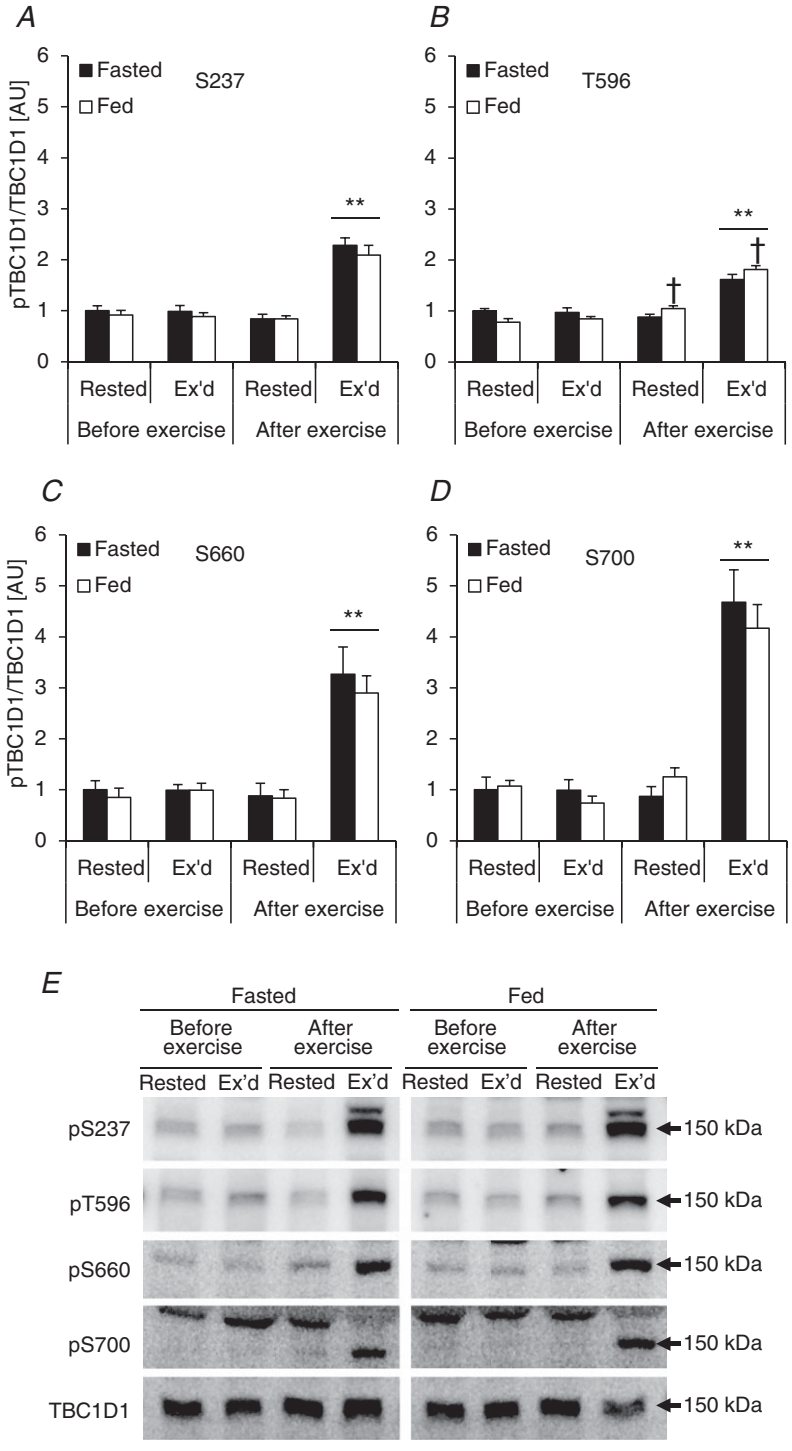

Figure 4. Phosphorylation signatures of TBC1D1 in human skeletal muscle in response to exercise and physiological insulin stimulation
Using site- and phospho-specific antibodies against TBC1D1, phosphorylation patterns induced by exercise in the fasted (black bars; $n = 7$) and the fed (open bars; $n = 8$) state were measured. *A*, S237; *B*, T596; *C*, S660; *D*, S700. *E*, representative Western blot images with molecular weight marker indications. All values are shown as means ± SEM. † indicates effect of the meal ($P < 0.05$); ** indicates effect of exercise ($P < 0.01$).

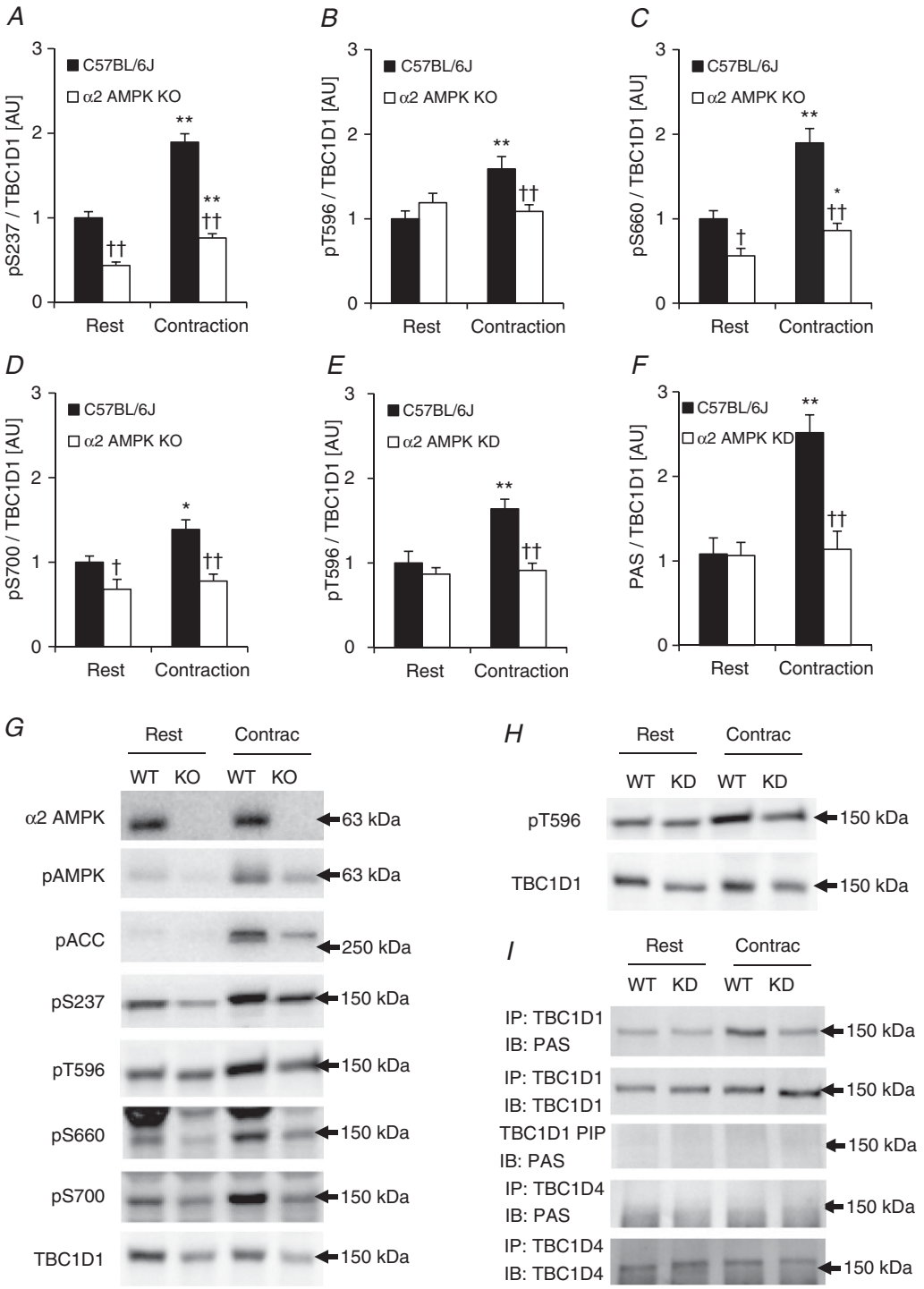

Figure 5. TBC1D1 phosphorylation signature in response to *in vitro* contraction in mouse skeletal muscle
EDL muscles from AMPK α2 knockout (KO; open bars) and wild-type (C57BL/6J) littermates (black bars; *A–D*) or AMPK α2 kinase dead (KD; open bars) and wild-type (C57BL/6J) littermates (black bars; *E* and *F*) were dissected and incubated *in vitro* for 40 min. Subsequently, muscles were stimulated to contract for 10 min (ten 10 s trains min^{-1} at 100 Hz; 0.2 ms pulse width). Muscles were then processed for Western blot analyses and developed with site- and phospho-specific antibodies against TBC1D1. For *A–E* the sample size is $n = 8$–12. Data in *F* was generated by immunoprecipitation experiments of total TBC1D1 protein from 200 μg of total protein lysate and subsequent Western blot analyses probing for PAS and total TBC1D1 protein. Due to lack of lysate, 8–10 samples were pooled in pairs to give $n = 4$–5 in each group. Statistical analyses revealed significant ($P < 0.05$) interaction effects in all datasets (*A–F*). All values are shown as means ± SEM; † and ††indicate effect of genotype ($P < 0.05$ and 0.01, respectively); * and ** indicate effect of contraction ($P < 0.05$ and 0.01, respectively).

