REVIEW

RabGAPs in skeletal muscle function and exercise

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Abstract

The two closely related RabGAPs TBC1D1 and TBC1D4 are key signaling factors of skeletal muscle substrate utilization. In mice, deficiency in both RabGAPs leads to reduced skeletal muscle glucose transport in response to insulin and lower GLUT4 abundance. Conversely, *Tbc1d1* and *Tbc1d4* deficiency results in enhanced lipid use as fuel in skeletal muscle, through yet unknown mechanisms. In humans, variants in *TBC1D1* and *TBC1D4* are linked to obesity, insulin resistance and type 2 diabetes. While the specific function in metabolism of each of the two RabGAPs remains to be determined, TBC1D1 emerges to be controlling exercise endurance and physical capacity, whereas TBC1D4 may rather be responsible for maintaining muscle insulin sensitivity, muscle contraction, and exercise. There is growing evidence that TBC1D1 also plays an important role in skeletal muscle development, since it has been found to be associated to meat production traits in several livestock species. In addition, TBC1D1 protein abundance in skeletal muscle is regulated by both, insulin receptor and insulin-like growth factor-1 (IGF-1) receptor signaling. This review focuses on the specific roles of the two key signaling factors TBC1D1 and TBC1D4 in skeletal muscle metabolism, development and exercise physiology.

Key Words

- ▶ growth factors
- ▶ insulin action
- ▶ muscle
- ▶ metabolism

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Skeletal muscle metabolism is crucial in controlling systemic metabolic flexibility and substrate utilization

Metabolic flexibility represents the functional ability of an organism to accordingly adapt its energy substrate preference to alterations in energy demand. In the healthy state, postprandial insulin secretion leads to enhanced glucose oxidation, while lipid oxidation is inhibited (Chomentowski *et al.* 2011). Conversely, the insulin-resistant state is mostly characterized by reduced use of glucose as fuel, ectopic lipid accumulation and mitochondrial dysfunction (Morino *et al.* 2006, Petersen & Shulman 2006).

In addition to the nutritional status such as fasting or postprandial conditions, physical activity requires a high degree of metabolic flexibility in order to ensure fuel

availability and utilization in response to the increase in required energy (Egan & Zierath 2013, Hawley *et al.* 2014).

Exercise is widely accepted as a useful means for both the prevention and treatment of metabolic diseases (Ostman *et al.* 2017). The underlying mechanisms of the beneficial impact of exercise on metabolic health are not yet fully understood. However, it is clear that regular physical activity has a strong influence on whole-body energy metabolism and, more specifically, on skeletal muscle substrate utilization (Madsen *et al.* 2015, Maillard *et al.* 2016, Lao *et al.* 2019).

Two putative key regulators of skeletal muscle substrate utilization are the closely related Rab guanosine

triphosphatase-activating proteins (RabGAPs) TBC1D1 and TBC1D4, the latter also known as AKT substrate of 160 kDa, AS160 (Dokas *et al.* 2013, Chadt *et al.* 2015). This review focuses on the specific roles of the two signaling factors TBC1D1 and TBC1D4 in skeletal muscle function, development and exercise physiology.

RabGAPs are key regulators of glucose metabolism in insulin-responsive tissues

RabGAPs control GLUT4-mediated glucose uptake into skeletal muscle and adipocytes

Glucose uptake into insulin-responsive tissues such as skeletal muscle, adipose tissue and the heart is accomplished via the facilitative glucose transporter type 4 (GLUT4) (Joost *et al.* 2002, Thorens & Mueckler 2010). In the basal state, that is, the absence of stimulatory elements like insulin or muscle contraction, GLUT4 is sequestered in intracellular storage vesicles (GSV) in the cytoplasm (Lauritzen 2013, Richter & Hargreaves 2013). In response to insulin, GLUT4 translocation to the plasma membrane is accelerated via a phosphorylation cascade downstream of the insulin receptor (IR) involving activation of AKT (Foley *et al.* 2011). Likewise, GLUT4 translocation is increased as a result of multiple stimuli/mechanisms like calcium-mediated processes, nitric oxide, reactive oxygen species and muscle contraction, the latter being associated with activation of AMP-activated kinase (AMPK) (Constable *et al.* 1988, Bryant *et al.* 2002, Tanaka *et al.* 2003, Horie *et al.* 2008, Angin *et al.* 2014).

The process of regulating the subcellular localization of GLUT4 is exceedingly complex and has been reviewed recently (Klip *et al.* 2019). A number of 21 kDa Rab GTPases have been implicated to be involved in GLUT4 vesicle traffic, and their activation state, GTP-bound or GDP-bound, is presumably regulated by the RabGAPs TBC1D1 and TBC1D4 (Zerial & McBride 2001). More than 60 different Rab proteins are described to date, exerting their main function in vesicle formation, transport, docking and fusion (Stenmark 2009, Pfeffer 2017). In adipocytes and muscular tissue, Rab8a, Rab10 and Rab14, all substrates for TBC1D1 and TBC1D4 *in vitro*, are associated with GLUT4 storage vesicles (Miinea *et al.* 2005, Roach *et al.* 2007, Reed *et al.* 2013).

While the exact function of the RabGAPs in the different steps of GLUT4 translocation is not fully understood, mutational analyses indicate that TBC1D1 and TBC1D4 exert an inhibitory effect on GLUT4 translocation that is relieved by phosphorylation at

specific residues (Mafakheri *et al.* 2018a). Several serine (Ser) and threonine (Thr) phosphorylation sites (Tables 1, 2, 3 and 4) are described in both, TBC1D1 and TBC1D4, to be targeted by AKT and AMPK (Sano *et al.* 2003, Kramer *et al.* 2006, Roach *et al.* 2007, Chen *et al.* 2008). TBC1D1 is phosphorylated by AKT at Ser²³¹ and Thr⁵⁹⁰ (Mafakheri *et al.* 2018b), whereas TBC1D4 has at least six phosphorylation motifs for AKT (Sano *et al.* 2003, Kramer *et al.* 2006).

Upon phosphorylation, 14-3-3 proteins bind to TBC1D1 and TBC1D4 (Ramm *et al.* 2006, Pehmoller *et al.* 2009). However, there is so far no direct evidence that either phosphorylation or 14-3-3 binding affects the RabGAP activity of the proteins *in vitro* (Mafakheri *et al.* 2018b). However, insertion of a constitutive 14-3-3 binding site into a phosphorylation-deficient dominant-negative TBC1D4 mutant reversed the inhibitory effect of the mutation on GLUT4 translocation *in vivo* (Ramm *et al.* 2006, Pehmoller *et al.* 2009). Presumably, phosphorylation-dependent recruitment of the RabGAPs to their cognate Rab GTPases may play an important role in regulating GLUT4 trafficking.

RabGAPs are crucial regulators of whole-body glycemia

Deficiency in only one of the two RabGAP leads to only moderate impairments in insulin sensitivity and glucose tolerance in rodents, indicating a possible compensatory function of the other respective isoform (Chadt *et al.* 2008, Lansey *et al.* 2012, Dokas *et al.* 2013, Wang *et al.* 2013). In double-deficient *Tbc1d1/Tbc1d4*-knockout mice, insulin- as well as AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide)-stimulated glucose uptake into skeletal muscle and adipose cells are almost completely abrogated and whole-body glycemia is affected to a greater extent compared to deficiency in only one of the RabGAPs (Chadt *et al.* 2015). Both muscle-specific *Tbc1d4*-knockout mice and homozygous allele carriers of a muscle-specific human loss-of-function mutation in *TBC1D4* demonstrate elevated postprandial blood glucose levels, supporting the greater postulated role for this RabGAP in insulin action (Moltke *et al.* 2014, Xie *et al.* 2016). Interestingly, mutations in *TBC1D1* have been linked to obesity-related traits in humans (Stone *et al.* 2006, Meyre *et al.* 2008) and mice (Chadt *et al.* 2008). Moreover, TBC1D1's role in beta-cell function may also influence fasting glucose homeostasis. Glucose-stimulated insulin secretion in isolated pancreatic islets from *Tbc1d1*-knockout mice was greater compared to WT littermates, suggesting

Table 1 TBC1D1 phosphorylation sites.

