Archives of Biochemistry and Biophysics 679 (2020) 108223

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

WNK1 phosphorylation sites in TBC1D1 and TBC1D4 modulate cell surface expression of GLUT1

Andreia F.A. Henriques[a,b], Paulo Matos[a,b], Ana Sofia Carvalho[c], Mikel Azkargorta[d], Felix Elortza[d], Rune Matthiesen[c], Peter Jordan[a,b,*]

[a] Department of Human Genetics, National Health Institute 'Dr. Ricardo Jorge', Lisbon, Portugal
[b] BioISI - Biosystems & Integrative Sciences Institute, Faculty of Sciences, University of Lisbon, Lisbon, Portugal
[c] CEDOC-Chronic Diseases Research Centre, Nova Medical School, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisbon, Portugal
[d] Center for Cooperative Research in Biosciences (CIC bioGUNE), Building 800, Science and Technology Park of Bizkaia, 48160, Derio, Spain

ARTICLE INFO

Keywords:
Glucose cotransport
GLUT1
Membrane traffic
Protein phosphorylation
TBC1D
WNK1

ABSTRACT

Glucose uptake by mammalian cells is a key mechanism to maintain cell and tissue homeostasis and relies mostly on plasma membrane-localized glucose transporter proteins (GLUTs). Two main cellular mechanisms regulate GLUT proteins in the cell: first, expression of GLUT genes is under dynamic transcriptional control and is used by cancer cells to increase glucose availability. Second, GLUT proteins are regulated by membrane traffic from storage vesicles to the plasma membrane (PM). This latter process is triggered by signaling mechanisms and well-studied in the case of insulin-responsive cells, which activate protein kinase AKT to phosphorylate TBC1D4, a RAB-GTPase activating protein involved in membrane traffic regulation. Previously, we identified protein kinase WNK1 as another kinase able to phosphorylate TBC1D4 and regulate the surface expression of the constitutive glucose transporter GLUT1. Here we describe that downregulation of WNK1 through RNA interference in HEK293 cells led to a 2-fold decrease in PM GLUT1 expression, concomitant with a 60% decrease in glucose uptake. By mass spectrometry, we identified serine (S) 704 in TBC1D4 as a WNK1-regulated phosphorylation site, and also S565 in the paralogue TBC1D1. Transfection of the respective phosphomimetic or unphosphorylatable TBC1D mutants into cells revealed that both affected the cell surface abundance of GLUT1. The results reinforce a regulatory role for WNK1 in cell metabolism and have potential impact for the understanding of cancer cell metabolism and therapeutic options in type 2 diabetes.

1. Introduction

The small family of WNK [with no lysine (K)] protein kinases includes four members in the human genome [1], which encode serine/threonine kinases with a specific sequence signature that includes substitution of a highly conserved lysine (hence their name) in the subdomain II of the catalytic domain [2,3] by a cysteine. This conserved lysine residue mediates ATP binding in most kinases but WNKs use an alternative lysine from subdomain I for this function [4,5].

The four human *WNK* genes (*WNK1, WNK2*, *WNK3*, *WNK4*) are located on different chromosomes [1] and show tissue-specific expression patterns, with WNK1 being almost ubiquitous. WNK proteins only share about 40% amino acid identity, mostly in their catalytic domains and some coiled-coil domains [6–8].

Mutations in the *WNK1* and *WNK4* genes cause the human monogenic disease familiar hyperkalemic hypertension (FHHt, also known as pseudohypoaldosteronism type 2 or Gordon syndrome), characterized by excessive renal sodium and potassium retention [6]. WNK1 (similar to WNK3 and WNK4) was identified to act as a multifunctional upstream activator of ion transport proteins in different segments of the nephron, in particular of the sodium-chloride cotransporter NCC [9] and the sodium-potassium-chloride cotransporter NKCC2 [10,11]. Both cotransporters become activated following their phosphorylation at two threonine residues in their amino terminal cytoplasmic domain, by either the STE20/SPS1-related proline-alanine-rich protein kinase (SPAK) or the oxidative stress-responsive kinase (OSR) 1. In addition, phosphorylation decreases endocytosis rates, promoting cotransporter retention in the plasma membrane (PM) [12]. The role of WNK1 is to

Abbreviations: FBS, fetal bovine serum; GLUT, glucose transporter; PM, plasma membrane; siRNA, small interfering RNA; TBC1D, (Tre-2, BUB2, CDC16) domain family member; WNK, With-no-lysine protein kinase; wt, wild type

* Corresponding author. Departamento de Genética Humana, Instituto Nacional de Saúde Doutor Ricardo Jorge, Avenida Padre Cruz, 1649-016, Lisboa, Portugal.
E-mail address: peter.jordan@insa.min-saude.pt (P. Jordan).

https://doi.org/10.1016/j.abb.2019.108223
Received 6 August 2019; Received in revised form 13 November 2019; Accepted 5 December 2019
Available online 06 December 2019
0003-9861/

phosphorylate and activate SPAK or OSR1 in hypotonic low-chloride conditions [9,13–15] due to its ability to act as a sensor of low chloride concentration in cells. Chloride usually binds to the WNK1 catalytic domain, leading to inhibition of autophosphorylation and kinase activation [16].

We previously reported that WNK1 is not only linked to ion transport regulation but also has a role in regulating the cell surface expression of another PM transporter, the *SLC2A1* gene product glucose transporter GLUT1 [17]. Members of the GLUT family are essential for uptake of extracellular food-derived glucose as the main energy source for most mammalian cell types. Besides an energy-coupled, sodium-gradient driven sugar cotransport in the intestine or kidney, most other tissues rely on facilitative transport by GLUT proteins along a glucose gradient that is established by cells through rapid phosphorylation and glycolysis of glucose [18]. GLUT1 represents the most ubiquitously expressed isoform. Two main cellular mechanisms regulate GLUT1 proteins in the cell: first, expression of the *SLC2A1* gene is under dynamic transcriptional control and is used by cancer cells to increase glucose availability; second, GLUT proteins are regulated by membrane traffic from storage vesicles to the PM. The best-studied example is insulin-stimulated glucose uptake of muscle cells and adipocytes, which occurs through rapid GLUT4 translocation to the PM and proportional increase in glucose uptake [19]. When insulin binds to its receptor on these cells, the phosphatidylinositol-3-kinase (PI3K) gets stimulated and triggers activation of protein kinase AKT that then phosphorylates its substrates (Tre-2-BUB2-CDC16) domain family member (TBC1D) 4 [20–22] or TBC1D1 [21,23–25]. These are two paralogue GTPase-activating proteins for RAB-family GTPase, which subsequently mediate translocation of GLUT4 storage vesicles to the PM in oxidative muscle and adipose cells or in glycolytic muscle fibers, respectively [26,27]. Impaired GLUT4 translocation is a hallmark characteristic of insulin resistance leading to diabetes mellitus.

Besides AKT, WNK1 was also identified to phosphorylate TBC1D4 *in vitro*. WNK1 expression in HEK293 cells promoted binding of TBC1D4 to regulatory 14-3-3 proteins, preventing the inhibitory interaction of TBC1D4 with the small GTPase RAB8A, a positive regulator of exocytic vesicle traffic. In consequence, GLUT1-containing storage vesicles can fuse with the PM and increase the cell surface expression of GLUT1 [17]. Similarly, WNK1 was shown to regulate GLUT4 PM expression in skeletal muscle cells [28].

In addition to WNK1, the serum-glucocorticoid-regulated kinase (SGK) 1 can also participate in the regulation of TBC1D4. SGK1 was found to phosphorylate TBC1D4 and increase the cell surface expression of GLUT1 upon activation by PI3K [29]. Also, SGK1 phosphorylation of TBC1D4 has a role in regulating the endocytosis of the epithelial sodium channel (ENaC) [30]. Thus, WNK1 and SGK1 may represent surrogate pathways to modulate TBC1D4 and glucose uptake in response to insulin.