primarily detects pT642 in cell-based models (Sano *et al.* 2003; Ramm *et al.* 2006; Geraghty *et al.* 2007). In line with this observation, we found that both PAS and T642 phosphorylation increased with exercise and insulin, and that the two stimuli combined induced a synergistic effect on the phosphorylation level (Figs 7*A* and *E* and 8). The phosphorylation patterns of S341 and S704 were similar to that of T642 (Figs 7*C* and *G* and 8) in that these sites responded significantly to both insulin and exercise. Furthermore, there was an additive (S704) and even a synergistic (S341) response when insulin and exercise were combined. S704 phosphorylation was found earlier to respond to both contraction and maximal insulin stimulation in rodent skeletal muscle (Treebak *et al.* 2010), and to insulin during hyperinsulinaemic euglycaemic clamp conditions in human skeletal muscle (Pehmøller *et al.* 2012). Here we show that this also occurs in human skeletal muscle under physiological conditions. We have previously found that both S588 and S751 can be phosphorylated in response to high insulin concentrations during a hyperinsulinaemic euglycaemic clamp (Treebak *et al.* 2009*b*; Vind *et al.* 2011; Biensø *et al.* 2012; Pehmøller *et al.* 2012). In the present study, however, S588 and S751 were only phosphorylated after exercise and did not respond to a physiological insulin stimulus (Figs 7*D* and *H* and 8). Finally, S666 did not respond to either exercise or insulin (Figs 7*F* and 8) and S318 responded only to insulin (Figs 7*B* and 8).

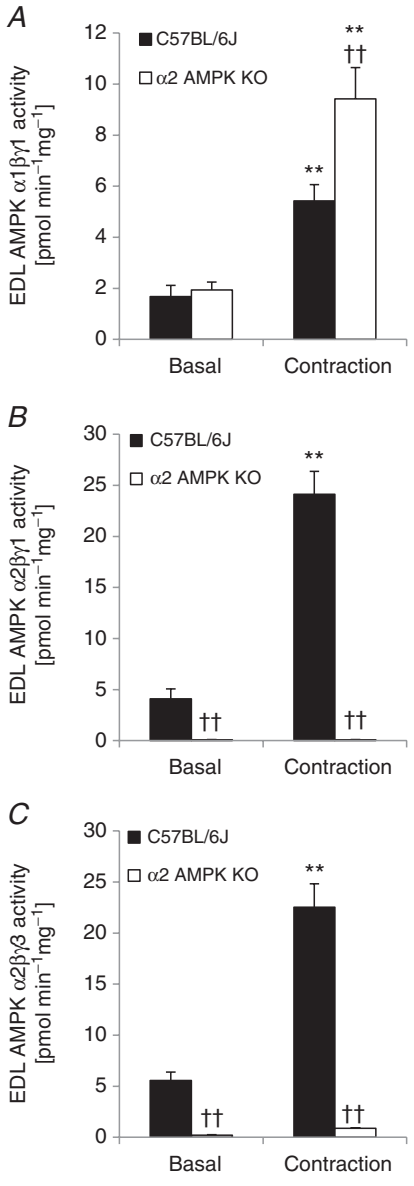

Figure 6. *In vitro* contraction of mouse skeletal muscle activates γ1 and γ3 AMPK trimers
AMPK trimer activity was measured in response to *in vitro* contraction of EDL muscles from α2 AMPK KO ($n = 5$–6) and C57BL/6J ($n = 8$–9) wild-type littermates. AMPK-trimers were isolated by sequential immunoprecipitation with isoform-specific antibodies from 300 μg of lysate. In the assay we did not distinguish between β1 and β2 AMPK complexes. *A*, α1βγ1 trimer activity; *B*, shows α2βγ1 trimer activity; *C*, α2βγ3 trimer activity. All assays showed significant interaction effects: *A*, $P < 0.05$; *B*, $P < 0.01$; *C*, $P < 0.01$. †† indicates effect of genotype ($P < 0.01$). ** indicates effect of contraction ($P < 0.01$).

AMPK trimer activity correlates with phosphorylation level on specific TBC1D1 and TBC1D4 sites in response to exercise in humans

To further explore the possibility that AMPK is regulating phosphorylation of specific sites on TBC1D1 and TBC1D4 in human skeletal muscle, we performed correlation analyses between specific AMPK trimer activities and phosphorylation levels of those phosphorylation sites on TBC1D1 and TBC1D4 that were phosphorylated in response to exercise (i.e. in the exercised leg after exercise in the fasted state). On TBC1D1 all four sites investigated were phosphorylated in response to exercise, and there was a significant correlation between α2/β2/γ3 AMPK trimer activity and phosphorylation of S237 ($P < 0.05$) and T596 ($P < 0.01$; Fig. 9*A* and *B*). Although phosphorylation of S660 and S700 correlated with borderline significance with α2/β2/γ3 AMPK activity ($P = 0.12$; $R^2 = 0.49$; $n = 7$ for S660; $P = 0.09$; $R^2 = 0.55$; $n = 7$ for S700; data not shown) these analyses may be biased by the distribution of the data and the low number of subjects. Interestingly, of the three existing human AMPK trimers, exercise-induced TBC1D1 phosphorylation at S237 and T596 correlated *only* with activity associated with the α2/β2/γ3 trimer. On the other hand, only α2/β2/γ1 trimer activity correlated with specific exercise-induced phosphorylation sites on TBC1D4. We found that several TBC1D4 sites were phosphorylated in response to exercise (i.e. S341, S588, T642, S704, and S751), but only S341 and S704 correlated significantly with α2/β2/γ1 trimer activity (Fig. 9*C* and *D*). Phosphorylation of S588 and S751 that was highly induced by exercise

correlated only with borderline significance ($P = 0.12$; $R^2 = 0.41$; $n = 7$ for S588; $P = 0.1$; $R^2 = 0.45$; $n = 7$ for S751; data not shown). We designed this study in a paired fashion which enabled us to correlate the increase in phosphorylation with the increase in AMPK trimer activity in response to exercise (i.e. delta *vs.* delta correlation). When changes in TBC1D1 phosphorylation were correlated with changes in activity of the AMPK trimer complexes, this slightly improved the correlation between S700 and AMPK $\alpha 2/\beta 2/\gamma 3$ activity ($P = 0.07$; $R^2 = 0.58$; $n = 7$; data not

