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Review

Rho GTPases – Emerging regulators of glucose homeostasis and metabolic health

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Abstract: Rho guanosine triphosphatases (GTPases) are key regulators in a number of cellular functions, including actin cytoskeleton remodeling and vesicle traffic. Traditionally, Rho GTPases are studied because of their function in cell migration and cancer, while their roles in metabolism are less documented. However, emerging evidence implicates Rho GTPases as regulators of processes of crucial importance for maintaining metabolic homeostasis. Thus, the time is now ripe for reviewing Rho GTPases in the context of metabolic health. Rho GTPase-mediated key processes include the release of insulin from pancreatic β-cells, glucose uptake into skeletal muscle and adipose tissue, and muscle mass regulation. Through the current review, we cast light on the important role of Rho GTPases in skeletal muscle, adipose tissue, and the pancreas and mechanisms by which Rho GTPases act to regulate glucose metabolism in health and disease. We also describe challenges and goals for future research.

Keywords: Rho GTPases; Metabolism; Glucose homeostasis, GLUT4 translocation, skeletal muscle, pancreas, insulin, diabetes, ageing

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1. Introduction

The Rho family of small guanosine triphosphatases (GTPases) is a distinct branch within the superfamily of Ras-related small GTPases. Twenty mammalian genes encoding Rho GTPases have been identified of which Rac1, Cdc42 and RhoA are the prototypes and therefore the best characterized. Rho GTPases are crucial organizers of the actin cytoskeleton with essential functions in cell migration, cell–cell contacts, proliferation, differentiation, and many other fundamental cellular processes [1,2]. Not surprisingly, this family of molecules play central roles in maintenance of health and their dysregulation often results in disease [3]. In the last few years, important and often surprising insight into the *in vivo* function of Rho GTPases has been gained. An essential function of Rho GTPases, only recently elucidated, is the regulation of processes important for the maintenance of whole body metabolic homeostasis, in particularly glucose metabolism and blood glucose control

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via their actions in metabolically active tissues, such as skeletal muscle and adipose tissue, as well as the pancreas.

Today metabolic diseases significantly contribute to early death in western society. More than 422 million people worldwide are estimated to have diabetes causing 3% of global deaths [4]. Metabolic diseases also contributes to 1/3 of all cancers [5] and cancer related deaths [6]. Type 2 diabetes is a metabolic disease that is associated with obesity, reduced insulin-stimulated glucose uptake by skeletal muscle and adipose tissue, and impaired β-cell function [7]. Because of their important roles in those processes, Rho GTPases represent a hitherto understudied potential for novel ways to understand dysfunctions in metabolic disease. This review will discuss the key evidence for a role of Rho GTPases in metabolic control and delineate the underlying molecular mechanisms. Further, this is extrapolated to discuss the role of Rho GTPases in conditions of metabolic dysfunction, such as type 2 diabetes and ageing.

1.1. Mechanism of action

1.1.1. General mechanisms of activation

Wide interest in small GTPases was triggered by the discovery of the Ras oncogenes in 1982 [8] and with its mutations a few years later proven to cause human cancers [9,10]. Those discoveries were soon followed by the identification of related proteins now forming the Ras superfamily [11,12]. Rho GTPases function as molecular switches that cycle between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound state (Figure 1a). At least three major types of regulatory proteins/factors have been described for Rho GTPases as illustrated in Figure 1a. Activation of Rho GTPases is induced by guanine exchange factors (GEFs), which facilitate switching from a GDP- to a GTP-bound state. Inactivation results from GTPase-activating proteins (GAPs) that promote the GTPase intrinsic activity mediating GTP to GDP hydrolysis [13,14]. Additionally, Rho guanine dissociation inhibitors (RhoGDIs) bind to the inactive, GDP-bound form of Rho GTPases maintaining their inactive state [15,16]. Thus, in order for a biological event to activate Rho GTPases, the balance between the GEFs, GAPs, and RhoGDIs must be altered to promote the Rho GTPase GTP-bound state. Mammalian cells encode genes for numerous of GEFs and GAPs but only three RhoGDIs. Depending on the biological mode of regulation, release from RhoGDI must occur and GEFs must be activated to interact with the Rho GTPase and promote GTP binding. Conversely, inactivation occurs when GAPs promote the GTP hydrolysis. Rho GTPases can then be extracted from the membrane by RhoGDIs. Active Rho GTPases recruit downstream effector molecules to the plasma membrane and trigger their activation [17].