2. Materials and methods

2.1. Cell culture and transfections

HEK293 (human embryonic kidney 293) cells were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% (v/v) of heat inactivated fetal bovine serum (FBS) (all reagents from Gibco, Thermo Fisher Scientific, Waltham, USA). Cells were maintained at 37 °C with 5% CO2, and regularly checked for absence of mycoplasm infection by PCR amplification of a 16S ribosomal DNA fragment (primers F: 5′- ACT CCT ACG GGA GGC AGC AGT A and R: 5′-TGC ACC ATC TGT CAC TCT GTT AAC CTC) from lysates of cells harvested from the culture medium.

For ectopic expression of plasmid cDNAs, HEK293 cells were transfected at 80–90% confluence using Metafectene (Biontex, Munich, Germany) according to the manufacturer's instructions. Transfection efficiencies were found to be around 90%, as determined microscopically using a GFP expression vector. Total amounts of transfected plasmid DNA were kept constant at 6 μg per 60 mm cell culture dish or 2 μg per 35 mm dish and adjusted with empty vector, if required. Cells were analyzed after 20 h for biochemical assays.

For gene-specific transcript silencing, HEK293 cells at 30% confluence were transfected using Metafectene with 200 pmol of small interfering RNA oligonucleotides (siRNAs) per 35 mm dish or 400 pmol per 60 mm dish. Cells transfected with siRNAs were analyzed after 48 h, and the degree of target gene expression depletion was determined in each experiment by Western blot (WB) (see below). All results were confirmed in at least three independent experiments. The siRNA oligos were ordered from Eurofins Genomics (Ebersberg, Germany) with the following sequences: control siLUC, 5′-CGU ACG CGG AAU ACU UCG ATT; siWNK1: 5′-GCA GGA GUG UCU AGU UAU A.

2.2. DNA plasmids and constructs

All constructs were verified by automated DNA sequencing and all primer sequences are available in Table S1. TBC1D1 was amplified from pBluescript with the primers KpnTBD1-F and KpnTBD1-R, cloned into pCR2.1 TOPO-TA vector (Invitrogen) and subcloned as a *Kpn*I fragment into expression plasmid pcDNA3-Myc. This construct will be referred to as Myc-TBC1D1. To introduce the unphosphorylatable alanine (A) or phosphomimetic aspartate (D) codons, the plasmid was mutated at codon 565 from TCC to GCC or GAC to obtain the constructs Myc-TBC1D1 S565A/D, using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, USA) according to the manufacturer's instructions.

Previously published constructs used in this study were: (1) pCR3.1/AS160-2myc [31], here designated as Myc-TBC1D4. To introduce the unphosphorylatable alanine (A) or phosphomimetic aspartate (D) codons, the plasmid was mutated at codon 704 from TCT to GCT or GAT to obtain the constructs Myc-TBC1D4 S704A/D, using the Site-Directed Mutagenesis. (2) pIRES2-EGFP-SGK1 CA and KD [32], were amplified with Eco-SGK1-F and Kpn-SGK1-R, cloned into pCR2.1 TOPO-TA and subcloned as *Eco*RI/*Kpn*I fragments into pEGFP-C2 expression plasmids.

2.3. In vitro protein kinase assays

For each condition, 0.5 μg of the recombinant protein kinase (Flag/His-WNK1 (1111-0000-1; N-terminal fragment 171–529), GST-AKT1 (0132-0000-2; full-length protein), GST-AKT2 (0276-0000-2; C-terminal fragment 107–481), GST-SGK1 (0199-0000-2; full-length protein) or GST-SGK3 (0198-0000-2; full-length protein), all from ProQinase, Freiburg, Germany) were incubated in 20 μL kinase reaction buffer (30 mM Tris-HCl pH 7.5, 10% glycerol, 1 mM DTT, 1 mM Na_3VO_4, 10 mM $MgCl_2$, 100 μM ATP), mixed with their recombinant substrate (0.5 μg of rMyc-TBC1D1 or rMyc-TBC1D4 (both from Origene Technologies, Rockville, USA) or recombinant kinase-dead OSR1 as a WNK1 positive control [17]), and incubated in the presence of 5 μCi γ^{32}P-ATP at 30 °C for 30 min. Finally, 2x SDS sample buffer (100 mM Tris-HCl pH 6.8, 10% glycerol, 4% SDS, 130 mM DTT, 0.5 mg bromophenol blue) was added, samples boiled and separated by SDS-PAGE followed by WB. Membranes were exposed to X-ray films, and subsequently incubated with the indicated antibodies in order to document that comparable protein quantities were present in each assay.

2.4. TBC1D phosphopeptide identification

The above protein kinase assays were repeated under non-radioactive conditions using *in vitro* phosphorylation of 0.5 μg recombinant substrate with 0.5 μg recombinant kinase in duplicate, standard 20 μL-reactions for 1 h at 30 °C. Then, 20 μL iodoacetamide (IAA) for cysteine alkylation were added for 1 h at 21 °C (protected from light), followed by quenching of remaining IAA with 20 μL DTT for 15 min at 21 °C. Duplicates were incubated with either trypsin (cuts after arginine or

lysine) or enteropeptidase (cuts only after lysine) and digested overnight at 37 °C. Digested peptides were purified by retention on C12 hydrophobic reverse phase porous resin filled into previously activated and washed small columns. Resin columns were then shipped for mass spectrometry (MS) to the CIC bioGUNE center in Derio, Spain. Peptide separation was performed on a nanoACQUITY UPLC System (Waters Corporation, Milford, USA) on-line connected to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). An aliquot of each sample was loaded onto a Symmetry 300C18 UPLC Trap column (180 μm × 20 mm, 5 μm, Waters). MS raw data and a description of their analysis are available in the Supplementary data.

2.5. Biotinylation of cell surface proteins

HEK293 cells were first transfected with either siWNK1 or siLUC and incubated for 24 h. In a second transfection, the Myc-TBC1D1/4 S-A mutants were transfected into the control (siLUC) dishes, whereas the Myc-TBC1D1/4 S-D mutants into the siWNK1 dishes. For all conditions, cells were placed on ice, washed three times with ice cold PBS-CM (PBS pH 8.0 containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and left 5 min in cold PBS-CM. Cells were then incubated for 45 min with 0.5 mg of EZ-Link Sulfo–NHS–SS-Biotin (Santa Cruz Biotechnology, Heidelberg Germany) to label all cell surface proteins. Cells were rinsed twice and left for 15 min on ice with ice-cold Tris-Q [100 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glycine, 1% (w/v) BSA] to quench the reaction. Cells were again washed three times with cold PBS-CM and lysed in 250 μL of pull-down buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP40, supplemented with a protease inhibitor cocktail composed of 1 mM PMSF, 1 mM 1,10-phenanthroline, 1 mM EGTA, 10 μM E64, and 10 μg/mL of each aprotinin, leupeptin, and pepstatin A; all from Sigma-Aldrich Quimica, Madrid, Spain). Cell lysates were cleared at 9000 × *g* for 5 min at 4 °C. An aliquot of 40 μL, representing total protein levels, was removed and added to 2x SDS modified sample buffer (62.5 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 0.02% bromophenol blue, 196.4 mM DTT), while 200 μL lysate were added to 15 μL of protein G agarose beads (Roche Portugal), rotated for 1 h at 4 °C and centrifuged for 1 min at 3000 × *g*. The pre-cleared lysates were recovered, added to 20 μL streptavidin-agarose beads (Sigma-Aldrich), previously incubated for 1 h in 1 mL cold pull-down buffer containing 2% (w/v) milk, and washed three times in pull-down buffer. Lysate and beads were rotated for 1 h at 4 °C, centrifuged for 1 min at 3000 × *g*, and washed five times in cold wash buffer (100 mM Tris-HCl pH 7.5, 300 mM NaCl, 1% (v/v) Triton X-100). Captured proteins were recovered in 20 μL of 2x SDS modified sample buffer with 100 mM DTT and analyzed by WB with specific antibodies, as described below.