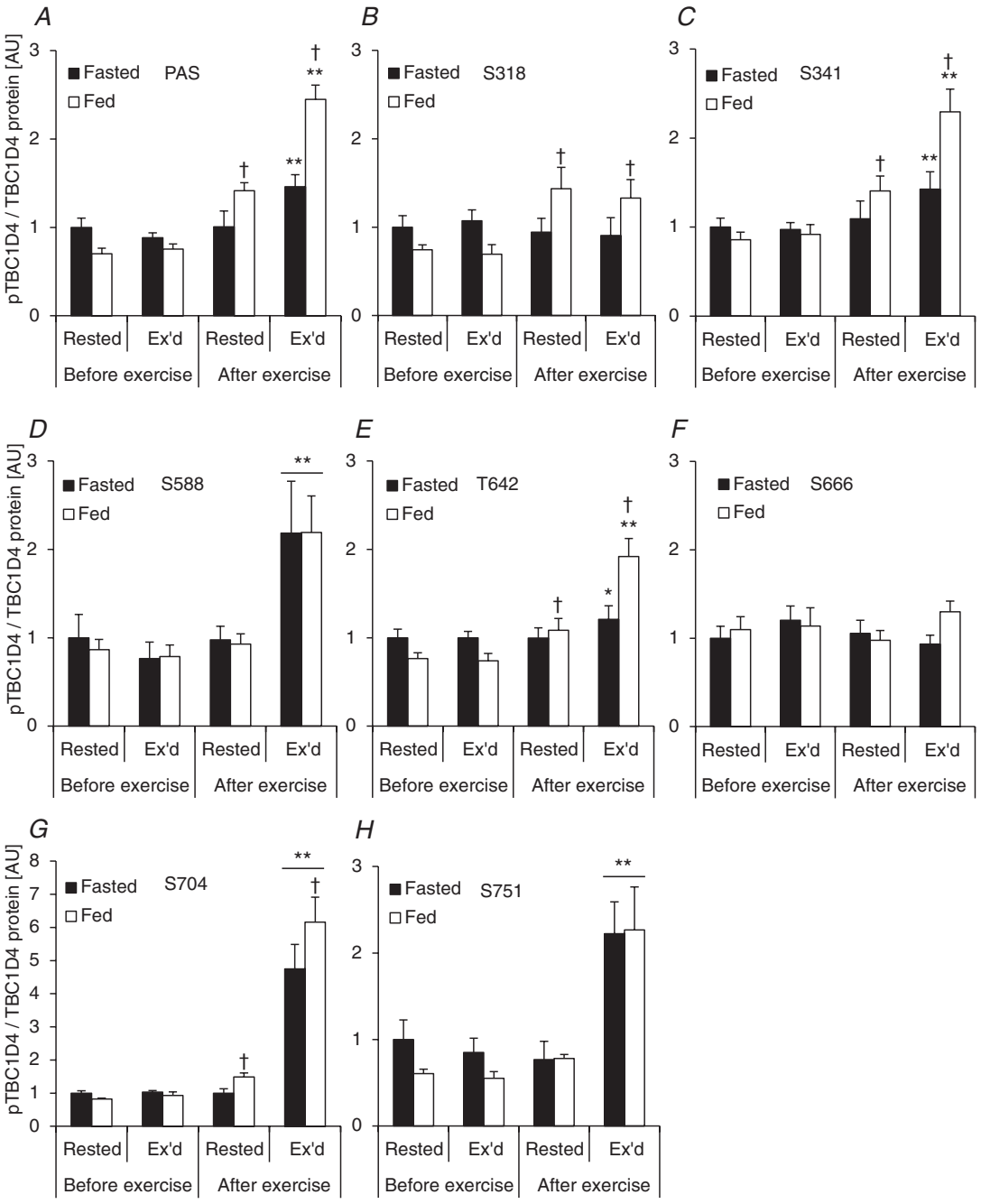

Figure 7. Phosphorylation signatures of TBC1D4 in human skeletal muscle in response to exercise and physiological insulin stimulation
Using site- and phospho-specific antibodies against TBC1D4, phosphorylation patterns induced by exercise in the fasted (black bars; $n = 7$) and the fed (open bars; $n = 8$) state were measured. In the PAS and pS341 datasets there were significant interaction effects ($P < 0.05$) indicating a synergistic effect on the specific phosphorylation site when exercise is performed in the presence of physiological insulin. All values are shown as means ± SEM; † indicates effect of the meal ($P < 0.05$); * and ** indicate effect of exercise ($P < 0.05$ and 0.01, respectively).

shown), but the only significant correlation remaining was between S237 phosphorylation and $\alpha2/\beta2/\gamma3$ trimer activity ($P < 0.05$; $R^2 = 0.60$; $n = 7$; data not shown). In addition, changes in TBC1D4 phosphorylation at S341 ($P < 0.01$; $R^2 = 0.77$; $n = 7$; data not shown) and S704 ($P < 0.01$; $R^2 = 0.86$; $n = 7$; data not shown) remained significantly correlated with changes in $\alpha2/\beta2/\gamma1$ AMPK complex activity. In short, correlation analyses support the idea that TBC1D1 (S237) and TBC1D4 (S341, S704) are phosphorylated by AMPK in human skeletal muscle during exercise.

Muscle contraction induces phosphorylation of TBC1D4 on specific sites in an AMPK-dependent manner

Exercise in humans led to increased phosphorylation of S341, S588, T642, S704, and S751 on TBC1D4 (Fig. 7). To explore the idea that AMPK is the upstream kinase mediating this effect we incubated soleus muscles from WT and $\alpha2$ AMPK KO mice and electrically stimulated them to contract. As shown before (Treebak *et al.* 2010), phosphorylation of S704 was increased 2-fold in response to contraction in WT mice and lack of AMPK led to an overall reduced level of phosphorylation (main effect of genotype; Fig. 10*C*). Interestingly, none of the other specific sites were phosphorylated by contraction and there was even a significant reduction in phosphorylation on sites T642 and S751 (Fig. 10*B* and *D*). Phosphorylation of S588 was generally lower in the knockout mice, but did not respond to contraction (Fig. 10*A*). Using a low percentage polyacrylamide gel (4.5%), and performing Western blot analyses with the PAS antibody, we were able to separate two bands, one running at 150 kDa and one running at 160 kDa. We have previously found that these bands correspond to TBC1D1 and TBC1D4, respectively (Taylor *et al.* 2008; Pehmøller *et al.* 2009). We found that *in vitro* contraction significantly increased phosphorylation of the 150 kDa band in both WT and $\alpha2$ AMPK KO animals (Fig. 10*E*). Although the increase in phosphorylation was not statistically different between the two genotypes, there was a generally lower level of phosphorylation in the $\alpha2$ AMPK KO mice. This difference between genotypes was also present for the 160 kDa band, but phosphorylation decreased significantly in response to contraction (Fig. 10*F*). Thus, it appears that in soleus the PAS antibody detects both TBC1D1 and TBC1D4, and that these proteins are differentially regulated during muscle contraction.

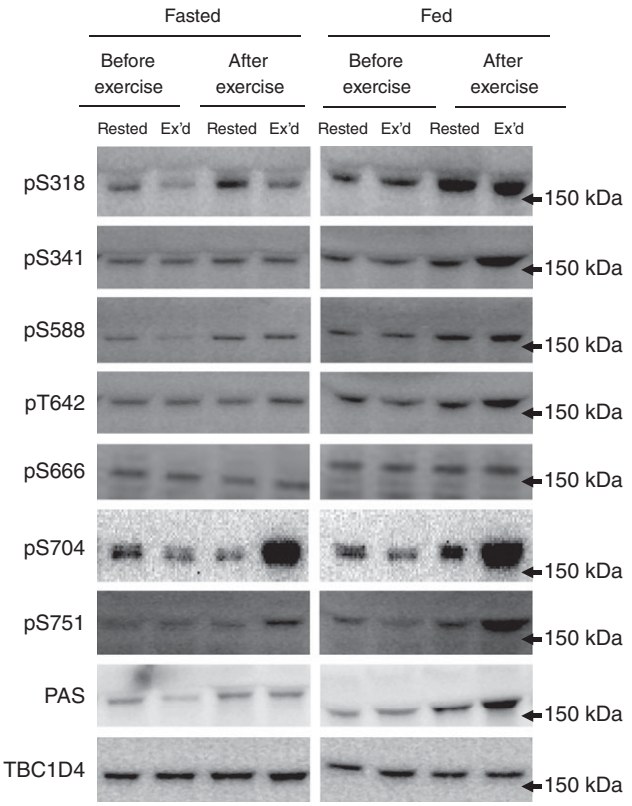

Figure 8. Representative Western blots for the data presented in Fig. 7 with molecular weight marker indications

In vitro contraction of mouse soleus muscle activates $\gamma1$ and $\gamma3$ AMPK trimers

In mouse skeletal muscle we have previously identified five different AMPK complexes of which the four AMPK $\gamma1$-containing complexes in soleus muscle constitute the majority (>95%) of AMPK complexes (Treebak *et al.* 2009*a*). Considering the finding that activity of AMPK $\gamma1$-containing complexes associates with phosphorylation of TBC1D4 on specific sites (i.e. S341 and S704) in human skeletal muscle, one possible explanation for the lack of TBC1D4 phosphorylation in mouse soleus muscle in response to contraction could be that activation of specific $\gamma1$-containing AMPK complexes does not occur in response to the experimental protocols we have used. We have previously shown that in response to both endurance type exercise (~70% $\dot{V}_{O_2peak}$) and to sprint type exercise in human skeletal muscle, the AMPK $\alpha2/\beta2/\gamma3$ complex is activated earlier and more potently compared with the $\alpha2/\beta2/\gamma1$ complex (Treebak *et al.* 2007). In fact, we found unchanged and even reduced activation of AMPK $\alpha2/\beta2/\gamma1$ in response to various types of sprint exercises ranging from 30 s to 20 min (Birk & Wojtaszewski, 2006; Treebak *et al.* 2007). In our hands, the stimulation protocol used in the present study to contract soleus and EDL muscles *in vitro* induces fatigue very rapidly. Thus, it is possible that conditions in our contraction experiments do not allow for AMPK $\gamma1$-mediated phosphorylation of TBC1D4 in mouse skeletal muscle. To investigate this we measured AMPK $\alpha1\beta(1/2)\gamma1$, $\alpha2\beta(1/2)\gamma1$,

and $\alpha2/\beta2/\gamma3$ trimer-specific activity in soleus from $\alpha2$ AMPK KD mice (Fig. 11). In this assay we did not distinguish between the different β AMPK trimer complexes. As shown in Fig. 11*B* and *C*, *in vitro* contraction of soleus activated $\gamma1$- and $\gamma3$-containing AMPK trimers in WT mice. In addition, $\alpha1$-containing AMPK trimers were also activated in the WT mice and as expected in the AMPK KD mice (Fig. 11*A*). Thus, lack of TBC1D4 phosphorylation on specific sites in soleus muscle in response to *in vitro* contraction is not due to lack of AMPK activation of specific trimers.