In addition to the regulation of the GTP-GDP cycling, post-translational modifications (e.g., isoprenylation, palmitoylation, phosphorylation, ubiquitination, sumoylation) of Rho GTPases appear to be required for optimal regulation of Rho GTPase function [18,19]. The intracellular spatiotemporal distribution of Rho GTPase is tightly controlled. In general,

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active Rho GTPases are targeted to the plasma membrane (or endosomal membranes) via a polybasic region and a prenyl group attached to a C-terminal cysteine residue, and several of them are also palmitoylated [18,20,21]. Prenylated cytosolic Rho GTPases are unstable and rapidly degraded [22]. Additionally, active Rho GTPases can also be targeted for proteasomal degradation by ubiquitylation [19,23].The molecular chaperone, RhoGDI, binds to prenylated Rho GTPases forming a cytosolic pool of mainly inactive GDP-loaded Rho GTPases and in addition protects Rho GTPases from ubiquitination and proteasomal degradation [22]. Thus, while release from RhoGDI is a necessary event for the Rho GTPases to be activated, the release may reduce Rho GTPases protein abundance. Likewise, GEFs, GAPs and RhoGDIs are also regulated by post-translational modifications, revealing the many modalities for regulation of these key molecular switches [19]. Yet another layer of complexity is added to the regulation of the spatiotemporal distribution and activation, as Rho GTPases can also interact to regulate each other's activity. For example, the Rho GTPases, Rac1/Cdc42 and RhoA have opposing roles and double-negative feedback loops exist between them [24–26]. The mechanisms underlying this feedback regulation between GTPases are not resolved but might involve Rho GTPase competitive binding to RhoGDI [22]. One study found that in vascular smooth muscle cells, phosphorylation of RhoA on S188 induced dissociation of Rac1 from RhoGDIα and thereby activation of Rac1 and vascular smooth muscle cell migration [27], while protecting RhoA from ubiquitin/proteasome-mediated degradation [28]. Moreover, a negative feedback loop exists between downstream effecter proteins and the Rho GTPases. The downstream effectors of Rac1/Cdc42, p21-activated kinases (PAKs), has been reported to negatively regulate GEFs [29,30], while PAK1 phosphorylation of RhoGDI at site S101 and S174 leads to RhoGDI dissocation from Rac1 [31]. Additionally, pharmacological inhibition (IPA-3) of PAK1-3 lowered Rac1 activity [24]. This also increased RhoA activity in agreement with the above mentioned negative regulation between Rho GTPases.

As outlined above, the regulation of Rho GTPases is highly complex making it challenging to determine the exact regulatory mechanisms operating under any one circumstance. Moreover, strategies to investigate their regulation *in vivo* in fully differentiated mature tissue are still wanting.

1.1.2. Downstream effects

Conserved structure and mechanism in multiple versions of Rho GTPases in bacteria, yeast, flies and vertebrates, suggest that all derive from a single primordial protein, repeatedly modified in the course of evolution to perform a variety of functions. Each Rho GTPase affects numerous of downstream proteins, all of which have specific roles in various cell processes [17,32]. Especially Rac1, Cdc42, and RhoA control pathways central in metabolic regulation. Rho GTPases are crucial organizers of the actin cytoskeleton (Figure 1b). Active GTP-bound RhoA, Rac1, and Cdc42 promote the assembly of actin stress fibers and focal adhesions, thin sheet-like lamellipodial protrusions and finger-like filopodial

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protrusions, respectively [33–38]. The regulation of the actin cytoskeleton by Rho GTPases are extensively reviewed in [1,39,40] and only a selected regulatory mechanisms investigated in relation to metabolic regulation will be presented here.

Actin remodeling is controlled through parallel actin polymerization and depolymerization. Generally, Rac1 and Cdc42 regulate comparable downstream events via activation of PAKs and activation of Wiskott - Aldrich syndrome family of proteins including WASP, N-WASP and WAVE1/2. WASP and N-WASP are critical downstream effectors of Cdc42 that mediate formation of filopodia through activation of the actin polymerization factor Arp2/3 [41]. Likewise, Rac1 exerts its effect on the cytoskeleton by binding to the WAVE complex, releasing WAVE and thereby promoting lamellipodia polymerization via activation of the Arp2/3 complex [42].