	Site	Target site	Tissue/cell line	Reference
Insulin stimulation				
Human	Thr ⁵⁹⁶	AKT	Skeletal muscle	Treebak <i>et al.</i> 2014
Mouse	Ser ²²⁹	AKT	C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ²³¹	AMPK	<i>In vitro</i> , cell-free extracts	Mafakheri <i>et al.</i> 2018a,b
	Thr ²⁵³		<i>In vitro</i> , TA muscle lysate	Taylor <i>et al.</i> 2008
	Ser ⁴⁸⁹	AKT	C2C12 myotubes	Peck <i>et al.</i> 2009, Mafakheri <i>et al.</i> 2018a,b
	Thr ⁴⁹⁹	AKT	C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ⁵⁰¹	AKT	C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ⁵²¹		C2C12 myotubes	Peck <i>et al.</i> 2009
	Thr ⁵⁹⁰	AKT	<i>In vitro</i> , cell-free extracts	Mafakheri <i>et al.</i> 2018a,b
	Ser ⁶²¹		<i>In vitro</i> , TA muscle lysate	Taylor <i>et al.</i> 2008, Peck <i>et al.</i> 2009
			C2C12 myotubes	
	Ser ⁶⁶⁰	AMPK	<i>In vitro</i> , TA muscle lysate	Taylor <i>et al.</i> 2008, Peck <i>et al.</i> 2009
			C2C12 myotubes	
	Ser ⁶⁹⁷		C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ⁶⁹⁸		C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ⁶⁹⁹		C2C12 myotubes	Peck <i>et al.</i> 2009
	Thr ¹²¹⁸		C2C12 myotubes	Peck <i>et al.</i> 2009
Fasting				
Human	Thr ⁵⁹⁶		Skeletal muscle	Vendelbo <i>et al.</i> 2012
Skeletal muscle contraction (during)				
Human	Ser ²³⁷	AMPK	Skeletal muscle cells	Ramm <i>et al.</i> 2006, Chen <i>et al.</i> 2008, Frosig <i>et al.</i> 2010, Treebak <i>et al.</i> 2014
				Chen <i>et al.</i> 2008
	Thr ⁴⁸⁹	AMPK	Bacterially expressed	Treebak <i>et al.</i> 2014
	Thr ⁵⁹⁶	AMPK	Skeletal muscle cells	Jessen <i>et al.</i> 2011, Treebak <i>et al.</i> 2014
	Ser ⁶⁶⁰	AMPK	Skeletal muscle cells	Treebak <i>et al.</i> 2014
	Ser ⁷⁰⁰	AMPK	Skeletal muscle cells	Treebak <i>et al.</i> 2014
Mouse	Ser ²³⁷	AMPK	<i>In vitro</i> , EDL muscle	Treebak <i>et al.</i> 2014
	Thr ⁴⁰⁴	AMPK	<i>In vitro</i> , cell-free extracts	Mafakheri <i>et al.</i> 2018a,b
	Thr ⁵⁹⁶	AMPK	<i>In vitro</i> , EDL muscle	Treebak <i>et al.</i> 2014
	Ser ⁶⁶⁰	AMPK	<i>In vitro</i> , EDL muscle	Treebak <i>et al.</i> 2014
	Ser ⁷⁰⁰	AMPK	<i>In vitro</i> , EDL muscle	Treebak <i>et al.</i> 2014
AICAR stimulation				
Mouse	Ser ¹⁴⁵		C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ¹⁴⁶		C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ²³¹		<i>In vitro</i> , TA muscle lysate	Taylor <i>et al.</i> 2008, Chen <i>et al.</i> 2016
	Thr ²⁵³	AMPK	<i>In vitro</i> , TA muscle lysate	Taylor <i>et al.</i> 2008
	Thr ⁴⁹⁹	AMPK	<i>In vitro</i> , TA muscle lysate	Taylor <i>et al.</i> 2008
	Ser ⁵⁰¹	AKT	C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ⁵²¹	AKT+AMPK	C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ⁵⁵⁹	AMPK	C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ⁵⁶⁰	AKT+AMPK	C2C12 myotubes	Peck <i>et al.</i> 2009
	Thr ⁵⁹⁰	AKT	C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ⁶⁰⁸	AKT+AMPK	C2C12 myotubes	Peck <i>et al.</i> 2009
	Ser ⁶²¹	AKT	<i>In vitro</i> , TA muscle lysate	Taylor <i>et al.</i> 2008, Peck <i>et al.</i> 2009
			C2C12 myotubes	
	Ser ⁶⁶⁰	AMPK	<i>In vitro</i> , TA muscle lysate	Taylor <i>et al.</i> 2008, Peck <i>et al.</i> 2009
			C2C12 myotubes	
	Ser ⁷⁰⁰	AMPK	<i>In vitro</i> , TA muscle lysate	Taylor <i>et al.</i> 2008, Peck <i>et al.</i> 2009
			C2C12 myotubes	
Human	Ser ⁴⁷³	AKT	Enhanced IGFBP-2 activation, activated AKT signaling in skeletal muscle	Yau <i>et al.</i> 2014
IGF-1 stimulation				
Human	Ser ⁴⁷³	AKT	Enhanced IGFBP-2 activation, activated AKT signaling in skeletal muscle	Yau <i>et al.</i> 2014
Mouse	Thr ¹⁷²	AKT	IGF-1 and IGFBP-2 increase AMPK phosphorylation at Thr ¹⁷² in 3T3-L1 adipocytes	Assefa <i>et al.</i> 2017
	Ser ²³⁷	AMPK	IGFBP-2 stimulation in 3T3-L1 adipocytes	Assefa <i>et al.</i> 2017
	Ser ⁴⁷³	AKT	IGF-1 and IGFBP-2 stimulate AKT Ser ⁴⁷³ in 3T3-L1 adipocytes	Assefa <i>et al.</i> 2017

Table 2 TBC1D4 phosphorylation sites.