Protein lysates were separated by 9% (w/v) SDS-PAGE in 1% (v/v) glycerol-containing gels run at 4 °C. For detection of specific proteins, SDS-PAGE gels were transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, USA). WB membranes were blocked in 5% (w/v) milk powder in TBS with 0.1% (v/v) Triton X-100, probed using primary antibodies listed below, washed 3x, and incubated with a secondary peroxidase-conjugated antibody. Bands were visualized by chemiluminescence on X-rays films and quantified on digitalized images by densitometric analysis with ImageJ software (NIH). Primary antibodies used were: mouse anti-PCNA (clone PC10; NA03) from MerckBiosciences (Nottingham, UK); mouse anti-transferrin receptor (clone H68.4; 13–6800), mouse anti-His (Invitrogen 37–2900) and rabbit anti-GST (Chemicon AB3282) from Thermo Fisher Scientific; mouse anti-Tubulin (clone B-5-1-2; T5168) and mouse anti-MYC (clone 9E10; M5546) from Sigma-Aldrich, rabbit anti-GLUT1 (ab652) from Abcam (Cambridge, UK), and sheep anti-WNK1 (S062B) from Dundee University, UK.

2.6. Glucose uptake assays

HEK293 cells were treated as described and glucose uptake was measured using Glucose Uptake-Glo™ Assay (Promega, Madison, USA), according to the manufacturer's instructions. Briefly, this assay is a fluorescence-based method measuring the amount 2-deoxyglucose (2DG) transported into cells during a period of 10 min 2DG is transported into cells and phosphorylated to produce 2-deoxyglucose-6-phosphate (2DG6P). The addition of Stop Buffer stops 2DG transport, lyses cells, destroys any NADPH within the cells and denatures cellular proteins. A neutralization buffer is added before the 2DG6P detection reagent, containing glucose-6-phosphate dehydrogenase (G6PDH) that oxidizes 2DG6P to 6-phosphodeoxygluconate (6PDG) and reduces NADP + to NADPH. The reductase uses the NADPH to convert the proluciferin to luciferin, which is then used by luciferase to produce light measured in the Lucy-2 luminometer (Anthos Mikrosysteme, Friesoythe, Germany). The relative light units (RLU) were then normalized to the total protein levels based on the PCNA levels in lysates detected by WB.

2.7. Statistical analysis

Data were analyzed using Student's t-tests for paired samples or ANOVA tests followed by post-hoc Tukey's tests when comparing multiple treatments. $P < 0.05$ was accepted as the level of statistical significance. Shown data reflect the mean ± SEM from at least three independent experiments.

3. Results

3.1. WNK1 modulates glucose uptake in HEK293 cells

Previously, we identified a role for WNK1 in regulating glucose transporter GLUT1 expression at the PM in HEK293 cells [17]. In pursuit of these studies, we determined whether the observed increase in GLUT1 at the PM would indeed correspond to an increase in glucose uptake. For this, we transfected cells with specific siRNAs to deplete endogenous WNK1 in HEK293 cells and 48 h later measured both, their glucose uptake and GLUT1 expression at the PM. The efficiency of WNK1 depletion was around 80% as confirmed by WB (Fig. 1B). Under these conditions, the levels at the PM of the transferrin receptor, which undergoes constitutively endosomal uptake and recycling, were not affected; however, the GLUT1 levels decreased (Fig. 1A) and cellular glucose uptake was reduced by 40% (Fig. 1C). A comparable reduction was also observed upon WNK1 depletion in HT29 colorectal cells (data not shown). Thus, the presence of WNK1 contributes to cellular glucose uptake.

3.2. Phosphorylation of TBC1D proteins by kinases WNK1, AKT and SGK

The mechanism by which WNK1 affected GLUT1 was shown to involve phosphorylation of the RAB-GAP protein TBC1D4 [17]. Phosphorylation of TBC1D4, but also of its paralog TBC1D1, is a key regulatory step in the kinase cascades leading to changes in glucose uptake [21,22]. AKT was the first protein kinase described to phosphorylate the RAB-GAPs TBC1D1 and TBC1D4 in response to insulin stimulation [20,22].

We therefore compared the sites of phosphorylation in TBC1D4 and TBC1D1 that the kinases AKT and WNK1 would recognize, and also included SGK1 for comparison. For this, we performed *in vitro* protein kinase assays using recombinant RAB-GAP proteins TBC1D1 and TBC1D4 as substrates and incubated them either alone or together with one of the following recombinant protein kinases: AKT1, AKT2, WNK1, SGK1 and SGK3. As shown in Fig. 2A, AKT1 autophosphorylated and in addition phosphorylated TBC1D1 and TBC1D4. As expected both TBC1D proteins were not radio-labelled in the presence of γ32P-ATP

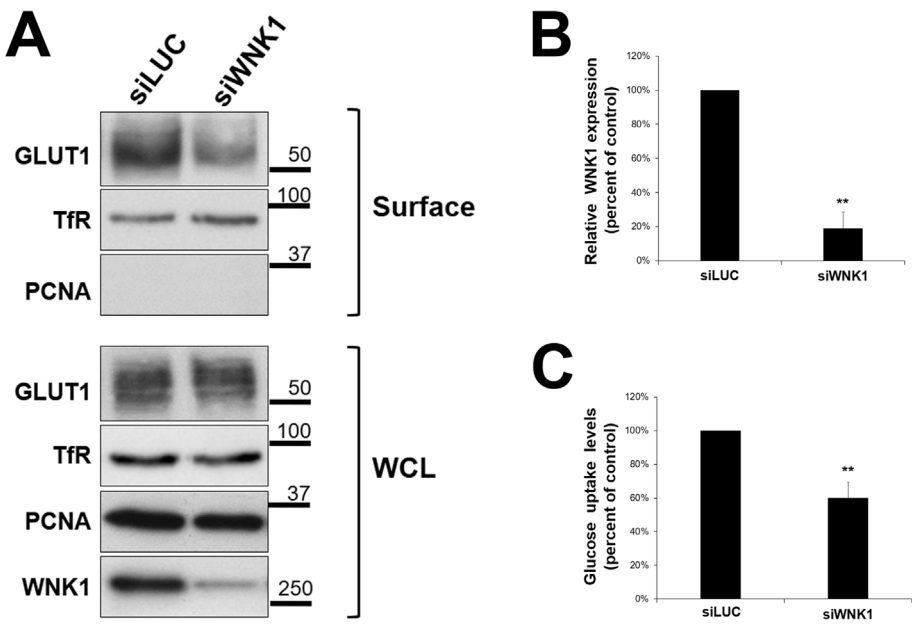

Fig. 1. WNK1 modulates glucose uptake in HEK293 cells. Cells were transfected with either siLUC (control) or siWNK1 and incubated for 48 h. Then cell surface proteins were biotinylated before cells were lysed and biotinylated proteins captured with Streptavidin-beads followed by SDS-PAGE and transfer to PVDF blotting membranes. (A) Detection of the indicated proteins in the whole-cell lysate (WCL) or in the biotinylated protein fraction (Surface). In both fractions, the PCNA protein served as a control to document either the amount of total protein loaded (WCL), or the absence of contaminating cytosolic proteins (Surface). The transferrin receptor (TfR) served to exclude unspecific effects on endocytosis or recycling (Surface). (B) Quantification of WNK1 depletion. Band intensities from the densitometry of membranes shown in (A) from at least three independent experiments were quantified and graphically displayed. (C) Quantification of glucose uptake levels. The glucose uptake assay was performed using the Glucose Uptake-Glo™ Assay (Promega) following the manufacturer's instructions and glucose values determined by relative light units (RLUs) normalized to total protein values using ImageJ (NIH) for quantification of the PCNA bands shown in (A). All data shown represent means ± SEM; ** = $P < 0.01$.

alone. For further validation, a phospho-specific antibody for TBC1D4 threonine (T) 642 was used, one of the first phosphosites described for AKT [20]. Using this antibody, we were able to confirm that the *in vitro* kinase assay reproduced the specific phosphorylation of TBC1D4 by AKT1 at an already described phosphosite (Fig. 2A). Identical results were obtained with recombinant AKT2 (data not shown).