Among other downstream effectors, PAKs activate LIM-domain-containing-kinases (LIMKs) promoting phosphorylation and inactivation of cofilin [41]. Active cofilin severs and depolymerizes actin filaments and leads to an increase in uncapped barbed ends promoting actin polymerization [41,43]. To counteract, LIMK-dependent inactivation of cofilin, Rac1 activates the cofilin phosphatase, slingshot1 (SSH1) promoting cofilin dephosphorylation and activation [44–46]. Thereby phosphorylation of cofilin is tightly regulated by Rac1 by two opposite mechanisms and Arp2/3. Rac1 is also an essential component for the assembly of the NADPH oxidase complex (NOX2) by binding to p67 PHOX and thereby facilitates the production of reactive oxygen species (ROS) at cell membranes [47,48].

Critical downstream effectors of RhoA are Rho-associated protein kinase (ROCK) and the Formin family of proteins that promotes the formation of straight, unbranched actin fibers. ROCK phosphorylates a large cohort of actin-binding proteins and intermediate filament proteins to modulate their functions. For example ROCK phosphorylates LIMK leading to the phosphorylation and inactivation of the actin severing protein cofilin. Formins capable of actin nucleation promote the elongation of pre-existing filaments by removing barbed end capping proteins [49]. Diaphanous formin (mDIa) is the most extensively studied and suggested to regulate microtubule stabilization in addition to its actin polymerization activity [50,51].

Whereas both downstream pathways of Rac1 and Cdc42 seems to be involved in metabolic control, RhoA may via downstream effectors be involved in muscle mass regulation and thereby play an important role in conditions of muscle mass loss. This will be reviewed below with emphasis on knowledge gained from *in vivo* studies and humans.

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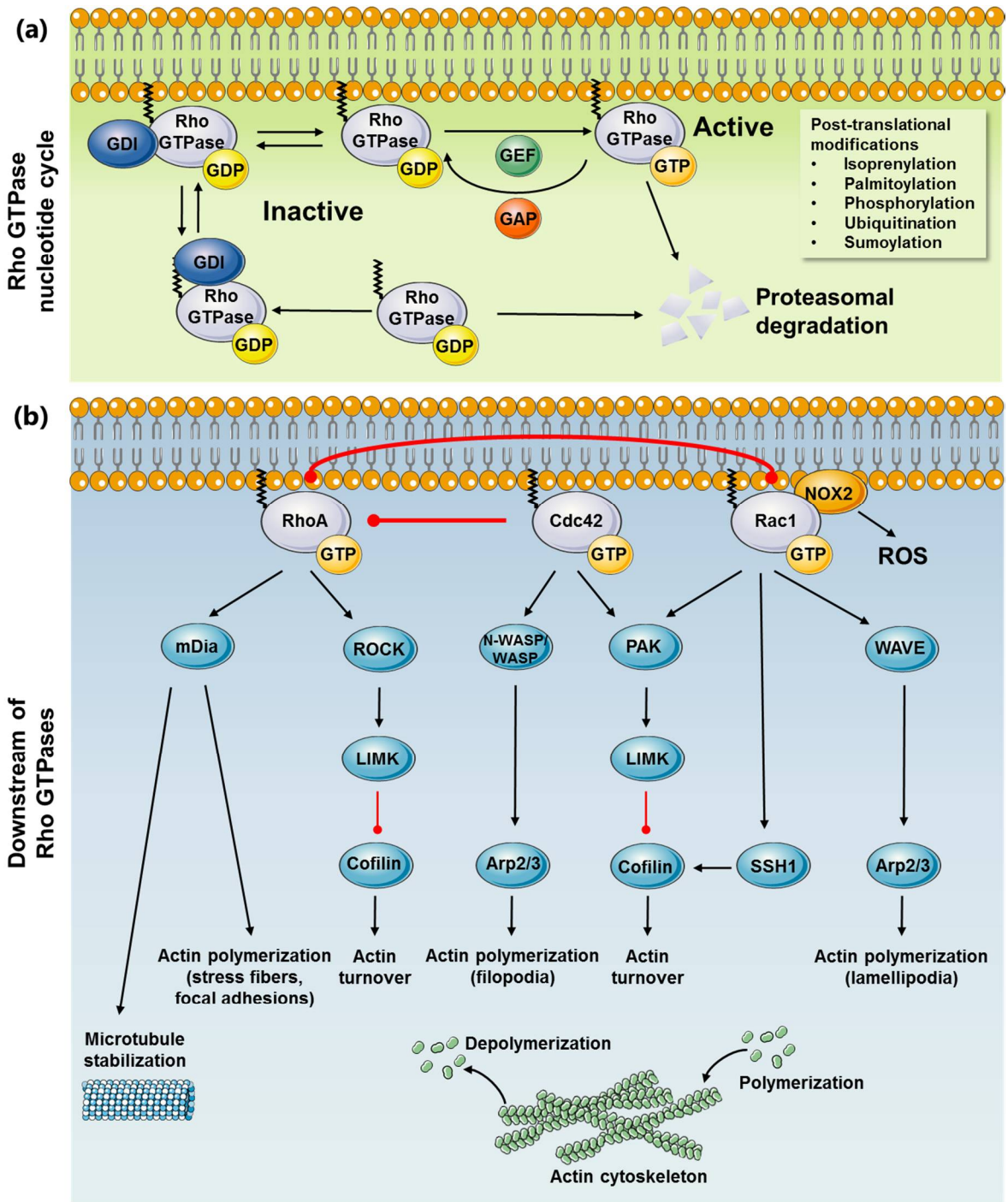