	Site	Target site	Tissue/cell line	Reference
Insulin stimulation	Human	Ser ³¹⁸	Skeletal muscle cells	Treebak <i>et al.</i> 2009, 2014
		Ser ³⁴¹	Skeletal muscle cells	Treebak <i>et al.</i> 2009, 2014
		Ser ⁵⁸⁸	Skeletal muscle cells	Treebak <i>et al.</i> 2009
		Thr ⁶⁴²	Skeletal muscle cells	Treebak <i>et al.</i> 2014
		Ser ⁷⁰⁴	Skeletal muscle cells	Treebak <i>et al.</i> 2014
	Mouse	Ser ⁷⁵¹	Skeletal muscle cells	Treebak <i>et al.</i> 2009
		Ser ³¹⁸	3T3-L1 adipocytes	Sano <i>et al.</i> 2003
		Ser ³⁴¹	3T3-L1 adipocytes	Sano <i>et al.</i> 2003
		Ser ⁵⁷⁰	3T3-L1 adipocytes	Sano <i>et al.</i> 2003
		Ser ⁵⁸⁸	3T3-L1 adipocytes	Sano <i>et al.</i> 2003
		Thr ⁶⁴²	3T3-L1 adipocytes	Sano <i>et al.</i> 2003, O'Neill <i>et al.</i> 2015
			Skeletal muscle	
		Ser ⁷¹¹	Soleus muscle	Treebak <i>et al.</i> 2010
		Thr ⁷⁵¹	3T3-L1 adipocytes	Sano <i>et al.</i> 2003
Insulin stimulation after contraction	Human	Ser ³¹⁸	Skeletal muscle cells	Pehmoller <i>et al.</i> 2009, Treebak <i>et al.</i> 2009, Vind <i>et al.</i> 2011
		Ser ³⁴¹	Skeletal muscle cells	Treebak <i>et al.</i> 2009, 2014
		Ser ⁵⁸⁸	Skeletal muscle cells	Treebak <i>et al.</i> 2009, 2014
		Ser ⁷⁰⁴	Skeletal muscle cells	Treebak <i>et al.</i> 2014
		Ser ⁷⁵¹	Skeletal muscle cells	Treebak <i>et al.</i> 2009, 2014
		Thr ⁶⁴²	Skeletal muscle cells	Treebak <i>et al.</i> 2009, 2014
	Fasting	Ser ⁶⁶⁶	Skeletal muscle cells	Treebak <i>et al.</i> 2014
		Ser ⁵⁸⁸	Skeletal muscle	Vendelbo <i>et al.</i> 2012
		Ser ⁷⁰⁴	Skeletal muscle	Vendelbo <i>et al.</i> 2012
		Ser ⁷⁵¹	Skeletal muscle	Vendelbo <i>et al.</i> 2012
Skeletal muscle contraction (during)	Human	Ser ³¹⁸	Skeletal muscle	Treebak <i>et al.</i> 2009
		Ser ³⁴¹	Skeletal muscle	Ramm <i>et al.</i> 2006, Chen <i>et al.</i> 2008, Frosig <i>et al.</i> 2010, Treebak <i>et al.</i> 2014
		Ser ⁵⁸⁸	Skeletal muscle	Treebak <i>et al.</i> 2009, 2014
		Thr ⁶⁴²	Skeletal muscle	Treebak <i>et al.</i> 2009, 2014
		Ser ⁶⁶⁶	Skeletal muscle	Treebak <i>et al.</i> 2009
		Ser ⁷⁰⁴	Skeletal muscle	Treebak <i>et al.</i> 2014
	Mouse	Ser ⁷⁵¹	Skeletal muscle	Treebak <i>et al.</i> 2009, 2014
		Thr ⁶⁴²	<i>In vivo</i> exercise, Gastrocnemius	Kramer <i>et al.</i> 2006
		Ser ⁷⁰⁴	<i>Ex vivo</i> contraction, EDL	Kramer <i>et al.</i> 2006
		Ser ⁷¹¹	<i>In situ</i> contraction TA	Treebak <i>et al.</i> 2010
		Thr ⁶⁴²	Epitrochlearis	Funai <i>et al.</i> 2009
	Rat			
Skeletal muscle contraction (post)	Human	Thr ⁶⁴²	Skeletal muscle	Guerra <i>et al.</i> 2010
	Mouse	Ser ²³⁷	<i>In vivo</i> exercise, EDL muscle	Treebak <i>et al.</i> 2014
		Ser ⁶⁶⁰	<i>In vivo</i> exercise, EDL muscle	Treebak <i>et al.</i> 2014
		Ser ⁷⁰⁰	<i>In vivo</i> exercise, EDL muscle	Treebak <i>et al.</i> 2014
		Ser ⁵⁸⁸	<i>In vivo</i> exercise, Epitrochlearis	Schweitzer <i>et al.</i> 2012, Castorena <i>et al.</i> 2014, Wang <i>et al.</i> 2018
	Rat	Thr ⁶⁴²	<i>In vivo</i> exercise, Epitrochlearis	Funai <i>et al.</i> 2009, 2010, Schweitzer <i>et al.</i> 2012, Castorena <i>et al.</i> 2014, Wang <i>et al.</i> 2018
		Ser ⁷⁰⁴	<i>In vivo</i> exercise, Epitrochlearis	Wang <i>et al.</i> 2018
AICAR stimulation	Mouse	Ser ⁶⁸⁰	<i>In vitro</i> gastrocnemius muscle lysates	Treebak <i>et al.</i> 2010
		Ser ⁷¹¹	<i>In vitro</i> gastrocnemius muscle lysates	Treebak <i>et al.</i> 2010
			Soleus muscle	
		Ser ⁷⁶¹	<i>In vitro</i> gastrocnemius muscle lysates	Treebak <i>et al.</i> 2010
		Ser ⁷⁶⁴	<i>In vitro</i> gastrocnemius muscle lysates	Treebak <i>et al.</i> 2010
		Ser ¹¹³⁵	<i>In vitro</i> gastrocnemius muscle lysates	Treebak <i>et al.</i> 2010

Table 3 Cross-species reference of phosphorylation sites in TBC1D1.^a

Human	Mouse	Rat	Stimulus	Reference
Ser ¹⁴⁵	Ser ¹⁴⁵	Ser ¹⁴⁵	AICAR	Peck <i>et al.</i> 2009
Ser ¹⁴⁶	Ser ¹⁴⁶	Ser ¹⁴⁶	AICAR	Peck <i>et al.</i> 2009
Ser ²³⁵	Ser ²²⁹	Ser ²²⁹	AKT	Chen <i>et al.</i> 2008, Peck <i>et al.</i> 2009
Ser ²³⁷	Ser ²³¹	Ser ²³¹	AMPK	Chen <i>et al.</i> 2008, Jessen <i>et al.</i> 2011, Treebak <i>et al.</i> 2014, Mafakheri <i>et al.</i> 2018a,b
–	Thr ²⁵³	Thr ²⁵³	AKT	Taylor <i>et al.</i> 2008
Thr ⁴¹⁰	Thr ⁴⁰⁴	Thr ⁴⁰⁴	AMPK	Mafakheri <i>et al.</i> 2018a,b
–	Ser ⁴⁸⁹	Ser ⁴⁸⁹	AKT	Peck <i>et al.</i> 2009, Mafakheri <i>et al.</i> 2018a,b
Thr ⁵⁰⁵	Thr ⁴⁹⁹	Thr ⁴⁹⁹	AKT	Peck <i>et al.</i> 2009
Ser ⁵²⁷	Ser ⁵²¹	Ser ⁵²¹	AKT/AICAR	Peck <i>et al.</i> 2009
Ser ⁵⁶⁵	Ser ⁵⁵⁹	Ser ⁵⁶⁰	AICAR	Peck <i>et al.</i> 2009
Ser ⁵⁶⁵	Ser ⁵⁶⁰	Ser ⁵⁶⁰	AICAR	Peck <i>et al.</i> 2009
Thr ⁵⁹⁶	Thr ⁵⁹⁰	Thr ⁵⁹⁰	AKT/Insulin/Exercise	Zhou <i>et al.</i> 2008, Jessen <i>et al.</i> 2011, Treebak <i>et al.</i> 2014
Ser ⁶⁶⁷	Ser ⁶²¹	Ser ⁶²¹	Insulin/AICAR	Taylor <i>et al.</i> 2008
Ser ⁶⁶⁷	Ser ⁶⁶⁰	Ser ⁶⁶²	Contraction/AICAR/AMPK	Peck <i>et al.</i> 2006, 2009, Taylor <i>et al.</i> 2008, Jessen <i>et al.</i> 2011, Treebak <i>et al.</i> 2014, Mafakheri <i>et al.</i> 2018a,b
–	Ser ⁷⁰⁰	Ser ⁷⁰²	Contraction/AICAR/AMPK	Taylor <i>et al.</i> 2008, Treebak <i>et al.</i> 2014, Mafakheri <i>et al.</i> 2018a,b

^aAccessions: TBC1D1: human (UniProt Q86T10), mouse (Q60949 UniProt), rat (UniProt D4AC16).

that TBC1D1 is a negative regulator of insulin secretion (Paglialunga *et al.* 2017, Stermann *et al.* 2018).

TBC1D1 and TBC1D4 are differentially expressed in insulin-target tissues, indicating complementary roles in signaling

Despite a high degree of homology (~47% overall amino acid identity, more than 76% in their functional GAP domain), the two RabGAPs show a differential expression pattern throughout insulin-responsive target tissues (Park *et al.* 2011). In mice, *Tbc1d4* is mainly expressed in adipose tissues, oxidative skeletal muscle fibers (*Soleus*) and the heart (Wang *et al.* 2013). In contrast, *Tbc1d1*

is shown to be nearly absent in adipose tissue, but predominantly expressed in glycolytic skeletal muscle fibers (*Extensor digitorum longus*=EDL) (Chadt *et al.* 2008, An *et al.* 2010, Szekeres *et al.* 2012). In humans, however, no significant differences are described concerning TBC1D1 and TBC1D4 protein abundance in the different skeletal muscle subtypes (Jensen *et al.* 2012). It is postulated that TBC1D1 represents the more ancient RabGAP isoform, being present in invertebrates, while TBC1D4 evolved with the vertebrates (Chen *et al.* 2008). The basis of ongoing research has been the question concerning the specific role of each of the two isoforms and their respective contribution to substrate metabolism. Rodent studies indicate greater insulin-stimulated glucose