For the *in vitro* WNK1 kinase assay (Fig. 2B), recombinant kinase-dead OSR1 (a previously identified physiological WNK1 substrate), served as a positive control [17,33], and the negative control, that a kinase-dead WNK1 mutant cannot phosphorylate TBC1D4, was previously shown [17]. His-tagged WNK1 autophosphorylated in the presence of γ^{32}P-ATP, but also phosphorylated both TBC1D proteins *in vitro*. Positive phosphorylation was further observed when GST-SGK1 and GST-SGK3 were used as kinases (Fig. 2C), consistent with a previously described role in regulating GLUT translocation to the PM [29].

3.3. Identification of phosphorylated sites in TBC1D1 and TBC1D4

Having established the *in vitro* phosphorylation of both TBC1D1 and TBC1D4, we next determined the specific phosphorylated residues in both RAB-GAPs. Although for AKT, several sites of phosphorylation in both TBC1Ds were already known [20,22,34,35], it was unknown whether WNK1, SGK1 and SGK3 would target the same or unique phosphosites. Phosphorylation sites were determined by MS following kinase assays repeated under non-radioactive conditions and the results summarized in Table 1 (raw MS data and their analysis are available as Supplementary data and Table S2).

The data identified well-known phosphosites for AKT1, such as serine (S) 237 and threonine (T) 596 from TBC1D1 [36], and S588 from TBC1D4 [20]. Importantly, novel TBC1D1 phosphosites were identified: T505 as a SGK1-specific phosphosite, and S565 as a WNK1-specific phosphosite. In the case of TBC1D4, S704 was identified to be preferentially phosphorylated by WNK1, SGK1 and SGK3. Other previously unreported phosphosites included S609, S673 and S725.

3.4. Validation of the WNK1-regulated phosphosites as modulators of PM expression of GLUT1

For further studies of the WNK1-phosphorylated sites TBC1D1-S565 and TBC1D4-S704, their functional relevance for the regulation of PM expression of GLUT1 was validated. First, site-directed mutagenesis was used to introduce the respective phosphomimetic aspartate (D) or unphosphorylatable alanine (A) residues at codons 565 or 704. Then, these Myc-tagged constructs were transfected into HEK293 cells, followed by biotinylation of cell surface proteins and WB. The unphosphorylatable S565A and S704A mutants were expected to maintain the respective TBC1D proteins active, and thus lead to a decrease in the amount of GLUT1 at the PM. As a positive control and term of comparison, we used WNK1 depleted cells (see Fig. 1). By contrast, the phosphomimetic mutants S565D and S704D should inactivate the RAB-GAP activity of the TBC1D proteins and promote an increase in PM GLUT1. Moreover, the phosphomimetic mutants should also be able to rescue the loss of PM GLUT1 induced by WNK1 depletion alone if they act downstream of WNK1. The transferrin receptor (TfR) was labelled to exclude unspecific effects on endocytosis or recycling.

As shown in Fig. 3A, transfection of HEK293 cells with the non-phosphorylatable TBC1D1-S565A mutant led to a decrease in PM GLUT1. The quantified magnitude of this effect (Fig. 3B) was comparable to the one caused by the depletion of WNK1, used as a positive control. The same decrease in PM GLUT1 was observed in cells transfected with the non-phosphorylatable mutant TBC1D4-S704 (Fig. 3C and D). The effect of these non-phosphorylatable mutants was not further increased by simultaneous depletion of endogenous WNK1 (data not shown).

When cells were then transfected with the phosphomimetic mutants TBC1D1-S565D or TBD1D4-S704D (Fig. 3A and C, respectively), both were able to increase PM GLUT1 when transfected alone (not shown) and, most importantly, to counteract the inhibitory effect of siWNK1, as quantified in Fig. 3B and D.

Thus, both phosphorylation sites, TBC1D1-S565 and TBC1D4-S704 were identified as novel regulatory phosphosites in these RAB-GAP proteins that are involved in the regulation of GLUT1 PM levels.

Another phosphorylated TBC1D1 residue identified in Table 1 was T505, which was recognized by SGK1. When the respective unphosphorylatable and phosphomimetic mutants were transfected into HEK293 cells, the analysis of biotinylated cell surface proteins revealed that neither the TBC1D1-T505A nor the -T505D mutant caused any significant change in the amount of GLUT1 present at the PM (Fig. S1). Thus, in contrast to the two sites TBC1D1-S565 and TBC1D4-S704, the TBC1D1-T505 residue does not seem to play a role in the regulation of

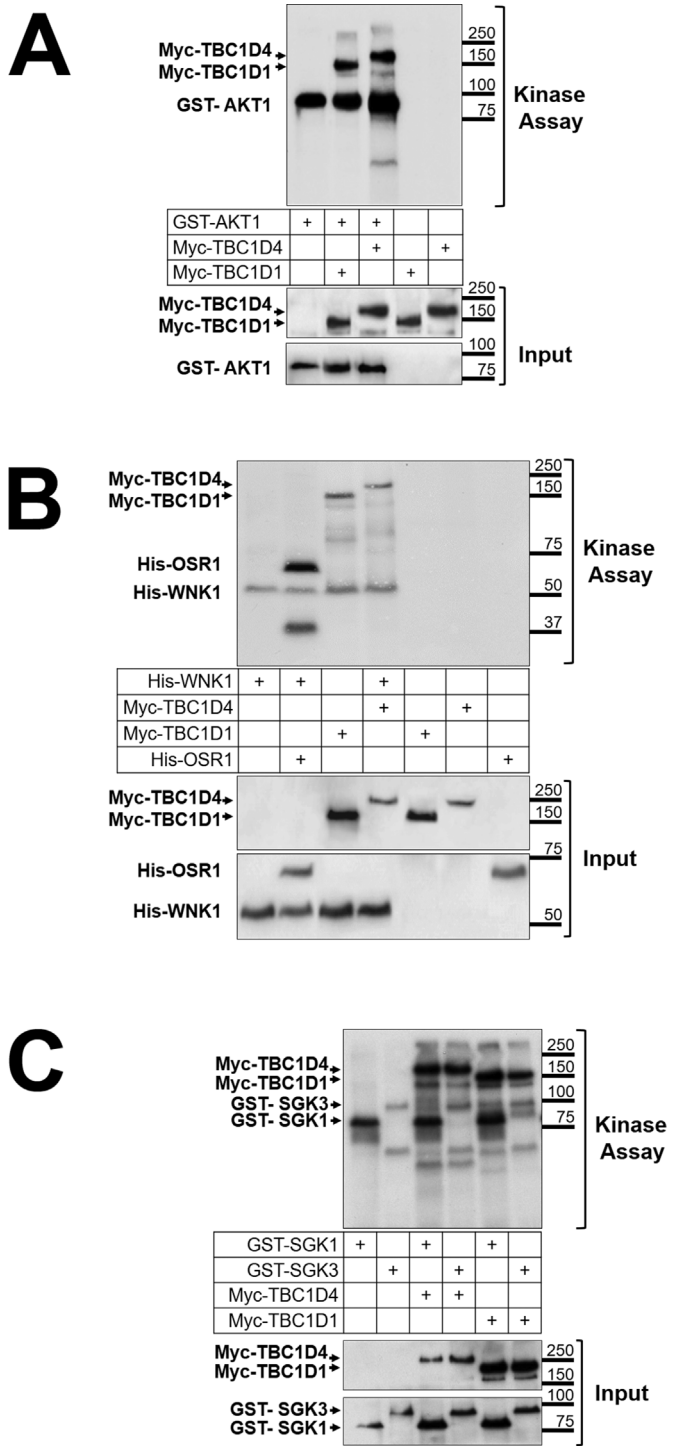

Fig. 2. *In vitro* protein kinase assays to determine the phosphorylation of TBC1D1 and TBC1D4 by the protein kinases (A) AKT1, (B) WNK1 and (C) SGK1 and SGK3. Recombinant tagged kinases were incubated in kinase buffer containing γ^{32}P-ATP, for 30 min at 30 °C, either alone or with one of the Myc-tagged recombinant TBC1D1 or TBC1D4 substrates. In (B), a recombinant kinase-dead mutant of OSR1 (HIS-OSR1) was used as a positive control for WNK1 kinase activity. After incubation with the radiolabeled ATP, the protein samples were separated by SDS-PAGE and transferred to PVDF blotting membranes. First, the incorporated radioactive γ^{32}P-phosphate was detected by exposing the membranes for 8 or 24 h to X-ray films (Kinase assay). Subsequently, the quantities of the recombinant proteins present on the radioactive membranes were documented by using anti-GST, anti-Myc and anti-His antibodies. Note that all studied kinases autophosphorylated and also phosphorylated the substrate proteins, TBC1D1 and TBC1D4.