Treadmill running induces muscle-specific changes in TBC1D1 and TBC1D4 phosphorylation patterns in mouse skeletal muscle

To test whether the decreased TBC1D4 phosphorylation of T642 and S751 in response to muscle contraction (Fig. 10*B* and *D*) was due to an *in vitro* rather than *in vivo* experimental approach, we ran male C57BL/6J mice ($n = 12$) on a treadmill (1 h; 18 m min^{-1}; 5 deg slope), dissected out soleus and EDL muscles immediately after exercise, and compared the phosphorylation level of TBC1D4 and TBC1D1 on specific sites with rested control mice. This exercise protocol activated AMPK as indicated by significantly increased phosphorylation of T172 of AMPK and of S212 of ACC, but only in EDL muscles (Fig. 12*A* and *B*). In soleus muscle TBC1D4 phosphorylation was either unchanged (Fig. 13*B* and *D*) or significantly decreased (Fig. 13*A*, *C* and *E*). We speculated that this decrease in TBC1D4 phosphorylation level with exercise could be due to putatively reduced insulin levels compared with the rested control mice. We measured Akt phosphorylation in soleus and EDL muscles and found significantly reduced pT308 and pS473 levels in both muscles with exercise (Fig. 12*C* and *D*). In addition, in the exercised soleus muscles there were significant ($P < 0.01$; $n = 6$) positive correlations between Akt phosphorylation status of S473, and phosphorylation of TBC1D4 on sites T642 and S751 ($R^2 = 0.85$ for Akt S473 *vs.* T642; $R^2 = 0.82$ for Akt S473 *vs.* S751). There were additional borderline significant correlations between Akt T308 and S473 phosphorylation and specific phosphorylation sites on TBC1D4 (data not shown). Thus, it is possible that Akt is responsible for maintaining basal TBC1D4 phosphorylation levels on specific sites in mouse skeletal muscle. However, since the conditions under which we incubate muscles *in vitro* are not influenced by circulating insulin levels, contractions *per se* may still, independently of AMPK activation,

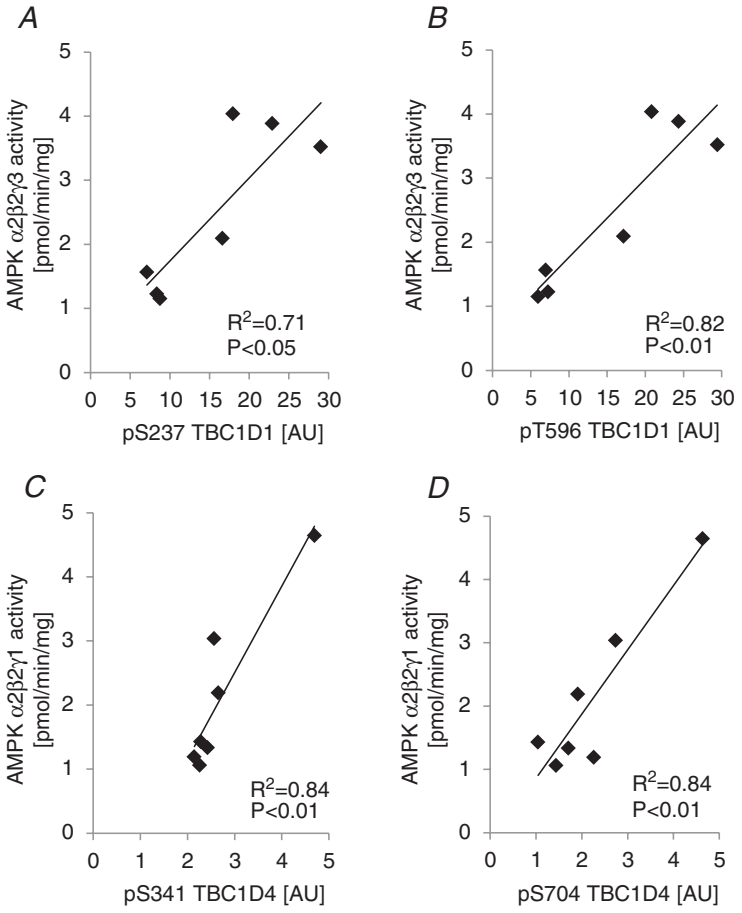

Figure 9. AMPK trimer activity correlates with phosphorylation of TBC1D1/4 on specific sites in human skeletal muscle
All correlations were performed on data from the exercised leg after exercise in the fasted state. *A* and *B* show correlations between AMPK $\alpha2/\beta2/\gamma3$ trimer activity and TBC1D1 pS237 and pT596, respectively. *C* and *D* show correlations between AMPK $\alpha2/\beta2/\gamma1$ trimer activity and TBC1D4 pS341 and pS704, respectively. Samples size is $n = 7$ for all figures. R^2 and significance level are shown in the respective panels.

lead to alterations in activity of upstream TBC1D4 kinases or phosphatases. A distinct possibility to explain the lack of TBC1D4 phosphorylation in both soleus and EDL with exercise could also be lack of muscle recruitment. In these samples, we did not measure glycogen levels. However, in a separate experiment where mice ran for 30 min at various intensities with 5 deg incline, we observed significant reductions (∼25%) in glycogen levels in both soleus and EDL already at 14 m min^{-1} (data not shown). Therefore, we believe that both muscles were recruited during the current exercise bout. In accordance with the TBC1D1 phosphorylation data obtained in the incubated EDL

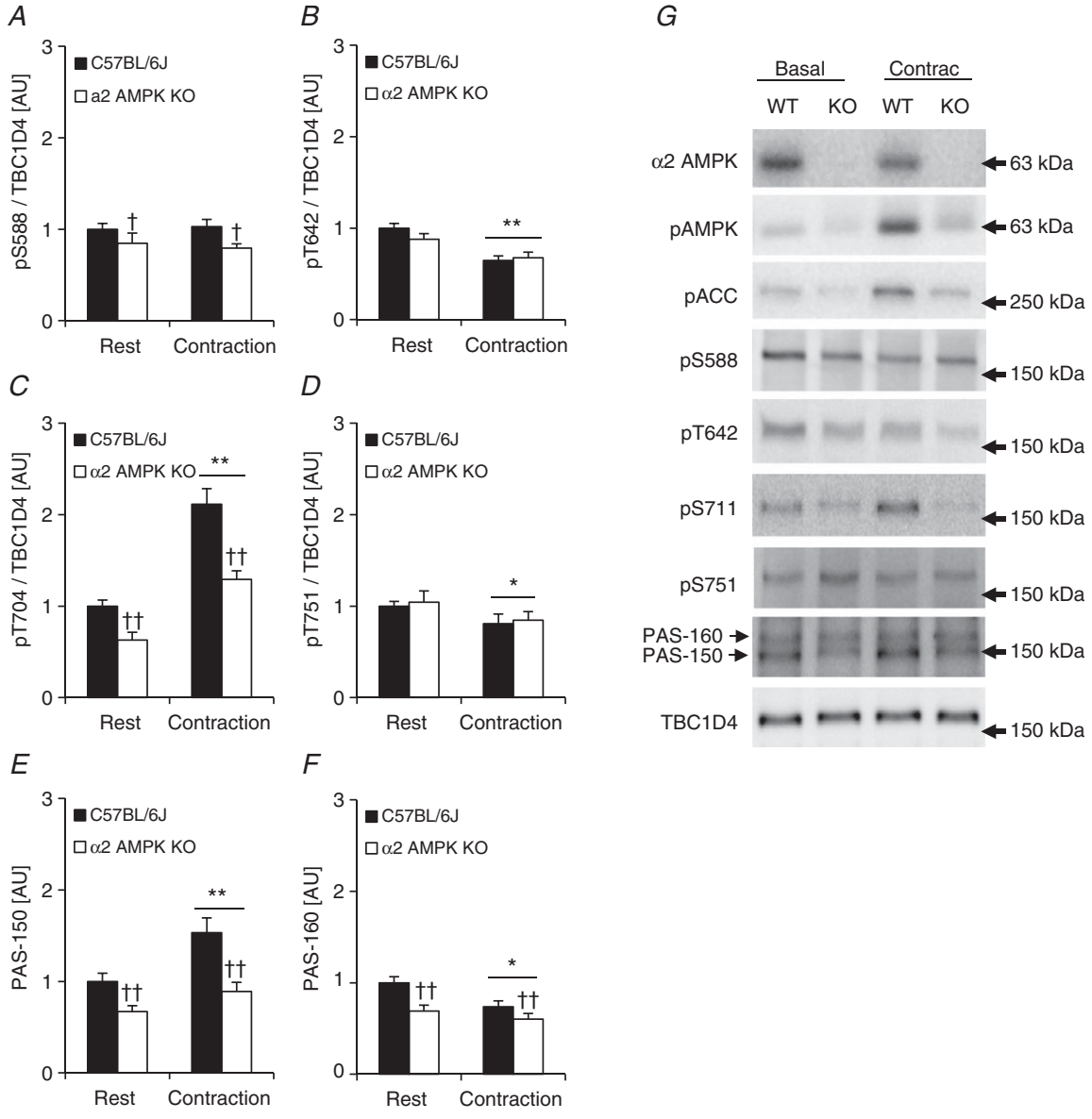

Figure 10. TBC1D4 phosphorylation signatures in response to *in vitro* contraction in mouse skeletal muscle
Soleus muscles from AMPK α2 knockout (KO; open bars) and wild-type (C57BL/6J) littermates (black bars; *A–F*) were dissected and incubated *in vitro* for 40 min. Subsequently, muscles were stimulated to contract for 10 min (ten 10 sec trains min^{-1} at 100 Hz; 0.2 ms pulse width). Muscles were then processed for Western blot analyses and developed with site- and phospho-specific antibodies against TBC1D4. Sample size for panels *B–F* is $n = 8–12$. Data in *A* was generated by immunoprecipitation experiments of total TBC1D4 protein from 800 μg of total protein lysate and subsequent Western blot analyses probing for pS588 and total TBC1D4 protein. Due to lack of lysate, 8–10 samples were pooled in pairs to give $n = 4–5$ in each group. All values are shown as means ± SEM; † and †† indicate effect of genotype ($P < 0.05$ and 0.01, respectively); * and ** indicate effect of contraction ($P < 0.05$ and 0.01, respectively).

muscles, phosphorylation of S237, S660, and S700 increased in response to exercise (Fig. 13*F*, *H* and *I*). It is possible that the lack of T596 phosphorylation in response to exercise in this muscle could be due activation/deactivation of opposing upstream kinases (Fig. 13*G*). To support this idea, it has previously been shown that both Akt and AMPK are upstream kinases of TBC1D1 on this site (Pehmøller *et al.* 2009; Vichaiwong *et al.* 2010).