Table 4 Cross-species reference of phosphorylation sites in TBC1D4.^a

Human	Mouse	Rat	Stimulus	Reference
Ser ³¹⁸	Ser ³²⁴	Ser ³²⁶	AKT/Insulin/Exercise	Sano <i>et al.</i> 2003, Geraghty <i>et al.</i> 2007, Treebak <i>et al.</i> 2009, 2014
Ser ³⁴¹	Ser ³⁴⁸	Ser ³⁵⁰	AICAR/Insulin/Exercise	Geraghty <i>et al.</i> 2007, Treebak <i>et al.</i> 2014
Ser ⁵⁷⁰	Ser ⁵⁷⁷	Ser ⁵⁷⁹	Insulin/AKT	Sano <i>et al.</i> 2003, Geraghty <i>et al.</i> 2007
Ser ⁵⁸⁸	Ser ⁵⁹⁵	Ser ⁵⁹⁷	Insulin/AKT/AICAR/Exercise	Sano <i>et al.</i> 2003, Geraghty <i>et al.</i> 2007, Treebak <i>et al.</i> 2009, 2014
Ser ⁵⁹¹	Ser ⁵⁹⁸	Ser ⁶⁰⁰	Contraction	Treebak <i>et al.</i> 2010
Ser ⁶⁰⁹	Ser ⁶¹⁶	Ser ⁶¹⁸	Contraction	Treebak <i>et al.</i> 2010
Thr ⁶⁴²	Thr ⁶⁴⁹	Thr ⁶⁵¹	Insulin/AKT/AICAR/Exercise	Sano <i>et al.</i> 2003, Geraghty <i>et al.</i> 2007, Treebak <i>et al.</i> 2009, 2014
Ser ⁶⁶⁶	Ser ⁶⁷³	Ser ⁶⁷⁵	IGF-1	Geraghty <i>et al.</i> 2007
Ser ⁶⁷³	Ser ⁶⁸⁰	Ser ⁶⁷⁵	AICAR	Treebak <i>et al.</i> 2010
Ser ⁷⁰⁴	Ser ⁷¹¹	Ser ⁷¹³	Insulin/AKT/AICAR/Exercise	Treebak <i>et al.</i> 2010, 2014
Thr ⁷⁵¹	Thr ⁷⁵⁸	Thr ⁷⁶⁰	Insulin/AKT/AICAR/Exercise	Sano <i>et al.</i> 2003, Geraghty <i>et al.</i> 2007, Treebak <i>et al.</i> 2009, 2014
Ser ⁷⁵⁴	Ser ⁷⁶¹	Ser ⁷⁶³	AICAR	Treebak <i>et al.</i> 2010
Ser ⁷⁵⁷	Ser ⁷⁶⁴	Ser ⁷⁶⁶	AICAR	Treebak <i>et al.</i> 2010
Ser ⁷⁸²	Ser ⁷⁸⁹	Ser ⁷⁹¹	Contraction	Treebak <i>et al.</i> 2010
Ser ¹¹²⁶	Ser ¹¹³⁵	Ser ¹¹³⁷	AICAR	Treebak <i>et al.</i> 2010

^aAccessions: TBC1D4: human (UniProt O60343), mouse (UniProt W8BYJ6), rat (RefSeq XP_017460442.1).

uptake in oxidative *Soleus* muscle compared to glycolytic *EDL* muscle (Bonen *et al.* 1981, James *et al.* 1985). A positive correlation between the relation of oxidative skeletal muscle and whole-body insulin sensitivity has been demonstrated in humans (Oberbach *et al.* 2006, Stuart *et al.* 2013). It is likely that human oxidative skeletal muscles are more important than glycolytic fibers for maintaining glucose homeostasis in response to insulin, since insulin-stimulated glucose transport in isolated human muscle strips was associated with the relative oxidative fiber content (Zierath *et al.* 1996). Indeed, a decreased proportion of oxidative skeletal muscle fibers has been found in individuals with metabolic syndrome, obesity and T2D (Marin *et al.* 1994, Gaster *et al.* 2001, Stuart *et al.* 2013). Differences in the insulin-mediated glucose metabolism between the muscle types are likely due to fiber type-specific expression and regulation of insulin signaling. Human oxidative skeletal muscle fibers exhibit greater protein levels of IR and GLUT4 and lower protein content of AKT, TBC1D1 and TBC1D4 compared to glycolytic fibers. The phosphorylation response to insulin is not different between both fiber types. These data suggest that human oxidative skeletal muscle fiber types have a higher glucose-handling capacity but a similar sensitivity for phosphoregulation by insulin, compared to glycolytic skeletal muscle fiber types (Albers *et al.* 2015).

RabGAP deficiency leads to reduced GLUT4 protein abundance in insulin-target tissues

Consistent with the fiber-type-specific RabGAP expression pattern, stimulation of glucose transport is impaired mainly in glycolytic skeletal muscle of *Tbc1d1*-deficient mice, whereas *Tbc1d4*-knockout mice display defective glucose uptake after stimulation in oxidative skeletal muscle (An *et al.* 2010, Chadt *et al.* 2015). Moreover, insulin-stimulated glucose transport is also substantially reduced in adipocytes from *Tbc1d4*-deficient mice (Chadt *et al.* 2015, Zhou *et al.* 2017). While the expected increase in basal glucose transport due to RabGAP depletion is shown for cultured 3T3-L1 adipocytes (Roach *et al.* 2007), this finding is not consistently validated in intact skeletal muscles or primary adipocytes (Chadt *et al.* 2015). The main reason for this discrepancy is thought to be a concomitant decrease in GLUT4 protein abundance due to the RabGAP deficiency in the respective tissue type (Chadt *et al.* 2015, Hargett *et al.* 2016). The depletion of GLUT4 protein in insulin-target tissues occurs concomitantly with RabGAP deficiency in most studies. In mice reduced GLUT4 protein levels were mainly observed in glycolytic skeletal

muscle types upon *Tbc1d1* deficiency, whereas knockout of *Tbc1d4* leads to a substantial reduction of GLUT4 abundance in oxidative skeletal muscle and the adipose tissue (Stockli *et al.* 2015). Moreover, human allele carriers of a nonsense p.Arg684Ter *TBC1D4* variant demonstrate significantly reduced GLUT4 protein content in skeletal muscle (Moltke *et al.* 2014).

Of note, RabGAP deficiency-associated depletion of GLUT4 in skeletal muscle seems to occur as a result of posttranslational missorting processes since no alterations were detected on the mRNA level for *Slc2a4* the gene encoding GLUT4 (Chadt *et al.* 2015, Stockli *et al.* 2015, Xie *et al.* 2016). The direct causal relationship between RabGAP deficiency and GLUT4 transporter reduction has been demonstrated by a study conducting a rescue experiment of GLUT4 abundance via overexpression of *Tbc1d1* in skeletal muscle from *Tbc1d1*-knockout mice using *in vivo* electrotransfection (Stockli *et al.* 2015).

GLUT4 depletion and altered subcellular distribution contribute to impaired glucose uptake in RabGAP-deficient skeletal muscle

It has been speculated whether depletion in TBC1D1 or TBC1D4 specifically affects skeletal muscle and adipocyte glucose uptake solely by reducing GLUT4 protein abundance or whether trafficking of GLUT4-containing vesicles may be impaired as well. Cell-surface labeling of GLUT4 in skeletal muscle from *Tbc1d1*-deficient mice demonstrated that the amount of basal cell-surface GLUT4 in the *EDL* was comparable with that of WT mice despite a lower abundance of total GLUT4. In contrast, upon insulin stimulation, cell-surface GLUT4 was also reduced compared to WT mice. However, taking the reduced amount of total GLUT4 transporter into account, a higher proportion of GLUT4 was present on the cell surface in the basal state, whereas the fraction of GLUT4 present on the plasma membrane in insulin-stimulated cells was relatively normal (Chadt *et al.* 2015). These data suggest that RabGAP-mediated impairments in glucose transport are mainly based on the reduction in GLUT4 abundance and only secondary on defects in transporter trafficking.

Both RabGAPs interact physically with the GLUT4 vesicle-resident protein insulin-regulated aminopeptidase (IRAP) (Peck *et al.* 2006). In the basal state, the cytosolic domain of IRAP binds to the second PTB domain of TBC1D4 and may keep the RabGAP in proximity to the GSVs. In response to insulin, TBC1D4 dissociates from IRAP and is released into the cytoplasm (Larance *et al.* 2005). Likewise, TBC1D1 interacts with IRAP in a phosphorylation-

dependent manner. Immunoprecipitation of IRAP resulted in co-precipitation of TBC1D1, and this interaction was abolished after phosphorylation of TBC1D1 by AMPK or AKT, indicating that IRAP recruits the RabGAPs in a phosphorylation-dependent manner to GLUT4 vesicles (Mafakheri *et al.* 2018b). Interestingly, in skeletal muscle from double-deficient *Tbc1d1/Tbc1d4*-knockout mice. IRAP and GLUT4 proteins were reduced, whereas IRAP was not changed in the single knockouts (Chadt *et al.* 2015). Abrogation of TBC1D1 or TBC1D4 may result in the degradation of a subset of GLUT4-containing vesicles, indicating crosstalk in RabGAP-mediated trafficking.