Table 1
Major phosphorylated sites identified by mass spectrometry in the indicated human TBC1D family member after *in vitro* protein kinase reactions with the indicated kinases (S = serine; T = threonine).

Human TBC1D1							
AKT1	S237				S585		T596
WNK1	S237			S565	S585		
SGK1	S237	T505					T596
SGK3							T596

Human TBC1D4							
AKT1	T568	S588			S725	S751	T766
WNK1				S704	S725		
SGK1	T568	S588	S673	S704		S751	
SGK3	T568	S588		S704	S725		

GLUT1 abundance at the PM.

4. Discussion

The results presented in this work highlight that the regulation of GLUT1, the constitutive glucose transporter expressed in most cell types, through phosphorylation of the RAB-GAP proteins TBC1D4 and TBC1D1 may be more complex than previously reported. We identified novel phosphorylation sites in TBC1D4 and TBC1D1 and show the involvement of two WNK1-phosphorylated sites in the regulation of GLUT1 plasma membrane expression.

Although AKT has been predominantly studied as the kinase responsible for the phosphorylation of TBC1D4 and TBC1D1, other kinases including WNK1 were shown to contribute to the regulation of glucose uptake [17,28,29,35]. Indeed, phosphorylation at various serine/threonine residues in either TBC1D member is the functionally decisive modification leading to inactivation of their RAB-GAPs activity [21,23,35,37]. We identified WNK1-regulated phosphorylation sites in each member, and demonstrate their involvement in the regulation of GLUT1 PM levels. Consistently, neither the TBC1D1 phosphosite FKLLGS^{565}, nor the TBC1D4 motif LHTSFS^{704} contain the consensus AKT recognition motif (R/K)x (R/K)xxS/T. Whereas TBC1D1-S565 was exclusively recognized by WNK1, the phosphorylation of TBC1D4-S704 was detected preferentially in the presence of WNK1, SGK1 or SGK3.

The crucial role for TBC1D4 in GLUT regulation has solid support: experimental suppression of TBC1D4 leads to insulin-independent GLUT4 release to the PM and mutational inactivation of the GAP domain [38] or of major phosphorylation sites [39] impede insulin-stimulated GLUT4 translocation. Furthermore, rare human mutations in the *TBC1D4* gene severely affect the carriers' insulin response [40,41]. TBC1D1 shares high sequence homology and domain structure with TBC1D4 [42], including multiple homologous phosphorylation sites. Both contribute to insulin-dependent glucose uptake, although TBC1D1 seems to be predominantly expressed in glycolytic muscle [27]. Nevertheless, TBC1D1 has been reported to increase the GLUT1 expression levels in adipocytes [43] and TBC1D4 contributes to the muscle cell response [44].

Although S565A mutant transfection caused a significant decrease in GLUT1 PM expression, comparable with the effect observed in WNK1-depleted cells, transfection of S565D mutant after WNK1 depletion was not enough to fully restore the levels of PM GLUT1 to that observed in control conditions. Accordingly, we might infer that S565 phosphorylation in TBC1D1 is necessary but not sufficient for the inactivation of the RAB-GAP and that phosphorylation by WNK1 or other kinases on additional residues might be required for full TBC1D1 inhibition. However, it cannot be excluded that the S565D mutant is only partially phosphomimetic, for example if the phosphorylation site serves as a recognition motif for adaptor proteins. Then, the S-D alteration might change the binding pocket causing a reduced ability of

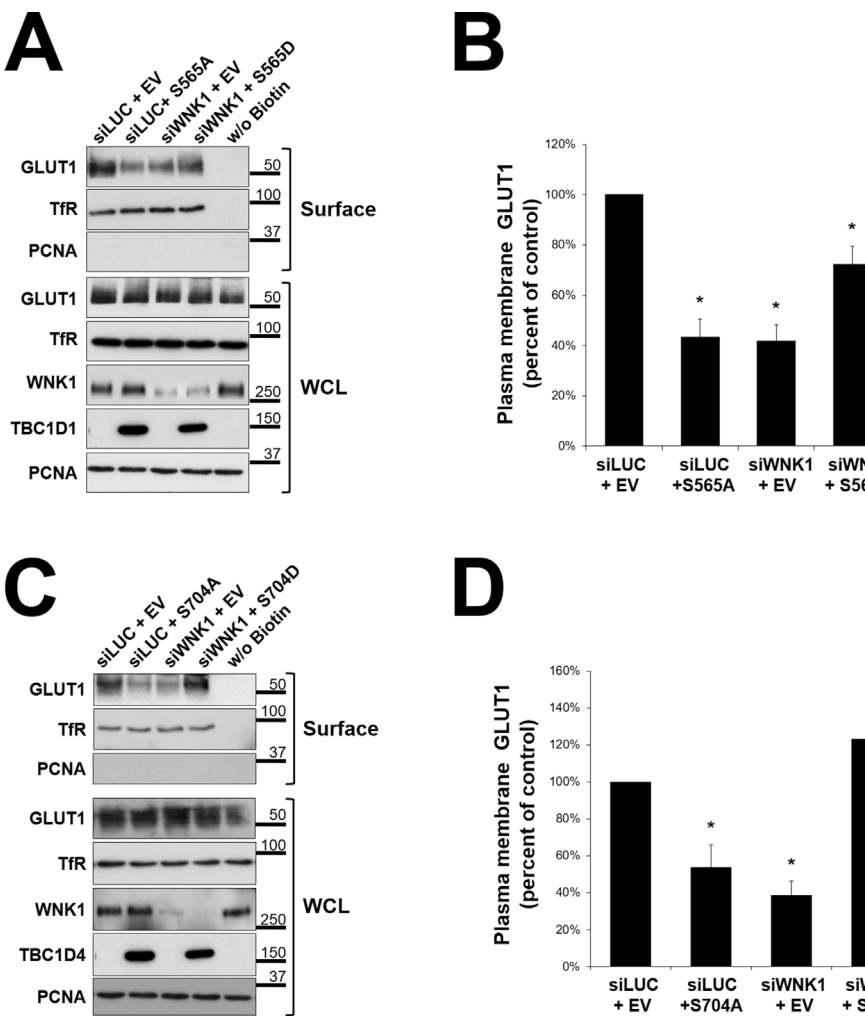

Fig. 3. Validation of the effect of the WNK1-targeted phosphosites TBC1D1-S565 (A–B) and TBC1D4-S704 (C–D) on GLUT1 PM expression. HEK293 cells were transfected as indicated either with siLUC and empty-vector (EV) as control, or siLUC and a non-phosphorylatable Myc-tagged TBC1D mutant [(A–B) TBC1D1-S565A, (C–D) TBC1D4-S704A)], or siWNK1 and EV as control, or siWNK1 and a phosphomimetic Myc-TBC1D mutant [(A–B) TBC1D1-S565D, (C–D) TBC1D4-S704D)]. After 48 h, cell surface proteins were biotinylated, then cells were lysed and proteins analyzed as described in the legend to Fig. 1. (A, C) Detection of the indicated proteins in the whole-cell lysates (WCL) or in the biotinylated protein fraction (Surface). As a further control, cells were not incubated with biotin (w/o biotin). (B, D) Corresponding quantification of GLUT1 detection in the biotinylated cell surface fraction, obtained from at least three independent experiments. Data are shown as fold change relative to siLUC + EV (control) and represent means ± SEM; * = $P < 0.05$ compared to respective control.

the mutant to bind the adaptor protein [45].