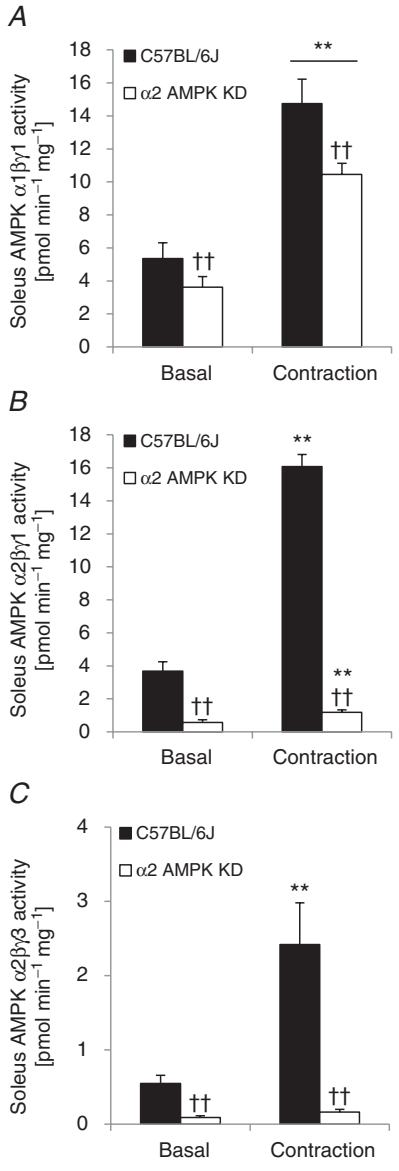

Figure 11. *In vitro* contraction of mouse skeletal muscle activates γ1 and γ3 AMPK trimers
AMPK trimer activity was measured in response to *in vitro* contraction of soleus muscles from α2 AMPK KD (n = 10–12) and C57BL/6J (n = 6–8) wild-type littermates. AMPK-trimers were isolated by sequential immunoprecipitation with isoform-specific antibodies from 300 μg of lysate. In the assay we did not distinguish between β1 and β2 AMPK complexes. *A*, $\alpha1\beta\gamma1$ trimer activity; *B*, $\alpha2\beta\gamma1$ trimer activity; *C*, $\alpha2\beta\gamma3$ trimer activity. In assays shown in *B* and *C* there are significant interaction effects ($P < 0.01$). †† indicates effect of genotype ($P < 0.01$). ** indicates effect of contraction ($P < 0.01$).

14-3-3 binding capacity to TBC1D1 and TBC1D4

We have previously shown that acute cycle ergometer exercise in human subjects and *in vitro* contraction of mouse EDL muscle increases TBC1D1 S237 phosphorylation and that this is associated with increased 14-3-3 binding capacity towards TBC1D1 *in vitro* (Pehmøller *et al.* 2009; Frøsig *et al.* 2010). In addition, it has been shown in L6 myotubes that pharmacological activation of AMPK results in S237 phosphorylation and increased 14-3-3 binding capacity towards TBC1D1 (Chen *et al.* 2008), and that insulin stimulation promotes T596 phosphorylation but without changes in 14-3-3 binding capacity in both L6 cells (Chen *et al.* 2008) and intact skeletal muscle (Pehmøller *et al.* 2009). Although we found a significant increase in S237 phosphorylation in the current study using the one-legged knee extensor exercise model we were not able to detect any changes in 14-3-3 binding capacity in response to either exercise or physiological insulin (Fig. 14*A*).

Others have shown that binding capacity of 14-3-3 to TBC1D4 increases in human skeletal muscle in response to acute aerobic exercise (Howlett *et al.* 2008), and we and others have shown that 14-3-3 binding capacity to TBC1D4 in human skeletal muscle can be induced by raising insulin levels to high physiological levels during hyperinsulinaemic clamp conditions (Howlett *et al.* 2008; Treebak *et al.* 2009*b*). In the present study we investigated the effect of exercise and physiological levels of insulin on 14-3-3 binding capacity to TBC1D4. Both stimuli alone induce significant increases in 14-3-3 binding capacity and there was a complete additive effect when the two stimuli were combined (Fig. 14*B*). Data from several *in vitro* cell culture models suggest that phosphorylation of S341 and/or T642 is necessary for 14-3-3 to bind to TBC1D4 (Ramm *et al.* 2006; Geraghty *et al.* 2007). In this current study the phosphorylation patterns of S341 and T642 are similar to the data obtained from the TBC1D4 14-3-3 overlay (compare Fig. 7*C* and *E* with Fig. 14*B*). However, correlations between 14-3-3 binding capacity and phosphorylation on either S341 or T642 in response to either stimulus were not significant (data not shown).

Phosphorylation level of Akt correlates with phosphorylation level of specific TBC1D1 and TBC1D4 sites in response to insulin in humans

We showed that Akt was phosphorylated on T308 and S473 in human skeletal muscle in response to physiological insulin (i.e. in the rested leg after exercise in the fed state;

Fig. 2*C* and *D*). In addition, we showed that several sites on TBC1D1/4 were phosphorylated in this condition (i.e. T596, S318, S341, T642, and S704; Figs 4 and 7). Except for S704, Akt has been proposed as an upstream kinase regulating these sites due to their location within the preferred Akt recognition sequence (RXRXXS/T; Sano *et al.* 2003; Roach *et al.* 2007). However, of these sites, recombinant Akt has been shown to phosphorylate only T596, S318, and T642 (Geraghty *et al.* 2007; Chen *et al.* 2008). Thus, we performed correlation analyses to examine whether any of the sites responding to physiological insulin stimulation could potentially be phosphorylated by Akt in human skeletal muscle. Akt2 has been shown to be responsible for the majority of the increase in Akt phosphorylation upon insulin stimulation in human skeletal muscle (Vind *et al.* 2012), and since Akt2 has been shown to be necessary for insulin to induce phosphorylation of T642 (Kramer *et al.* 2006*a*), it was surprising that phosphorylation of T642 did not correlate significantly with the phosphorylation level of any of the two Akt phosphorylation sites ($P > 0.05$; $R^2 = 0.19$ for Akt T308 *vs.* T642; $P > 0.05$; $R^2 = 0.45$ for Akt S473 *vs.* T642; $n = 8$). All other sites correlated significantly with the phosphorylation level of both T308 (Fig. 15) and S473 (Fig. 16). To further explore the pT642 data in relation to Akt phosphorylation, we correlated the delta changes of T642 phosphorylation to the delta changes of Akt T308 and S473 phosphorylation. However, we were still not able to obtain significant correlations (Supplemental Figs S1D and S2D). In addition, we correlated the delta changes in phosphorylation of all of the other TBC1D1/4 sites regulated by physiological insulin stimulation with delta changes in Akt phosphorylation. All delta correlations were either of similar strength or stronger compared with the non-delta correlations (Supplemental Figs S1 and S2). The PAS antibody has been shown to primarily detect phosphorylation of T642 (Sano *et al.* 2003; Ramm *et al.* 2006; Geraghty *et al.* 2007), and while phosphorylation of T642 did not correlate with the phosphorylation level of Akt, phosphorylation measured with the PAS antibody strongly correlated with Akt phosphorylation (Figs 15*E*, 16*E* and Supplemental Figs S1F, and S2F). Since all detectable PAS signal in human skeletal muscle can be accounted for in a TBC1D4 IP (Højlund *et al.* 2008), it is therefore possible that the PAS antibody detects other TBC1D4 sites than T642 in human skeletal muscle.