RabGAPs control lipid metabolism in skeletal muscle

Skeletal muscle lipid utilization is of high relevance for the maintenance of a metabolic flexible state and thus may prevent insulin resistance and ectopic lipid accumulation. Accumulation of toxic lipid intermediates such as diacylglycerols (DAGs) and ceramides in skeletal muscle are known to impair muscular insulin sensitivity by inhibiting the insulin signaling cascade, a process described as 'lipotoxocity' (Ragheb *et al.* 2009, Boucher *et al.* 2014, Morales *et al.* 2017). Both TBC1D1 and TBC1D4 have been implicated to exert an important function in skeletal muscle lipid use; therefore, these two factors can be considered as signaling hubs between glucose and lipid metabolism. The following section summarizes the specific impact of the two RabGAPs on the diverse steps of skeletal muscle lipid metabolism, such as transport of fatty acids into the cell, fatty acid oxidation (FAO) and lipid accumulation.

RabGAPs regulate FAO in skeletal muscle

Tbc1d1-, *Tbc1d4*- and double-deficient mice preferably use lipids as energy substrate, indicated by a lower respiratory quotient (RQ) and thus elevated fatty acid oxidation *in vivo*. In addition, palmitate oxidation is increased in isolated *Tbc1d1*-deficient skeletal muscle *ex vivo* (Chadt *et al.* 2008, 2015, Dokas *et al.* 2013). This phenotype is species-dependent and only present in mice, as the RQ is unaltered between *Tbc1d4*-deficient rats and WT littermates (Arias *et al.* 2019). Interestingly, isolated skeletal muscles from *Tbc1d4*-knockout mice demonstrated enhanced levels of FAO as well; however, whereas both glycolytic and oxidative skeletal muscles from *Tbc1d1*-deficient mice displayed enhanced basal FAO with no further increase by AICAR, FAO was also increased in glycolytic muscle

from *Tbc1d4*-knockout which had normal glucose uptake, indicating independent regulation of glucose versus lipid utilization by RabGAPs (Chadt *et al.* 2015). Moreover, deficiency of both *Tbc1d1* and *Tbc1d4* did not exert additive effects on FAO as muscle from double-deficient *Tbc1d1/Tbc1d4*-knockout mice resembles each single knockout. These data indicate that (at least in glycolytic EDL muscle) TBC1D1 and TBC1D4 equally contribute to the effect on FAO, and the same target distal to both RabGAPs is likely to mediate the increased FAO.

A previous study demonstrated that overexpression of *Tbc1d1* in mouse skeletal muscle by electrotransfection reduced the activity of β -HAD, a key enzyme in the β -oxidation pathway, resulting in impaired fatty acid oxidation (Maher *et al.* 2014). However, in skeletal muscle from *Tbc1d1*-knockout rats mitochondrial β -HAD activity was unaltered (Whitfield *et al.* 2017). The divergent results may be caused by compensatory mechanisms occurring during skeletal muscle development in the *Tbc1d1*-knockout rats as overexpression of *Tbc1d1* in mouse muscle was conducted in the adult state. Interestingly, the impact of RabGAPs on lipid metabolism is not restricted to skeletal muscle. In isolated mouse pancreatic islets *Tbc1d1* deficiency leads to both, enhanced insulin secretion and FAO *ex vivo*, presumably due to convergent RabGAP-mediated control of insulin vesicle and fatty acid transporter trafficking (Stermann *et al.* 2018).

To date, it is not clear whether RabGAPs have a direct regulatory connection to the oxidation of fatty acids or whether they exert their function exclusively on the level of fatty acid transport into the cell.

RabGAPs may regulate the transport of fatty acids and lipid accumulation into the skeletal muscle

Enhanced lipid use in RabGAP deficiency may result from altered trafficking and/or abundance of proteins involved in fatty acid transport and oxidation. Knockdown of *Tbc1d4* in rat L6 myotubes and knockdown of *Tbc1d1* in mouse C2C12 myotubes increased long-chain fatty acid uptake (Chadt *et al.* 2008, Miklosz *et al.* 2016). Similarly, both *Tbc1d1*- and *Tbc1d4*-knockout mice and *Tbc1d1*-knockout rats displayed increased fatty acid oxidation in isolated skeletal muscle (Chadt *et al.* 2015, Whitfield *et al.* 2017). However, while *Tbc1d4*-deficient L6 myotubes had increased expression levels of the fatty acid transporters fatty acid translocase (FAT/CD36) and fatty acid-binding protein (FABPpm), the abundance and plasma membrane localization of FAT/CD36 in skeletal muscle of *Tbc1d1*-knockout rats was not altered (Miklosz *et al.* 2016,

Whitfield *et al.* 2017). Thus, assuming a species-conserved mechanism present in RabGAP deficiency, alterations in FAT/CD36 may not explain the increased lipid use in the different knockout cells. Interestingly, however, knockdown of *Tbc1d4* in mouse HL-1 cardiomyocytes resulted in redistribution of FAT/CD36 to the cell surface in a Rab8-dependent manner and overexpression of a phosphorylation-deficient dominant-negative *Tbc1d4* mutant had the opposite effect (Samovski *et al.* 2012). Further studies should investigate an involvement of other additional fatty acid transporter proteins in RabGAP signaling, including FATP-1, FATP-4 or FABPpm which may contribute to the enhanced lipid utilization across species (Nickerson *et al.* 2009).

Silencing of *Tbc1d4* after palmitate incubation in L6 myotubes has been demonstrated to decrease lipotoxic effects by a reduction in DAG and lipid levels (Table 1) (Miklosz *et al.* 2017). Moreover, the intramyocellular lipid content in these cells was decreased due to an elevated FAO rate (Miklosz *et al.* 2017). There is a relationship between fatty acid-binding protein 3 (FABP3) content in skeletal muscle and the development of obesity and/or insulin resistance in mice. Mice with diet-induced obesity exhibit increased FABP3 content and fatty acid (FA) uptake in skeletal muscle. FABP3 may also contribute to TBC1D4 phosphorylation by insulin-dependent AKT activation in cells under palmitate-induced lipotoxic conditions (Kusudo *et al.* 2011). In contrast, an increase in muscle FABP3 with disturbed insulin action may result in i.m. fat deposition and could lead to insulin resistance (Kusudo *et al.* 2011). After a fasting period of 72 h, TBC1D4 phosphorylation of Ser⁵⁸⁸, Ser⁷⁰⁴ and Ser⁷⁵¹ is decreased, whereas TBC1D1 phosphorylation of Thr⁵⁹⁶ is unaltered in human skeletal muscle (Vendelbo *et al.* 2012). This is associated with an increase in lipid content (Vendelbo *et al.* 2012). Interestingly, fasting did not impact phosphorylation of AMPK or AKT. Therefore it can be speculated that changes in skeletal muscle lipid content after fasting is mediated by reduced TBC1D4 signaling (Vendelbo *et al.* 2012).

RabGAPs are crucial factors of exercise physiology

Exercise endurance is impaired in *Tbc1d1* deficiency

Contraction-stimulated GLUT4 translocation is thought to be insulin-independent, associated with AMPK activation and not reduced by insulin resistance (Marette *et al.* 1992, Richter & Hargreaves 2013). *Tbc1d1*-deficient mice

showed impaired exercise performance and endurance, reduced GLUT4 protein abundance and reduced glucose uptake in skeletal muscle (Dokas *et al.* 2013, Chadt *et al.* 2015, Stockli *et al.* 2015). The impairment in exercise endurance could not be attributed to changes in plasma non-esterified fatty acids (NEFAs) or muscle glycogen levels, since both are unaltered in knockout animals compared to WT littermates during exercise (Stockli *et al.* 2015). In *Tbc1d4*-deficient rats, voluntary physical activity was unaltered, even though GLUT4 protein abundance was decreased and AICAR-stimulated glucose uptake into skeletal muscle was reduced (Arias *et al.* 2019). However, exercise performance and exercise capacity were not determined in *Tbc1d4*-deficient rats or *Tbc1d4*-knockout mice. Thus, while the data support a role of TBC1D1 in determining exercise capacity, a possible involvement of TBC1D4 remains to be clarified.