By contrast, transfection of mutant TBC1D4 S704D reverted the decrease of GLUT1 PM expression caused by WNK1 depletion. Phosphorylation at S04 was also reported in skeletal muscle following physical exercise and shown to result from a PI3K-independent mechanism that requires activation of AMPK [46,47]. However, regarding non-exercise related phosphorylation little was known about this phosphosite. In the present work, we related the phosphorylation of S704 in TBC1D4 with the protein kinases WNK1, SGK1 and SGK3.

Interestingly, the transfection of the mutants TBC1D1 T505A (non-phosphorylatable mutant) and TBC1D1 T505D (phosphomimetic mutant) did not cause a significant change in the PM expression of GLUT1 (Fig. S1). Therefore, we were not able to validate T505 as a determinant phosphosite for GLUT1 localization. Nevertheless, the transfection of a constitutively active SGK1 mutant (SGK1 CA) increased GLUT1 PM expression, concordant with previous results on SGK1 regulating GLUT1 [29] (data not shown). Interestingly, proteogenomic studies revealed a TBC1D1 T505 phosphosite enrichment in breast cancer samples [48].

Together, these data provide additional insights into glucose uptake regulation through protein kinase WNK1-mediated phosphorylation of TBC1D1 and TBC1D4, two key regulators of glucose transporters. We showed that WNK1 contributed to the regulation of about 40% of the overall cellular glucose uptake (Fig. 1), consistent with the fact that several regulatory kinases, including AKT, AMPK and SGK, have been identified to converge on TBC1D proteins as a common regulatory target.

Dysregulation of glucose uptake in human tissues is of high importance in two major illnesses, type 2 diabetes and cancer. Our findings have thus potential biomedical implications. First, the biochemical response to insulin is lost in type 2 diabetes, a leading health problem worldwide. Therefore, more detailed knowledge about signaling and trafficking pathways regulating GLUT4 and GLUT1, and how they are coordinated, is essential to understand the pathogenesis of diabetes and to generate new therapeutic options for the restoration of insulin response in the growing population of type 2 diabetes patients. According to the International Diabetes Federation, the total number of patients with diabetes is predicted to rise to over 600 million by the year 2045 [49]. Second, reprogramming energy metabolism is one of the hallmarks of cancer cells in order to fuel their rapid cell growth and division [50]. Otto Warburg observed that cancer cells use the less energy-efficient glycolysis despite the presence of oxygen [51]. In this process, cancer cells compensate for the lower yield in ATP production by upregulating glucose transporters, markedly GLUT1, which substantially increases import of extracellular glucose into the cytoplasm [52]. Overexpression of GLUT1 is correlated with poor survival in most solid tumors [53]. Interestingly, S565 in TBC1D1, a phosphosite described in this work as a GLUT1 PM expression regulator, was found to be phosphorylated in samples from non-small-cell lung carcinoma (NSCLC) cell models [54]. WNK1 phosphorylates S565 and also affects several cancer signaling pathways, including MAPK cascades, PI3K-AKT and TGF-β [8,55]. Thus, deregulation of WNK1 expression or activity could conceivably promote tumor cell metabolism. It remains to be explored whether the other three WNK family members also participate in the regulation of GLUT1 PM expression. WNK-specific inhibitors that are being developed based on the distinct catalytic domain features

[56,57] may help to increase the knowledge about WNK signaling pathways in the regulation of glucose uptake and cancer development.

CRediT authorship contribution statement

Andreia F.A. Henriques: Investigation, Methodology, Writing - original draft. **Paulo Matos:** Visualization, Validation, Writing - review & editing. **Ana Sofia Carvalho:** Methodology, Investigation. **Mikel Azkargorta:** Methodology. **Felix Elortza:** Methodology. **Rune Matthiesen:** Methodology, Formal analysis, Writing - review & editing. **Peter Jordan:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors have no competing interests to declare.

Acknowledgements

This work was supported by Fundação para a Ciência e Tecnologia (FCT) [grants PTDC/SAU-MET/117236/2010 to PJ, grant UID/MULTI/04046/2019 to the research unit BioISI, and fellowship SFRH/BD/106080/2015 from the BioSYS PhD programme PD65-2012 to AFAH]. The authors acknowledge José Ferrão for regular mycoplasma testing in cultured cells and Patrícia Barros for critical reading of the manuscript. Anthony Albiston (Melbourne, Australia) and Florian Lang (University of Tubingen, Germany) kindly provided constucts pCR3.1/AS160-2myc or pIRES2-EGFP-SGK1, respectively, for this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2019.108223.