Discussion

Phosphorylation patterns on TBC1D1 and TBC1D4 for different stimuli constitute important information

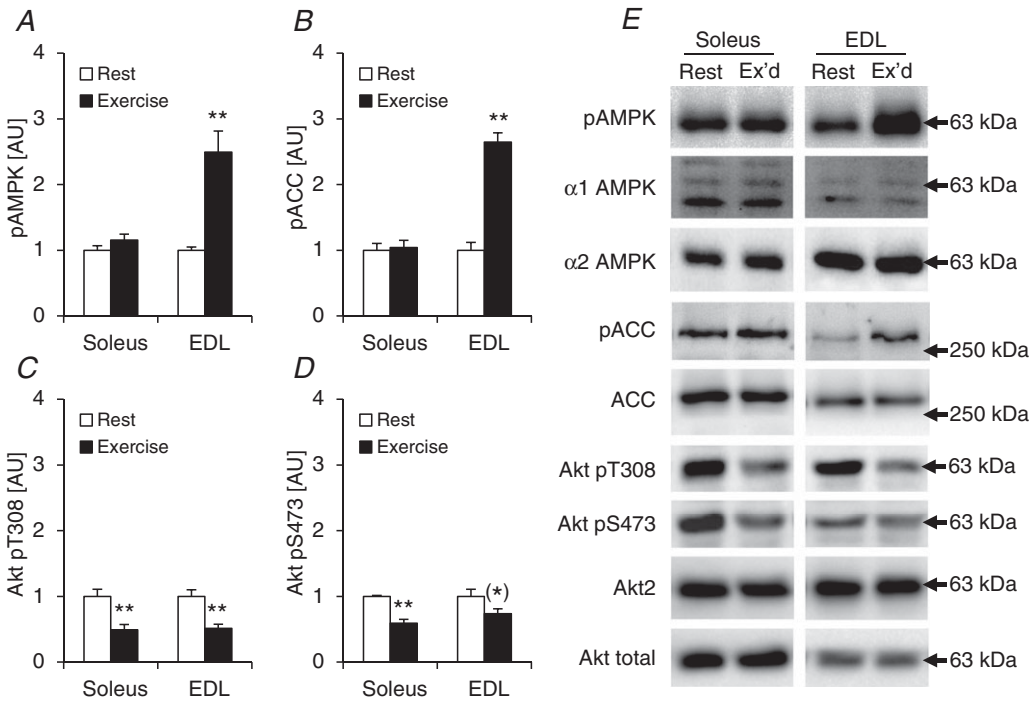

Figure 12. Exercise-induced phosphorylation of AMPK, ACC and Akt in mouse soleus and EDL muscles
C57BL/6J wild-type mice were either run ($n = 12$) on a motorized treadmill for 1 h (18 m min^{-1}; 5 deg incline) or were rested controls ($n = 12$). Phosphorylation of AMPK T172 (*A*), ACC S212 (*B*), Akt T308 (*C*), and Akt S473 (*D*) was measured to determine whether activity of these putative TBC1D1/4 kinases were altered in soleus and EDL muscles. All values are shown as means ± SEM; ** indicates effect of exercise ($P < 0.01$); * indicates borderline significant effect of exercise ($P = 0.07$).

regarding how these proteins are regulated and what sites may be important for specific stimuli to exert their effects through these proteins. Most studies that have investigated phosphorylation patterns on TBC1D1 and TBC1D4 have used rodent or cell culture-based models and stimulus conditions that are not physiological, and it is possible that results from these studies do not directly translate to what is seen in human skeletal muscle under physiological conditions. In the present study, TBC1D1 phosphorylation patterns between species were similar for the sites investigated. On the other hand, muscle contractions induced by *in vivo* exercise resulted in very different phosphorylation signatures on TBC1D4 in skeletal muscle from mice compared with humans. TBC1D4 was phosphorylated on a variety of residues (S341, S588, T642, S704, and S751) in human muscle during exercise, whereas several residues on TBC1D4 were either unchanged or underwent significant dephosphorylation during *ex vivo* contraction

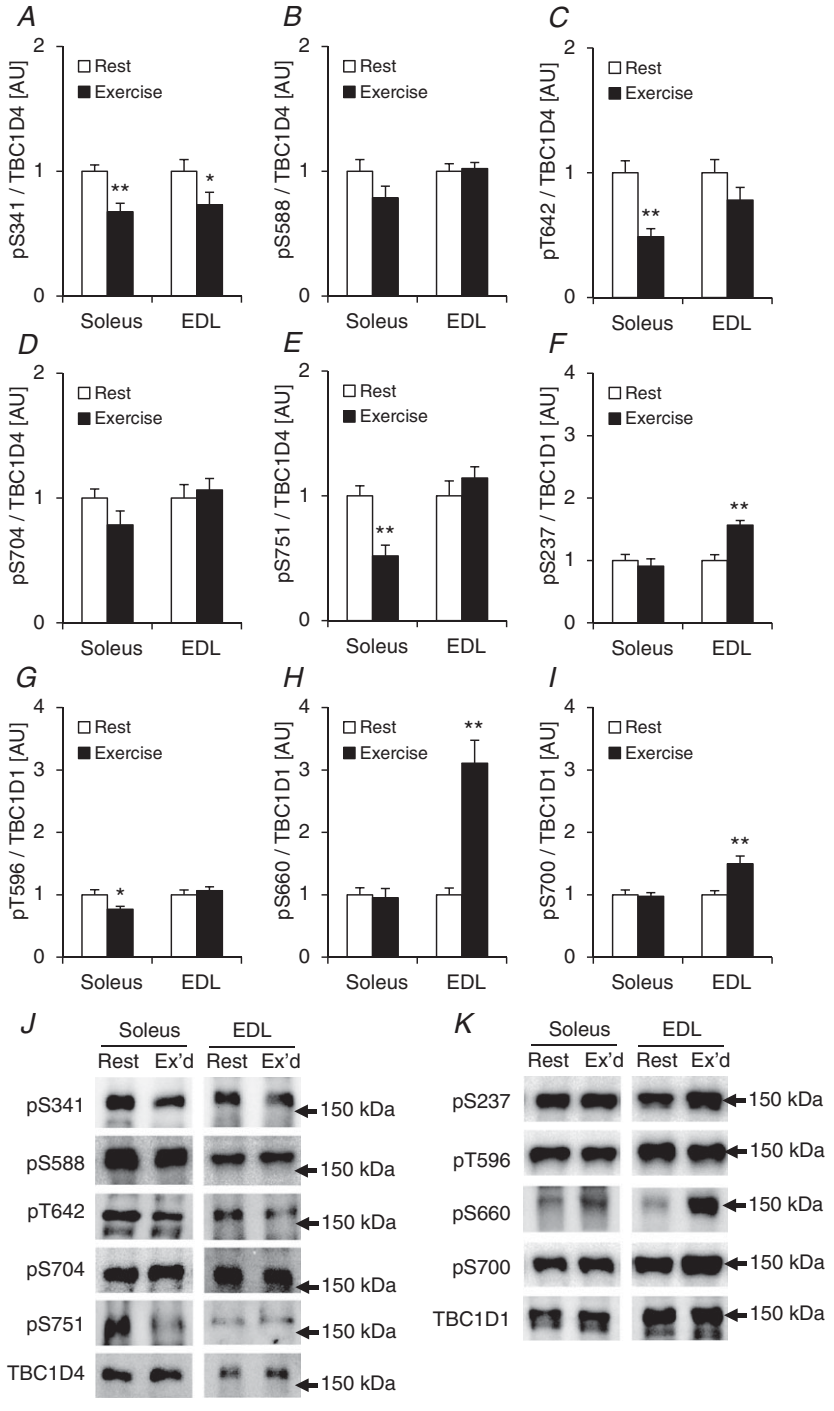

Figure 13. Exercise-induced phosphorylation signatures of TBC1D4 and TBC1D1 in mouse soleus and EDL muscles
C57BL/6J wild-type mice were either run ($n = 12$) on a motorized treadmill for 1 h (18 m min^{-1}; 5 deg incline) or were rested controls ($n = 12$). Phosphorylation of TBC1D4 (S341, S588, T642, S704, and S751) and TBC1D1 (S237, T596, S660, and S700) was measured in both soleus and EDL muscles and related to values obtained from rested controls. *J* and *K*, representative Western blot images for pTBC1D4 and pTBC1D1, respectively with molecular weight marker indications. All values are shown as means ± SEM; * and ** indicate effect of exercise ($P < 0.05$ and 0.01, respectively).

or during *in vivo* exercise. Along the same lines, a recent study reported increased S588 phosphorylation but unchanged S318, S341, and S751 phosphorylation after *in situ* contraction of the mouse gastrocnemius muscle (Ducommun *et al.* 2012). In the present study, we did not attempt to match exercise protocols used for the human volunteers and the cohort of mice in terms of intensity, duration, or mode. Furthermore, fibre type differences, and differences in substrate utilization during exercise may be contributing factors to explain the species difference on TBC1D4 phosphorylation patterns observed. However, the differential phosphorylation patterns of TBC1D4 among species highlight the potential risk associated with uncritical use of animal data to explain physiological phenomena in humans.