Interestingly, a recent study confirmed reduced exercise performance and impaired glucose tolerance in *Tbc1d1*-deficient rats, despite no changes in GLUT4 abundance thus supporting a critical role of TBC1D1 in exercise response independent of GLUT4 levels (Whitfield *et al.* 2017).

The different GLUT4 levels in these studies may derive from species-dependent differences between rats and mice, as TBC1D1 and TBC1D4 abundance did not differ among multiple types of rat skeletal muscles (Castorena *et al.* 2011, Jensen *et al.* 2012). However, Whitfield *et al.* examined GLUT4 protein abundance in white and red quadriceps muscle, whereas others could confirm a reduced GLUT4 protein content in mouse EDL muscle as well (Chen *et al.* 2008, Dokas *et al.* 2013, Stockli *et al.* 2015). Further studies are required to establish the relation of GLUT4 and TBC1D1 in different muscle types and species.

Exercise potentiates insulin-stimulated RabGAP phosphorylation

Human TBC1D1 is phosphorylated in response to contraction by AMPK at Ser²³⁷, Thr⁴⁸⁹, Thr⁵⁹⁶, Ser⁶⁶⁰ and Ser⁷⁰⁰ *in vitro* (Fig. 1 and Table 2) (Ramm *et al.* 2006, Chen *et al.* 2008, Frosig *et al.* 2010, Treebak *et al.* 2014). Recently, Thr⁴⁰⁴ (corresponding to Thr⁴¹⁰ in humans) could be detected as a novel AMPK-dependent phosphorylation site (Table 4) (Mafakheri *et al.* 2018b). In humans, AMPK has been shown to phosphorylate TBC1D4 at Ser³⁴¹, Ser⁵⁸⁸ and Ser⁷⁵¹, *in vitro* (Fig. 1 and Tables 3, 5) (Ramm *et al.* 2006, Chen *et al.* 2008, Treebak *et al.* 2014).

In primary myotubes, *in vitro* electric pulse stimulation (EPS) mimics at least in part biological

Table 5 Summary of RabGAP action in lipid metabolism.

	TBC1D1-KO/Kd	TBC1D4-KO/Kd	Reference
Respiratory quotient (RQ) or Respiratory Exchange Ratio (RER)	↓ in mice ↓ ↔ RER in rats	↓ in mice	Chadt <i>et al.</i> 2008, 2015, Dokas <i>et al.</i> 2013, Whitfield <i>et al.</i> 2017, Arias <i>et al.</i> 2019 FAO in skeletal muscle
↑ basal (EDL mouse muscle)	↑ basal (EDL mouse muscle)	Chadt <i>et al.</i> 2008, 2015, Dokas <i>et al.</i> 2013	
↑ basal (Soleus mouse muscle)	↔ basal (Soleus mouse muscle)	Chadt <i>et al.</i> 2008, 2015, Dokas <i>et al.</i> 2013	
↔ AICAR (EDL + Soleus mouse muscles)	↔ AICAR (EDL + Soleus mouse muscles)	Chadt <i>et al.</i> 2008, 2015, Dokas <i>et al.</i> 2013	
↑ basal (EDL rat muscle)	n.d.	Whitfield <i>et al.</i> 2017	n.d.
↑ in C2C12 myotubes	Miklosz <i>et al.</i> 2016	FAU in skeletal muscle Intramyocellular lipid accumulation in C2C12 myotubes	n.d.
↓ after palmitate incubation	Miklosz <i>et al.</i> 2017	Intramyocellular DAG in C2C12 myotubes	n.d.
↓ after palmitate incubation	Miklosz <i>et al.</i> 2017	FAT/CD36 expression	↑ red Quadriceps rat muscle ↔ white Quadriceps rat muscle
↑ in C2C12 myotubes	Miklosz <i>et al.</i> 2016, Whitfield <i>et al.</i> 2017	FATP-1 expression	n.d.
↔ in C2C12 myotubes	Miklosz <i>et al.</i> 2016	FATP-4 expression	n.d.
↔ in C2C12 myotubes	Miklosz <i>et al.</i> 2016	FABPpm expression	n.d.
↑ in C2C12 myotubes	Miklosz <i>et al.</i> 2016	Mitochondrial β-HAD activity	↔ in rat skeletal muscle
n.d.	Whitfield <i>et al.</i> 2017	FAO in pancreatic islets	↑ basal (mouse islets)
n.d.	Stermann <i>et al.</i> 2018		

adaptions to endurance exercise (Nedachi *et al.* 2008). Similar to *in vivo* exercise, long-term EPS (≥ 24 h, low frequency) induces glucose uptake and oxidation in response to AMPK activation via increased TBC1D1 phosphorylation (Nedachi *et al.* 2008). In contrast, short-term EPS (≤ 8 h) treatment in fact leads to increased AMPK phosphorylation only at low frequency (≤ 5 Hz), whereas glucose uptake is increased at low and high frequency (≥ 30 Hz) (Goto-Inoue *et al.* 2016, Nikolic *et al.* 2017). However, no changes in TBC1D1 phosphorylation were observed after short-term EPS at both frequencies (Nikolic *et al.* 2017). In human skeletal muscle from lean subjects, acute exercise followed by insulin stimulation potentiated phosphorylation of TBC1D4 at Ser³¹⁸, Ser³⁴¹, Ser⁵⁸⁸, Ser⁷⁰⁴, Ser⁷⁵¹ and Thr⁶⁴² (Vind *et al.* 2011, Pehmoller *et al.* 2012). In contrast, human primary myotubes that were exposed to *in vitro* contraction via EPS increased insulin-mediated phosphorylation of Thr⁶⁴², Ser³⁴¹ and Ser⁵⁸⁸, but could not potentiate the phosphorylation of Ser³¹⁸ and Ser⁷⁰⁴. Therefore it is difficult to deduce additive stimulatory effects of insulin and contraction on TBC1D4 phosphorylation (Park *et al.* 2019). Myotubes from lean individuals that were first subjected to EPS displayed elevated TBC1D4

phosphorylation in response to insulin stimulation compared to obese persons. Therefore, *in vitro* contractile activity is able to potentiate insulin-stimulated TBC1D4 phosphorylation and improves insulin action, but to a lesser extent with cells from severely obese participants (Park *et al.* 2019).

TBC1D1 signaling is important for glucose uptake during recovery after acute exercise training

So far, it is not possible to deduce a specific role of each of the two RabGAPs in exercise signaling in skeletal muscle, since both exhibit phosphorylation sites that are exclusively phosphorylated in response to exercise (Treebak *et al.* 2014). In muscle-specific AMPK $\alpha 1\alpha 2$ (mdAMPK $\alpha 1\alpha 2$)-knockout mice where both α -subunits were abrogated, contraction-mediated glucose uptake into skeletal muscle was still normal, although TBC1D1 phosphorylation is severely impaired. This indicates that TBC1D1 may have a greater impact on glucose uptake during recovery after exercise than throughout the actual exercise training. However, in muscles from WT mice, TBC1D1 phosphorylation is increased 30 min and 1 h after *in situ* contraction and enhanced glucose uptake

Signaling pathway	Position
Exercise	Human
Insulin	(Mouse)
IGF-1

Figure 1

Domain structures and phosphorylation sites in TBC1D1 and TBC1D4. The two related proteins, TBC1D1 and TBC1D4, contain two amino-terminal PTB domains, a Ca²⁺/calmodulin-binding domain (CBD) and a catalytic RabGAP domain. Both TBC1D1 and TBC1D4 are phosphorylated at multiple Ser/Thr residues in response to insulin, IGF-1 and contraction/exercise by AKT and AMPK.

has been observed 1 h after the end of the contraction (Kjøbsted *et al.* 2019b).