References

[1] F. Veríssimo, P. Jordan, WNK kinases, a novel protein kinase subfamily in multi-cellular organisms, Oncogene 20 (2001) 5562–5569, https://doi.org/10.1038/sj.onc.1204726.
[2] S.K. Hanks, T. Hunter, Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification, FASEB J. 9 (1995) 576–596, https://doi.org/10.1096/fasebj.9.8.7768349.
[3] G. Manning, The protein kinase complement of the human genome, Science 298 (2002) 1912–1934, https://doi.org/10.1126/science.1075762.
[4] B. Xu, J.M. English, J.L. Wilsbacher, S. Stippec, E.J. Goldsmith, M.H. Cobb, WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II, J. Biol. Chem. 275 (2000) 16795–16801.
[5] X. Min, B.-H. Lee, M.H. Cobb, E.J. Goldsmith, Crystal structure of the kinase domain of WNK1, a kinase that causes a hereditary form of hypertension, Structure 12 (2004) 1303–1311, https://doi.org/10.1016/j.str.2004.04.014.
[6] F.H. Wilson, S. Disse-Nicodème, K.A. Choate, K. Ishikawa, C. Nelson-Williams, I. Desitter, M. Gunel, D.V. Milford, G.W. Lipkin, J.M. Achard, M.P. Feely, B. Dussol, Y. Berland, R.J. Unwin, H. Mayan, D.B. Simon, Z. Farfel, X. Jeunemaitre, R.P. Lifton, Human hypertension caused by mutations in WNK kinases, Science 293 (2001) 1107–1112, https://doi.org/10.1126/science.1062844.
[7] B. Xu, X. Min, S. Stippec, B.-H. Lee, E.J. Goldsmith, M.H. Cobb, Regulation of WNK1 by an autoinhibitory domain and autophosphorylation, J. Biol. Chem. 277 (2002) 48456–48462, https://doi.org/10.1074/jbc.M207917200.
[8] S. Moniz, P. Jordan, Emerging roles for WNK kinases in cancer, Cell. Mol. Life Sci. 67 (2010) 1265–1276, https://doi.org/10.1007/s00018-010-0261-6.
[9] C. Richardson, F.H. Rafiqi, H.K.R. Karlsson, N. Moleleki, A. Vandewalle, D.G. Campbell, N.A. Morrice, D.R. Alessi, Activation of the thiazide-sensitive Na+-Cl- cotransporter by the WNK-regulated kinases SPAK and OSR1, J. Cell Sci. 121 (2008) 675–684, https://doi.org/10.1242/jcs.025312.
[10] A.N. Anselmo, S. Earnest, W. Chen, Y.-C. Juang, S.C. Kim, Y. Zhao, M.H. Cobb, WNK1 and OSR1 regulate the Na+, K+, 2Cl- cotransporter in HeLa cells, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 10883–10888, https://doi.org/10.1073/pnas.0604607103.
[11] C. Richardson, K. Sakamoto, P. de los Heros, M. Deak, D.G. Campbell, A.R. Prescott, D.R. Alessi, Regulation of the NKCC2 ion cotransporter by SPAK-OSR1-dependent and -independent pathways, J. Cell Sci. 124 (2011) 789–800, https://doi.org/10.1242/jcs.077230.
[12] L.L. Rosenbaek, M.L.A. Kortenoeven, T.S. Aroankins, R.A. Fenton, Phosphorylation decreases ubiquitylation of the thiazide-sensitive cotransporter NCC and subsequent clathrin-mediated endocytosis, J. Biol. Chem. 289 (2014) 13347–13361, https://doi.org/10.1074/jbc.M113.543710.
[13] A.C. Vitari, M. Deak, N.A. Morrice, D.R. Alessi, The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases, Biochem. J. 391 (2005) 17–24, https://doi.org/10.1042/BJ20051180.
[14] A.C. Vitari, J. Thastrup, F.H. Rafiqi, M. Deak, N.A. Morrice, H.K.R. Karlsson, D.R. Alessi, Functional interactions of the SPAK/OSR1 kinases with their upstream activator WNK1 and downstream substrate NKCC1, Biochem. J. 397 (2006) 223–231, https://doi.org/10.1042/BJ20060220.
[15] T. Moriguchi, S. Urushiyama, N. Hisamoto, S. Iemura, S. Uchida, T. Natsume, K. Matsumoto, H. Shibuya, WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1, J. Biol. Chem. 280 (2005) 42685–42693, https://doi.org/10.1074/jbc.M510042200.
[16] A.T. Piala, T.M. Moon, R. Akella, H. He, M.H. Cobb, E.J. Goldsmith, Chloride sensing by WNK1 involves inhibition of autophosphorylation, Sci. Signal. 7 (2014), https://doi.org/10.1126/scisignal.2005050 ra41–ra41.
[17] A.I. Mendes, P. Matos, S. Moniz, P. Jordan, Protein kinase WNK1 promotes cell surface expression of glucose transporter GLUT1 by regulating a Tre-2/USP6-BUB2-Cdc16 domain family member 4 (TBC1D4)-Rab8A complex, J. Biol. Chem. 285 (2010) 39117–39126, https://doi.org/10.1074/jbc.M110.159418.
[18] M.B. Calvo, A. Figueroa, E.G. Pulido, R.G. Campelo, L.A. Aparicio, Potential role of sugar transporters in cancer and their relationship with anticancer therapy, Internet J. Endocrinol. 2010 (2010) 1–14, https://doi.org/10.1155/2010/205357.
[19] D. Leto, A.R. Saltiel, Regulation of glucose transport by insulin: traffic control of GLUT4, Nat. Rev. Mol. Cell Biol. 13 (2012) 383–396, https://doi.org/10.1038/nrm3351.
[20] S. Kane, H. Sano, S.C.H. Liu, J.M. Asara, W.S. Lane, C.C. Garner, G.E. Lienhard, A method to identify serine kinase substrates: akt phosphorylates A novel adipocyte protein with A rab GTPASE-ACTIVATING protein (GAP) domain, J. Biol. Chem. 277 (2002) 22115–22118, https://doi.org/10.1074/jbc.C200198200.
[21] K. Sakamoto, G.D. Holman, Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic, Am. J. Physiol. Endocrinol. Metab. 295 (2008) E29–E37, https://doi.org/10.1152/ajpendo.90331.2008.
[22] H. Zaid, C.N. Antonescu, V.K. Randhawa, A. Klip, Insulin action on glucose transporters through molecular switches, tracks and tethers, Biochem. J. 413 (2008) 201–215, https://doi.org/10.1042/BJ20080723.
[23] W.G. Roach, J.A. Chavez, C.P. Mîinea, G.E. Lienhard, Substrate specificity and effect on GLUT4 translocation of the Rab GTPase-activating protein Tbc1d1, Biochem. J. 403 (2007) 353–358, https://doi.org/10.1042/BJ20061798.
[24] G.R. Peck, J.A. Chavez, W.G. Roach, B.A. Budnik, W.S. Lane, H.K.R. Karlsson, J.R. Zierath, G.E. Lienhard, Insulin-stimulated phosphorylation of the rab GTPase-activating protein TBC1D1 regulates GLUT4 translocation, J. Biol. Chem. 284 (2009) 30016–30023, https://doi.org/10.1074/jbc.M109.035568.
[25] D. An, T. Toyoda, E.B. Taylor, H. Yu, N. Fujii, M.F. Hirshman, L.J. Goodyear, TBC1D1 regulates insulin- and contraction-induced glucose transport in mouse skeletal muscle, Diabetes 59 (2010) 1358–1365, https://doi.org/10.2337/db09-1266.
[26] J.S. Bogan, Regulation of glucose transporter translocation in health and diabetes, Annu. Rev. Biochem. 81 (2012) 507–532, https://doi.org/10.1146/annurev-biochem-060109-094246.
[27] A. Chadt, A. Immisch, C. de Wendt, C. Springer, Z. Zhou, T. Stermann, G.D. Holman, D. Loffing-Cueni, J. Loffing, H.-G. Joost, H. Al-Hasani, Deletion of both rab-GTPase–activating proteins TBC1D1 and TBC1D4 in mice eliminates insulin- and AICAR-stimulated glucose transport, Diabetes 64 (2015) 746–759, https://doi.org/10.2337/db14-0368.
[28] J.-H. Kim, H. Kim, K.-H. Hwang, J.S. Chang, K.-S. Park, S.-K. Cha, I.D. Kong, WNK1 kinase is essential for insulin-stimulated GLUT4 trafficking in skeletal muscle, FEBS Open Bio 8 (2018) 1866–1874, https://doi.org/10.1002/2211-5463.12528.
[29] M. Palmada, C. Boehmer, A. Akel, J. Rajamanickam, S. Jeyaraj, K. Keller, F. Lang, SGK1 kinase upregulates GLUT1 activity and plasma membrane expression, Diabetes 55 (2006) 421–427, https://doi.org/10.2337/diabetes.55.02.06.db05-0720.
[30] X. Liang, M.B. Buttertworth, K.W. Peters, R.A. Frizzel, AS160 modulates aldosterone-stimulated epithelial sodium channel forward traffickin, Mol. Biol. Cell 21 (2010) 2024–2033, https://doi.org/10.1091/mbc.E10.
[31] G.R. Peck, S. Ye, V. Pham, R.N. Fernando, S.L. Macaulay, S.Y. Chai, A.L. Albiston, Interaction of the akt substrate, AS160, with the glucose transporter 4 vesicle marker protein, insulin-regulated aminopeptidase, Mol. Endocrinol. 20 (2006) 2576–2583, https://doi.org/10.1210/me.2005-0476.
[32] Y. Feng, Q. Wang, Y. Wang, B. Yard, F. Lang, SGK1-mediated fibronectin formation in diabetic nephropathy, Cell. Physiol. Biochem. 16 (2005) 237–244, https://doi.org/10.1159/000089849.
[33] A. Zagórska, E. Pozo-Guisado, J. Boudeau, A.C. Vitari, F.H. Rafiqi, J. Thastrup, M. Deak, D.G. Campbell, N.A. Morrice, A.R. Prescott, D.R. Alessi, Regulation of activity and localization of the WNK1 protein kinase by hyperosmotic stress, J. Cell Biol. 176 (2007) 89–100, https://doi.org/10.1083/jcb.200605093.
[34] H.F. Kramer, C.A. Witczak, E.B. Taylor, N. Fujii, M.F. Hirshman, L.J. Goodyear, AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle, J. Biol. Chem. 281 (2006) 31478–31485, https://doi.org/10.1074/jbc.M605461200.
[35] S. Chen, J. Murphy, R. Toth, D.G. Campbell, N.A. Morrice, C. Mackintosh, Complementary regulation of TBC1D1 and AS160 by growth factors, insulin and AMPK activators, Biochem. J. 409 (2008) 449–459, https://doi.org/10.1042/BJ20071114.
[36] K.K.Y. Cheng, W. Zhu, B. Chen, Y. Wang, D. Wu, G. Sweeney, B. Wang, K.S.L. Lam, A. Xu, The adaptor protein APPL2 inhibits insulin-stimulated glucose uptake by