Using the one-legged knee extensor exercise model and having the same subjects complete an acute bout of exercise in the fasted and fed condition allowed us to utilize paired statistics for a more powerful evaluation of our research question. The meal ingested elicited regulation by phosphorylation of both TBC1D1 and TBC1D4, although in general the increases in phosphorylation were smaller compared with findings during hyperinsulinaemic euglycaemic clamp conditions (Treebak *et al.* 2009*b*; Pehmøller *et al.* 2012; Vendelbo *et al.* 2012). Moreover, the phosphorylation signatures on TBC1D1 and TBC1D4 in response to the meal were different to what we have previously reported during hyperinsulinaemic euglycaemic clamps (Treebak *et al.* 2009*b*; Pehmøller *et al.* 2012; Vendelbo *et al.* 2012), as we were not able to see regulation of S588

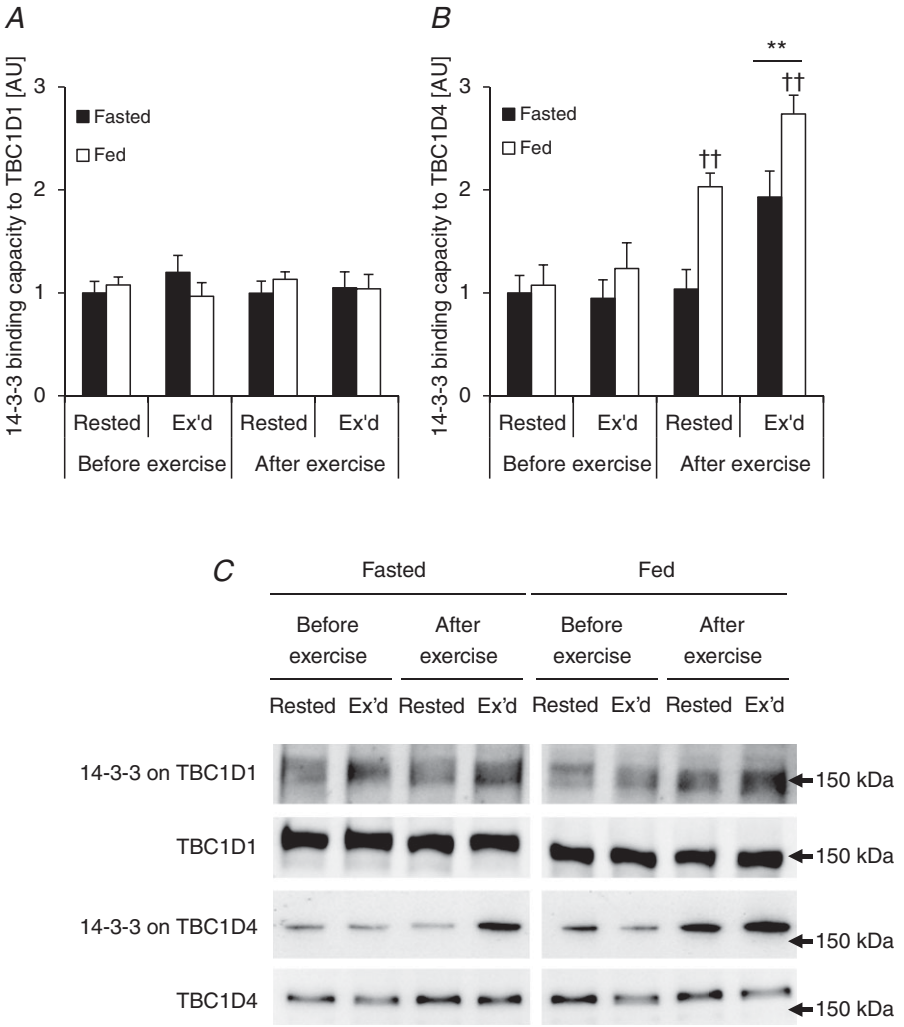

Figure 14. Exercise and physiological insulin stimulation results in differential 14-3-3 binding capacity to TBC1D1 and TBC1D4
14-3-3 overlays were used to evaluate the binding capacity to TBC1D1 (*A*) and TBC1D4 (*B*) induced by exercise in the fasted (black bars; $n = 7$) and the fed (open bars; $n = 8$) state. *C*, representative overlays and Western blots for total TBC1D1 and TBC1D4 protein are shown in with molecular weight marker indications. All values are shown as means ± SEM. †† indicates effect of the meal ($P < 0.01$);. ** indicates effect of exercise ($P < 0.01$).

and S751 on TBC1D4 in the current study. Also, in the present study we found that those sites regulated by both exercise in the fasted state and in response to the meal (i.e. T596, S341, T642, and S704) were all regulated in an additive manner by exercise in the fed state. 14-3-3 binding to TBC1D4 has been suggested to be necessary for insulin-mediated GLUT4 trafficking in adipocytes (Ramm *et al.* 2006). Here we showed that *in vitro* 14-3-3 binding capacity to TBC1D4 was up-regulated by exercise *per se*, by the meal *per se*, and synergistically by exercise in the fed

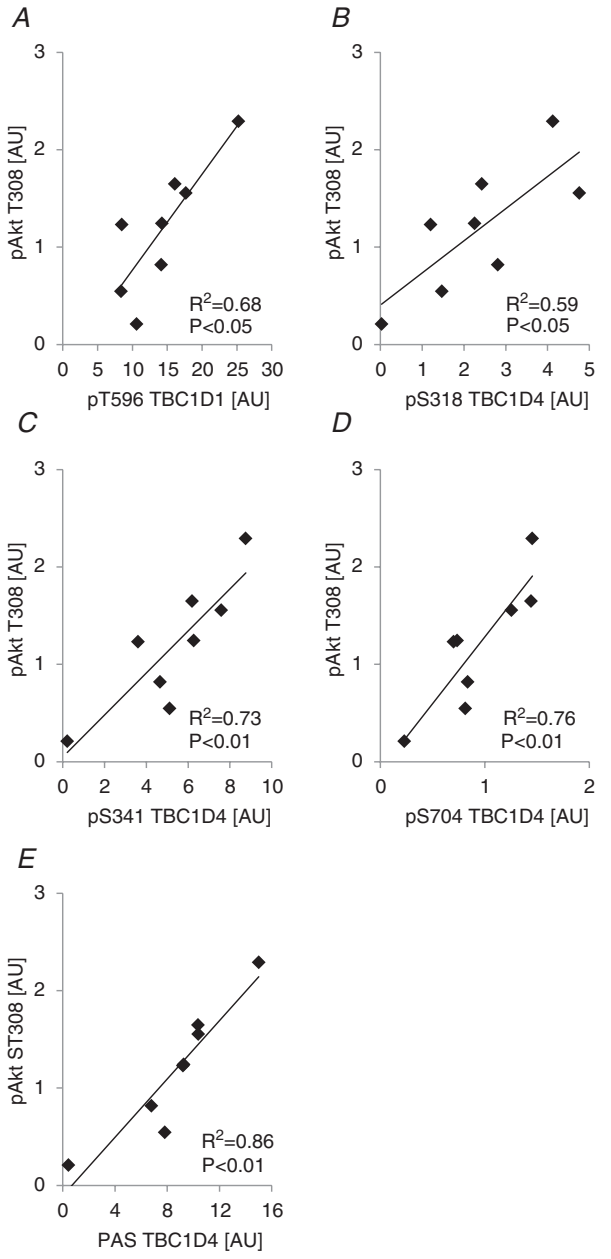

Figure 15. Akt phosphorylation status on T308 correlates with phosphorylation of TBC1D1/4 on specific sites in human skeletal muscle
All correlations were performed on data from the rested leg after exercise in the fed state (i.e. the insulin-stimulated leg). *A*, correlation between Akt pT308 and phosphorylation of TBC1D1 at pT596. *B–E*, correlations between Akt pT308 and phosphorylation of TBC1D4 at S318, S341, S704, and PAS, respectively. Samples size is $n = 8$ for all figures. R^2 and significance level are shown in the respective figures.

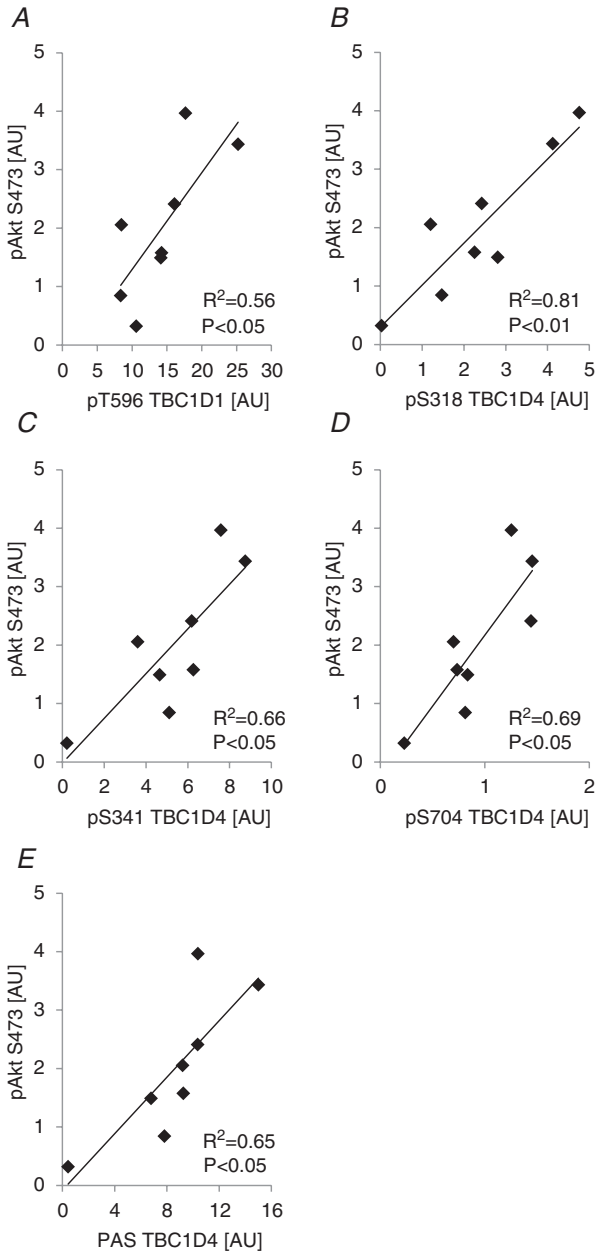

Figure 16. Akt phosphorylation status on S473 correlates with phosphorylation of TBC1D1/4 on specific sites in human skeletal muscle
All correlations are performed on data from the rested leg after exercise in the fed state (i.e. the insulin-stimulated leg). *A*, correlation between Akt pS473 and phosphorylation of TBC1D1 at pT596. *B–E*, correlations between Akt pS473 and phosphorylation of TBC1D4 at S318, S341, S704, and PAS, respectively. Samples size is $n = 8$ for all figures. R^2 and significance level are shown in the respective figures.