TBC1D4 enhances skeletal muscle insulin sensitivity and insulin-dependent glucose transport several hours after a single bout of exercise

Genetic evidence suggests that TBC1D4 is required to enhance insulin sensitivity in skeletal muscle after exercise. Three hours after contraction or 6 h after AICAR stimulation, insulin-stimulated glucose uptake was increased in muscles from WT but not from *Tbc1d4*-deficient mice (Kjøbsted *et al.* 2019a). Similarly, in rat skeletal muscle, TBC1D4 phosphorylation was enhanced after a single bout of exercise, resulting in increased insulin-dependent glucose transport for several hours post exercise (Arias *et al.* 2007). The enhanced insulin sensitivity in muscle after exercise was associated with increased insulin-stimulated phosphorylation of TBC1D4 but not TBC1D1 (Arias *et al.* 2007, Funai *et al.* 2009, Pehmoller *et al.* 2012, Kjøbsted *et al.* 2015, 2019b).

Exercise has been shown to induce increased insulin-stimulated glucose uptake in skeletal muscle fibers type I, IIA, IIB, and IIBX but not IIX fibers, leading to the hypothesis whether exercise effects on TBC1D4 phosphorylation correspond to fiber type-selective exercise effects on insulin-stimulated glucose uptake (Cartee *et al.* 2016). Immediately after exercise, phosphorylation of TBC1D4 at Ser⁷⁰⁴ has been observed in each fiber type. However, in insulin-stimulated muscles 3.5 h post exercise, phosphorylation of TBC1D4 at Thr⁶⁴² and Ser⁷⁰⁴ was only observed in skeletal muscle fiber type I, IIA, IIB, and IIBX, but not in IIX fibers. These results support the hypothesis that enhanced insulin-stimulated phosphorylation of TBC1D4 is linked to elevated insulin-stimulated glucose uptake post exercise in a fiber type-specific manner (Wang *et al.* 2019).

RabGAPs may regulate translocation of fatty acid transporter to the plasma membrane in response to contraction

Muscle contraction induces FAT/CD36 translocation to the plasma membrane and increases import of

NEFAs (Jordy & Kiens 2014). In the resting state, a direct mechanistic link between AMPK activation and FAT/CD36 translocation has been proposed, since AICAR stimulation induced an increase in fatty acid uptake in skeletal muscle. More specifically, this AMPK-induced lipid oxidation is reduced in *Cd36*-knockout mice compared to WT littermates (Bonen *et al.* 2007). Moreover, FAT/CD36 translocation to the cell membrane is shown to be blunted after AICAR stimulation in transgenic AMPK kinase dead (AMPK KD) mice in non-exercising conditions, indicating that AMPK represents an important regulator of FAT/CD36 translocation under specific physiological conditions (Jeppesen *et al.* 2011). AMPK is a heterotrimeric protein that consists of catalytic α -subunit and regulatory β - and γ -subunits (Hardie & Sakamoto 2006). The α -subunit is responsible for AMPK activation due to phosphorylation of Thr¹⁷² residue (McBride & Hardie 2009). AMPK α KD mice overexpress a kinase-dead mutant of AMPK α 2-protein, leading to a suppressed AMPK α 2 activity. Interestingly, exercise induced an equal increase in cell-surface membrane FAT/CD36 content in skeletal muscle from WT and AMPK α 2 KD2 KDa mice (Jeppesen *et al.* 2011). This result supports an AMPK-independent mechanism in the FAT/CD36 translocation to the cell surface (Jeppesen *et al.* 2011). As direct AMPK phosphorylation targets, TBC1D1 and TBC1D4 represent a probable link between contractive stimulation and the uptake of fatty acids, for instance by regulating FAT/CD36 translocation.

Muscle contraction has a stimulatory effect on the translocation of FABPpm, FATP-1 and FATP-4 transporters as well (Jain *et al.* 2009), whereby FATP-1 translocation is also induced by insulin stimulation (Jain *et al.* 2015). Moreover, a decrease of both, FAT/CD36 and FABPpm protein abundance, is observed in a mouse model lacking both AMPK α 1 and - α 2 in skeletal muscle (AMPK α 1 α 2 mdKO) under sedentary and trained conditions (Fentz *et al.* 2015). In AMPK α 1 α 2 mdKO, TBC1D1 phosphorylation at the AMPK-dependent phosphorylation site Ser²³⁷ is blunted in response to contraction compared to WT littermates. These changes may contribute to an impairment of fatty acid cellular membrane transport and therefore TBC1D1 Ser²³⁷ may account to FAT/CD36 and FABPpm translocation and fatty acid metabolism in response to exercise (Fentz *et al.* 2015). These results indicate that in mice exercise- and contraction-induced fatty acid utilization in skeletal muscle is disturbed only when both AMPK α 1 and AMPK α 2 subunits are ablated, due to missing phosphorylation of TBC1D1 Ser²³⁷.

TBC1D4 signaling is involved in the exercise-mediated improvements on lipid-induced insulin resistance

As already mentioned in a chapter above, accumulation of toxic lipid intermediates such as DAGs in skeletal muscle impair muscular insulin sensitivity by inhibiting the insulin signaling cascade and consequently, GLUT4 translocation (Ragheb *et al.* 2009, Boucher *et al.* 2014, Morales *et al.* 2017). Exercise has a prophylactic effect on lipid-induced insulin resistance. Even a single bout of exercise has the ability to prevent lipid-induced impairments in whole-body glucose tolerance (Schenk & Horowitz 2007). The prophylactic effect of exercise on lipid-induced insulin resistance may involve TBC1D4 signaling. After lipid-induced insulin resistance in exercised human, prior exercise normalized insulin-stimulated glucose uptake to the level that has been observed in the resting control leg that received saline injection (Pehmoller *et al.* 2012). Phosphorylation of TBC1D4 at Ser³⁴¹ was impaired after lipid-induced insulin resistance in exercise human. The impairment could not be fully rescued after a single bout of exercise. On the other hand, neither prior exercise nor intralipid infusion affected basal and insulin-induced phosphorylation of TBC1D1 at Thr⁵⁹⁶ and Ser²³⁷ (Pehmoller *et al.* 2012). These results indicate that the signaling nexus TBC1D4 could play a potential role in the enhanced insulin action observed after a single bout of exercise (Treebak *et al.* 2009, Pehmoller *et al.* 2012).

RabGAPs control skeletal muscle development

IGFBP-2 activation leads to enhanced insulin-stimulated glucose uptake in human skeletal myotubes via AKT and AMPK activation

Insulin-like growth factor-binding protein-2 (IGFBP-2) belongs to a family of seven structurally binding proteins that regulate the bioavailability of IGF-1 (Russo *et al.* 2005). Reduced IGFBP-2 plasma levels are associated with higher fasting glucose levels and reduced insulin sensitivity in humans (Heald *et al.* 2006) and therefore IGFBP-2 may play a role in the development of insulin resistance and T2DM. Treatment of 3T3-L1 adipocytes with IGFBP-2 stimulates glucose uptake in a synergistic activation of AKT and AMPK with subsequently increased phosphorylation of TBC1D1 at the AMPK-dependent phosphorylation site Ser²³⁷ (Assefa *et al.* 2017). Neither AKT-dependent TBC1D1 phosphorylation sites, nor the phosphorylation

state of TBC1D4 were measured. According to these data, it seems that IGFBP-2 activates the insulin-responsive phosphorylation sites of AKT at Ser⁴⁷³ and AMPK at Thr¹⁷² (Yau *et al.* 2014, Assefa *et al.* 2017).

TBC1D1 signaling affects plasma IGF-1 levels

IGF-1 exhibits structural homology to proinsulin and plays a critical role in proliferation and differentiation of skeletal muscle and adipocytes (Rinderknecht & Humbel 1978, Smith *et al.* 1988). Insulin-like metabolic effects are induced by IGF-1 in both skeletal muscle and adipose tissue (Monzavi & Cohen 2002). Mice that exhibit a *Tbc1d1*^{Ser231Ala} knockin mutation that prevents AMPK-dependent phosphorylation of TBC1D1 at Ser²³¹ display higher plasma IGF-1 levels (Chen *et al.* 2016). This phosphorylation site is necessary to mediate 14-3-3 interaction with TBC1D1 in response to AMPK stimulation. 14-3-3 proteins interact with phosphorylated serine and threonine residues in TBC1D1 and TBC1D4 to support glucose uptake (Ramm *et al.* 2006). In *Tbc1d1*^{Ser231Ala} knockin mice, where AMPK-dependent phosphorylation at Ser²³¹ and subsequent 14-3-3 interaction are inhibited, glucose uptake in response to AICAR-stimulation is reduced (Chen *et al.* 2016). TBC1D1 may mediate IGF-1 secretion, as TBC1D1 is located on IGF-1 storage vesicles (Chen *et al.* 2016). In *Tbc1d1*^{Ser231Ala} knockin mice, endocrinal and paracrine/autocrine IGF-1 secretion is enhanced in a Rab8a-dependent manner, resulting in a hypersecretion of IGF-1 that causes increased activation of the AKT-mammalian target of rapamycin (mTOR) pathway (Chen *et al.* 2016). These data provide evidence that TBC1D1 signaling interacts with the AKT-mTOR pathway via IGF-1 signaling (Fig. 2).