interacting with TBC1D1 in skeletal muscle, Diabetes 63 (2014) 3748–3758, https://doi.org/10.2337/db14-0337.
[37] J.A. Chavez, W.G. Roach, S.R. Keller, W.S. Lane, G.E. Lienhard, Inhibition of GLUT4 translocation by Tbc1d1, a rab GTPase-activating protein abundant in skeletal muscle, is partially relieved by AMP-activated protein kinase activation, J. Biol. Chem. 283 (2008) 9187–9195, https://doi.org/10.1074/jbc.M708934200.
[38] L. Eguez, A. Lee, J.A. Chavez, C.P. Miinea, S. Kane, G.E. Lienhard, T.E. McGraw, Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein, Cell Metabol. 2 (2005) 263–272, https://doi.org/10.1016/j.cmet.2005.09.005.
[39] S. Chen, D.H. Wasserman, C. MacKintosh, K. Sakamoto, Mice with AS160/TBC1D4-Thr649Ala Knockin mutation are glucose intolerant with reduced insulin sensitivity and altered GLUT4 trafficking, Cell Metabol. 13 (2011) 68–79, https://doi.org/10.1016/j.cmet.2010.12.005.
[40] S. Dash, H. Sano, J.J. Rochford, R.K. Semple, G. Yeo, C.S.S. Hyden, M.A. Soos, J. Clark, A. Rodin, C. Langenberg, C. Druet, K.A. Fawcett, Y.C.L. Tung, N.J. Wareham, I. Barroso, G.E. Lienhard, S. O'Rahilly, D.B. Savage, A truncation mutation in TBC1D4 in a family with acanthosis nigricans and postprandial hyperinsulinemia, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 9350–9355, https://doi.org/10.1073/pnas.0900909106.
[41] I. Moltke, N. Grarup, M.E. Jørgensen, P. Bjerregaard, J.T. Treebak, M. Fumagalli, T.S. Korneliussen, M.A. Andersen, T.S. Nielsen, N.T. Krarup, A.P. Gjesing, J.R. Zierath, A. Linneberg, X. Wu, G. Sun, X. Jin, J. Al-Aama, J. Wang, K. Borch-Johnsen, O. Pedersen, R. Nielsen, A. Albrechtsen, T. Hansen, A common Greenlandic TBC1D4 variant confers muscle insulin resistance and type 2 diabetes, Nature 512 (2014) 190–193, https://doi.org/10.1038/nature13425.
[42] G.D. Cartee, Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle, Diabetologia 58 (2015) 19–30, https://doi.org/10.1007/s00125-014-3395-5.
[43] Q.L. Zhou, Z.Y. Jiang, J. Holik, A. Chawla, G.N. Hagan, J. Leszyk, M.P. Czech, Akt substrate TBC1D1 regulates GLUT1 expression through the mTOR pathway in 3T3-L1 adipocytes, Biochem. J. 411 (2008) 647–655, https://doi.org/10.1042/BJ20071084.Akt.
[44] S. Ishikura, A. Klip, Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation, Am. J. Physiol. Cell Physiol. 295 (2008) C1016–C1025, https://doi.org/10.1152/ajpcell.00277.2008.
[45] N. Dephoure, K.L. Gould, S.P. Gygi, D.R. Kellogg, Mapping and analysis of phosphorylation sites: a quick guide for cell biologists, Mol. Biol. Cell 24 (2013) 535–542, https://doi.org/10.1091/mbc.e12-09-0677.
[46] J.T. Treebak, C. Pehmøller, J.M. Kristensen, R. Kjøbsted, J.B. Birk, P. Schjerling, E.A. Richter, L.J. Goodyear, J.F.P. Wojtaszewski, Acute exercise and physiological insulin induce distinct phosphorylation signatures on TBC1D1 and TBC1D4 proteins in human skeletal muscle, J. Physiol. 592 (2014) 351–375, https://doi.org/10.1113/jphysiol.2013.266338.
[47] R. Kjøbsted, A.J.T. Pedersen, J.R. Hingst, R. Sabaratnam, J.B. Birk, J.M. Kristensen, K. Højlund, J.F.P. Wojtaszewski, Intact regulation of the AMPK signaling network in response to exercise and insulin in skeletal muscle of male patients with type 2 diabetes: illumination of AMPK activation in recovery from exercise, Diabetes 65 (2016) 1219–1230, https://doi.org/10.2337/db15-1034.
[48] P. Mertins, D.R. Mani, K.V. Ruggles, M.A. Gillette, K.R. Clauser, P. Wang, X. Wang, J.W. Qiao, S. Cao, F. Petralia, E. Kawaler, F. Mundt, K. Krug, Z. Tu, J.T. Lei, M.L. Gatza, M. Wilkerson, C.M. Perou, V. Yellapantula, K. Huang, C. Lin, M.D. McLellan, P. Yan, S.R. Davies, R.R. Townsend, S.J. Skates, J. Wang, B. Zhang, C.R. Kinsinger, M. Mesri, H. Rodriguez, L. Ding, A.G. Paulovich, D. Fenyö, M.J. Ellis, S.A. Carr, Proteogenomics connects somatic mutations to signalling in breast cancer, Nature 534 (2016) 55–62, https://doi.org/10.1038/nature18003.
[49] N.H. Cho, J.E. Shaw, S. Karuranga, Y. Huang, J.D. da Rocha Fernandes, A.W. Ohlrogge, B. Malanda, IDF Diabetes Atlas: global estimates of diabetes prevalence for 2017 and projections for 2045, Diabetes Res. Clin. Pract. 138 (2018) 271–281, https://doi.org/10.1016/j.diabres.2018.02.023.
[50] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674, https://doi.org/10.1016/j.cell.2011.02.013.
[51] O. Warburg, On the origin of cancer cells, Science 123 (1956) 309–314, https://doi.org/10.1126/science.123.3191.309.
[52] R.G. Jones, C.B. Thompson, Tumor suppressors and cell metabolism: a recipe for cancer growth, Genes Dev. 23 (2009) 537–548, https://doi.org/10.1101/gad.1756509.
[53] J. Wang, C. Ye, C. Chen, H. Xiong, B. Xie, J. Zhou, Y. Chen, S. Zheng, L. Wang, Glucose transporter GLUT1 expression and clinical outcome in solid tumors: a systematic review and meta-analysis, Oncotarget 8 (2017), https://doi.org/10.18632/oncotarget.15171.
[54] M. Klammer, M. Kaminski, A. Zedler, F. Oppermann, S. Blencke, S. Marx, S. Müller, A. Tebbe, K. Godl, C. Schaab, Phosphosignature predicts dasatinib response in non-small cell lung cancer, Mol. Cell. Proteom. 11 (2012) 651–668, https://doi.org/10.1074/mcp.M111.016410.
[55] S. Gallolu Kankanamalage, A.S. Karra, M.H. Cobb, WNK pathways in cancer signaling networks, Cell Commun. Signal. 16 (2018) 72, https://doi.org/10.1186/s12964-018-0287-1.
[56] J. Zhang, X. Deng, K.T. Kahle, Leveraging unique structural characteristics of WNK kinases to achieve therapeutic inhibition, Sci. Signal. 9 (2016), https://doi.org/10.1126/scisignal.aaj2227 pe3–pe3.
[57] K. Yamada, H.-M. Park, D.F. Rigel, K. DiPetrillo, E.J. Whalen, A. Anisowicz, M. Beil, J. Berstler, C.E. Brocklehurst, D.A. Burdick, S.L. Caplan, M.P. Capparelli, G. Chen, W. Chen, B. Dale, L. Deng, F. Fu, N. Hamamatsu, K. Harasaki, T. Herr, P. Hoffmann, Q.-Y. Hu, W.-J. Huang, N. Idamakanti, H. Imase, Y. Iwaki, M. Jain, J. Jeyaseelan, M. Kato, V.K. Kaushik, D. Kohls, V. Kunjathoor, D. LaSala, J. Lee, J. Liu, Y. Luo, F. Ma, R. Mo, S. Mowbray, M. Mogi, F. Ossola, P. Pandey, S.J. Patel, S. Raghavan, B. Salem, Y.H. Shanado, G.M. Trakshel, G. Turner, H. Wakai, C. Wang, S. Weldon, J.B. Wielicki, X. Xie, L. Xu, Y.I. Yagi, K. Yasoshima, J. Yin, D. Yowe, J.-H. Zhang, G. Zheng, L. Monovich, Small-molecule WNK inhibition regulates cardiovascular and renal function, Nat. Chem. Biol. 12 (2016) 896–898, https://doi.org/10.1038/nchembio.2168.