state. This may suggest that signalling arising from insulin and contraction converges on TBC1D4. The additive effects on phosphorylation and 14-3-3 binding may lead to elevated glucose transporter translocation to the plasma membrane, and may along with the elevated extracellular glucose concentration mediate the expected increased glucose uptake during exercise in the fed state compared to the fasted state (DeFronzo *et al.* 1981). Although this may also be true for the TBC1D1, we were unable to detect regulation of the 14-3-3 binding capacity on this protein in either of the conditions. This contrasts our own observations during more intense exercise (Frøsig *et al.* 2010).

TBC1D1 has four confirmed phosphorylation sites and while S237, S660, and S700 were phosphorylated in response to exercise only, T596 was phosphorylated in response to both postprandial increase in insulin and exercise. Furthermore, the two stimuli together gave an additive effect on T596 phosphorylation, suggesting that both exercise- and insulin-stimulated kinases regulate this site. Our findings are partly in line with recent studies showing increased S237 and S660 phosphorylation in response to exercise in human skeletal muscle (Frøsig *et al.* 2010; Jessen *et al.* 2011). Exercise-induced TBC1D1 phosphorylation on S237 and T596 correlated significantly with activity of $\alpha2/\beta2/\gamma3$ AMPK trimer complexes indicating that this AMPK complex could be the *in vivo* upstream kinase for these sites. However, when correlation analyses on delta values were performed, the only significant correlation remaining was between S237 and $\alpha2/\beta2/\gamma3$ AMPK activity. We have previously shown that recombinant AMPK can phosphorylate S237 *in vitro* (Frøsig *et al.* 2010), further supporting the idea that AMPK is a direct upstream TBC1D1 kinase. We found that Akt phosphorylation of S473, but not T308, was significantly increased in the exercised leg indicating that Akt may have been activated in the exercised leg. This could have contributed to the positive correlation between phosphorylation level of T596 and $\alpha2/\beta2/\gamma3$ AMPK activity. In EDL muscle from $\alpha2$ AMPK KO mice we confirmed that S237, T596, and S660 on TBC1D1 are regulated exclusively by $\alpha2$ AMPK in response to contractions (Pehmøller *et al.* 2009; Vichaiwong *et al.* 2010), and we have identified S700 as another site regulated by AMPK in contracting skeletal muscle.

TBC1D4 has nine confirmed phosphorylation sites and we were able to measure the phosphorylation status of seven of these. Since S341, S588, T642, S704, and S751 all were exercise-responsive sites, we correlated phosphorylation level with AMPK activity. We have previously shown that recombinant AMPK can phosphorylate S704 *in vitro* (Treebak *et al.* 2010). Here we showed that S341 and S704 correlated significantly with activity of $\alpha2/\beta2/\gamma1$ AMPK complexes, suggesting that this trimer may be responsible for phosphorylating these sites *in vivo*. Interestingly, and in contrast to TBC1D1, $\alpha2/\beta2/\gamma3$ AMPK trimer activity did not correlate with phosphorylation level of any of the exercise-responsive TBC1D4 sites. We have previously found that cycle ergometer exercise induced PAS phosphorylation in human muscle and that this correlated with activation of $\alpha2/\beta2/\gamma1$ AMPK complexes (Treebak *et al.* 2007); however, in the present study where the exercise mode was different, we did not find this correlation.

The idea that different AMPK trimers target specific Rab-GAPs is intriguing and suggests that AMPK and TBC1D1/4 could be localized to distinct compartments in the cell, and that TBC1D1 and TBC1D4 may play distinct roles in inhibiting vesicle mobilization. It is possible, for instance, that each Rab-GAP controls different Rab-GTPases responsible for separate steps in the mobilization of GLUT4 containing vesicles. To understand the function of TBC1D1 and TBC1D4 phosphorylation in response to AMPK activation, one would need specific AMPK activators that target specific AMPK heterotrimers. Although, A-769662 has been shown to target specific AMPK trimers *in vitro* (Cool *et al.* 2006; Scott *et al.* 2008), off-target effects in intact skeletal muscle have been reported (Treebak *et al.* 2009*a*). The fact that phosphorylation of S588 and S751 did not correlate significantly with AMPK $\alpha2/\beta2/\gamma1$ activity could be due to other kinases than AMPK mediating the phosphorylation of these sites. A recent study has shown that phosphorylation of S588 and T642 in response to insulin occurs on different pools of TBC1D4 in 3T3-L1 adipocytes and that protein kinase Cζ (PKCζ) is a likely upstream kinase for S588 (Ng *et al.* 2010). Furthermore, PKCζ is activated in response to exercise in human skeletal muscle (Richter *et al.* 2004; Rose *et al.* 2004), supporting the idea that this kinase could potentially target TBC1D4 *in vivo*.

Overexpression of the TBC1D4 mutant in which four key residues (S318, S588, T642, and S751) have been mutated to alanine (4P mutant) has been shown to decrease contraction-stimulated glucose uptake in mouse skeletal muscle (Kramer *et al.* 2006*b*). However, phosphorylation of T642 does not appear to be required for this effect of contraction (Ducommun *et al.* 2012), and since TBC1D4 phosphorylation has been reported to be unchanged or even reduced on three of the four sites in the 4P mutant (present study and Ducommun *et al.* 2012), it is possible that TBC1D4 does not mediate contraction-stimulated glucose uptake in muscle. On the other hand, phosphorylation of S588, one of the sites mutated in the 4P mutant, was reported to increase in response to *in situ* contraction of the gastrocnemius muscle (Ducommun *et al.* 2012). Whether phosphorylation of S588 alone is sufficient to increase glucose uptake, or whether aggregate mutation of multiple sites on TBC1D4 is necessary to disrupt function and glucose uptake is not known. Future studies using transgenic or knock-in mouse models are required to answer these questions.

The correlation analyses performed on the raw pTBC1D1/4 and pAkt data from the rested leg after

exercise in the fed state (insulin-stimulated) together with the analyses performed on delta values (i.e. rested leg in the fed trial after exercise minus rested leg in the fed trial before exercise) showed significant correlations between both Akt sites (T308/S473) and T596, S318, S341, S704, and PAS. T596, S318, and S341 are all within the Akt consensus sequence and it is likely that Akt is responsible for phosphorylating these sites in response to insulin. Indeed, phosphorylation of T596 in response to insulin has been shown to be ablated in Akt2 knockout mice (Vichaiwong *et al.* 2010). As previously shown, phosphorylation of S704 in response to insulin is completely ablated if mouse skeletal muscles are pre-incubated with the PI3 kinase inhibitor wortmannin (Treebak *et al.* 2010). In addition, the S704 phosphorylation was similar between WT and Akt2 knockout mice in response to a maximal dose of insulin, and recombinant Akt1/2 and PKCζ did not phosphorylate S704 *in vitro* (Treebak *et al.* 2010). Thus, although we found strong correlations between Akt phosphorylation and S704 in human skeletal muscle in the present study we do not know whether this relationship is causal. It would be interesting to study the effect on S704 phosphorylation in Akt2 knockout animals in response to a submaximal/physiological insulin dose, as muscles from these mice do not respond the same way to a maximal compared with a submaximal insulin doses in terms of glucose uptake (Cho *et al.* 2001).

The strength of the current study is that we have investigated the phosphorylation signatures of TBC1D1 and TBC1D4 in human skeletal muscle in response to physiological stimuli in a paired manner. We have applied transgenic animal models to further substantiate our findings from human skeletal muscle. However, data from these experiments indicate that it is not always possible to transfer findings across species, underlining the necessity to study intact muscle from human beings. Our findings show that in human skeletal muscle TBC1D1 and TBC1D4 are points of convergence of signalling events activated during postprandial physiological increase in insulin and acute exercise, and that the proposed upstream kinases AMPK and Akt may mediate this effect. Our working hypothesis is that TBC1D1 and TBC1D4 may be involved in the insulin-sensitizing effect of exercise, and perhaps other insulin-sensitizing agents (Pehmøller *et al.* 2012).

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Additional information

Competing interests

The authors report no competing interests

Author contributions

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