RabGAPs are linked with skeletal muscle development via IR and IGF1R signal transduction

Ablation of one or both RabGAPs in mice did not result in substantial changes in lean mass (Chadt *et al.* 2015). However, in states of strongly altered muscle mass changes in TBC1D1/TBC1D4 abundance and/or signaling have been observed. Both, insulin receptor (IR) and the IGF-1 receptor (IGF1R) signaling are playing a role in muscle growth and glucose homeostasis. Ablation of IR and IGF1R specifically in murine skeletal muscle (MIGIRKO) led to a >60% decrease in muscle mass, severe muscle atrophy with spinal deformities and obvious kyphosis (O'Neill *et al.* 2015). MIGIRKO mice exhibit hypoglycemia

under fasted conditions and increased basal glucose uptake due to increased expression and translocation of GLUT4, indicating that whole-body adaptations like increased basal glucose uptake into scWAT are responsible for normal glucose and insulin tolerance. It has been hypothesized that alternative tyrosine kinases may activate the insulin receptor substrate (IRS)-phosphoinositide 3-kinase (PI3K)-AKT pathway in the absence of IR and IGF1R. Indeed, total EGF receptor (EGFR) levels are increased in MIGIRKO muscles, which may contribute to the observed increase in AKT phosphorylation. As a direct result of the elevated AKT activation, basal phosphorylation of TBC1D4 (Thr⁶⁴²) is increased in *EDL* and *Soleus* from fasted MIGIRKO mice (O'Neill *et al.* 2015). Despite an enhanced AMPK activation, TBC1D1 phosphorylation is not altered and TBC1D1 abundance is decreased (O'Neill *et al.* 2015). Increased AMPK phosphorylation might contribute to the decreased muscle mass in MIGIRKO mice (Bolster *et al.* 2002). Reduced TBC1D1 expression correlates with less muscle growth while increased TBC1D4 signaling seems to be responsible for enhanced basal glucose uptake in response to increased AKT activation in the basal state.

TBC1D4 is associated with skeletal muscle growth, as TBC1D4 signaling is enhanced upon inactivation of the myokine myostatin (Kocsis *et al.* 2017). Myostatin has been shown to be a negative regulator of muscle growth and development that inhibits proliferation and differentiation in myogenic cells as well as protein synthesis in existing muscle fibers (Chen & Lee 2016). Mice that exhibit a loss of myostatin activity displayed hypermuscular changes and increased muscle weight. Moreover, phosphorylation of TBC1D4 (Thr⁶⁴²) is higher in comparison to WT littermates. However, neither phosphorylation state nor protein abundance of TBC1D1 were determined in mice exhibiting loss of myostatin activity. Therefore the hypermuscular effects could also be mediated by TBC1D1, and TBC1D4 is rather responsible for the improvements in glucose tolerance as AKT phosphorylation is increased as well (Kocsis *et al.* 2017).

Thus, even though both RabGAPs are associated with states of altered skeletal muscle growth, the causal contribution of TBC1D1 and TBC1D4 in skeletal muscle development remains to be determined. It is possible that RabGAP signaling primarily supports altered energy substrate metabolism of the growing muscle.

Moreover, while fiber type differences have a strong impact on skeletal muscle development (Schiaffino & Reggiani 2011), the role of RabGAPs in fiber type-specific development remains to be established.

Figure 2

RabGAPs in skeletal muscle signaling. The RabGAPs TBC1D1 and TBC1D4 are crucial mediators in skeletal muscle signaling in response to insulin, IGF-1 and contraction. After binding of insulin or IGF-1 to its receptor, the IRS-PI3K-AKT signaling cascade is activated, leading to phosphorylation of TBC1D1 and TBC1D4 and subsequent translocation of GLUT4 from storage vesicles (GSV) to the plasma membrane. Phosphorylation of TBC1D4 may also regulate trafficking of fatty acid transporters FAT/CD36 and FATPpm. In response to contraction, LKB1 is also activated, leading to phosphorylation of AMPK and stimulation of fatty acid oxidation in mitochondria. TBC1D1 is associated with IGF-1 containing vesicles and might be involved in secretion of the hormone in skeletal muscle, where IGF-1 act in a paracrine/autocrine manner promoting skeletal muscle growth, differentiation and protein synthesis through the mTORC1 pathway (Geraghty *et al.* 2007, Taylor *et al.* 2008, Zhou *et al.* 2008, Peck *et al.* 2009, Funai *et al.* 2010, Guerra *et al.* 2010, Treebak *et al.* 2010, Jessen *et al.* 2011, Schweitzer *et al.* 2012, Castorena *et al.* 2014, Wang *et al.* 2018).

TBC1D1 is associated with increased muscle mass in livestock animals

From genetic studies of livestock it is known that TBC1D1 is closely associated with meat production and, as a consequence, with skeletal muscle development. A strong quantitative trait locus (QTL) for growth in chicken has been identified in the genomic region containing

TBC1D1 (Rubin *et al.* 2010). Moreover, specific SNPs in the chicken *TBC1D1* gene are associated with body weight (g.69340070CNT) and leg muscle weight (g.69307744CNT) (Wang *et al.* 2014). During chicken development, *Tbc1d1* mRNA increases in thigh muscle until week 10 and is unaltered afterward, whereas in breast muscle *Tbc1d1* mRNA expression is generally unaltered during chicken development (Peng *et al.* 2015). These differences may occur

due to the physiology of muscle composition, as the chicken thigh is more oxidative, compared to the more glycolytic breast and therefore the latter more closely resembles the glycolytic EDL (Williamson *et al.* 2006). However, muscle weight has not been measured in the latter study; therefore, it is unclear whether *Tbc1d1* mRNA expression equally correlates with growth in different types of muscle. In addition, a variant (g.219G>A) in the porcine *TBC1D1* gene was associated with meat production in Italian Large White and Italian Duroc breeds, whereas another variant (c.214G>A) was associated with postnatal growth traits in rabbits (Fontanesi *et al.* 2011, 2012, Yang *et al.* 2013). The latter studies however did not resolve the contribution of the variants to fat deposition and muscle mass. Despite the genetic evidence for an association of TBC1D1 with muscle growth, it remains to be further investigated whether TBC1D1 signaling induces muscle growth or hypertrophy activates TBC1D1 expression and signaling.

Conclusion

In this review, we summarize studies of the two RabGAPs TBC1D1 and TBC1D4 in skeletal muscle function, development and exercise physiology. The importance of TBC1D1 and TBC1D4 for GLUT4-mediated glucose uptake is well established. However, the mechanisms how these RabGAPs regulate lipid utilization in skeletal muscle are now well understood. In analogy to the regulation of glucose transporters, TBC1D1 and TBC1D4 might regulate RabGTPase-dependent trafficking of fatty acid transporters.

While both RabGAPs maybe involved in the exercise-stimulated glucose uptake where deficiency impairs exercise endurance, the individual contribution of each RabGAP is not yet known. TBC1D1 may have a greater impact on glucose uptake during recovery after exercise than throughout the actual exercise training, whereas there is genetic evidence of TBC1D4 signaling to enhance muscle insulin sensitivity following contraction. Moreover, genetic cross species studies indicate that TBC1D1, the evolutionary more ancient isoform, is closely associated with meat production and thus muscle development, implicating an essential function of this RabGAP in muscle biology.

In summary, the contributions of each of the two RabGAPs to skeletal muscle function and development are complex and not restricted to purely metabolic signaling pathways. There is rising evidence for a diversified role of TBC1D1 and TBC1D4 in the distinct processes, and it is clear that up to today, we are far from understanding the whole picture.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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