Figure 1. Rho GTPase nucleotide cycle (a) and downstream mechanisms of Rho GTPase action (b). (a) Activation of Rho GTPases require i) release from RhoGDI (GDI) and ii) activation of guanine exchange factors (GEFs), which facilitate switching from an inactive GDP- to an active GTP-bound state and translocation to the plasma membrane. Inactivation occurs when GTPase-activating proteins (GAPs) stimulate GTP to GDP hydrolysis and the Rho GTPase re-bound to RhoGDI. Free prenylated Rho GTPases are unstable and degraded and also active Rho GTPases can be targeted to proteasomal degradation. (b) Downstream effector

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proteins of the major Rho GTPases, Rac1, Cdc42 and RhoA. Downstream effector proteins of Rac1 and Cdc42 include Wiskott - Aldrich syndrome family of proteins (WASP, N-WASP, WAVE1/2) and p21-activated kinases (PAKs). WASP, N-WASP and WAVE promote actin polymerization via activation of the Arp2/3 complex. PAKs activate LIM-domain-containing-kinases (LIMKs) promoting phosphorylation and inactivation of the actin severing protein cofilin. Rac-dependent activation of slingshot1 (SSH1) promote cofilin dephosphorylation and actin depolymerization (actin turnover). Downstream effector proteins of Rac1 also includes the NADPH oxidase complex (NOX2) to produce reactive oxygen species (ROS) at the cellular membrane. Downstream effector proteins of RhoA include Rho-associated protein kinase (ROCK) and the Formin family of proteins (mDia). Among other downstream effector proteins, ROCK regulate the actin turnover via the LIMK-Cofilin pathway.

2. Rho GTPases in regulation of glucose homeostasis

Rho GTPases have important functions during cell development and differentiation (reviewed in [1,52–54]). However, they also play crucial roles in fully differentiated tissues, including the pancreas, skeletal muscle, and adipose tissue (Figure 2). Following a meal, nutrients are absorbed in the intestines and enter the circulation. This results in a postprandial transient increase in blood glucose and free fatty acids. Blood glucose quickly returns to baseline because of an increased secretion of the hormone insulin from the pancreatic β cells. When in the circulation, insulin stimulates glucose uptake into skeletal muscle and the adipose tissue. Together with inhibition of hepatic glucose production, insulin returns blood glucose back to baseline [55]. Those processes are significantly dysregulated in metabolically dysfunctional conditions, such as type 2 diabetes and obesity [56–58]. Both the pancreatic release of insulin and resulting insulin-stimulated uptake of glucose into skeletal muscle and adipose tissue are tightly regulated by complex processes that involve Rho GTPases. Rho GTPases likely also play insulin-independent roles in the maintenance of glucose homeostasis, including exercise-stimulated glucose uptake and the beneficial adaptations to exercise training. In the following sections, we will delineate and discuss the current evidence for a role of Rho GTPases with a focus on their roles in maintaining glucose homeostasis.

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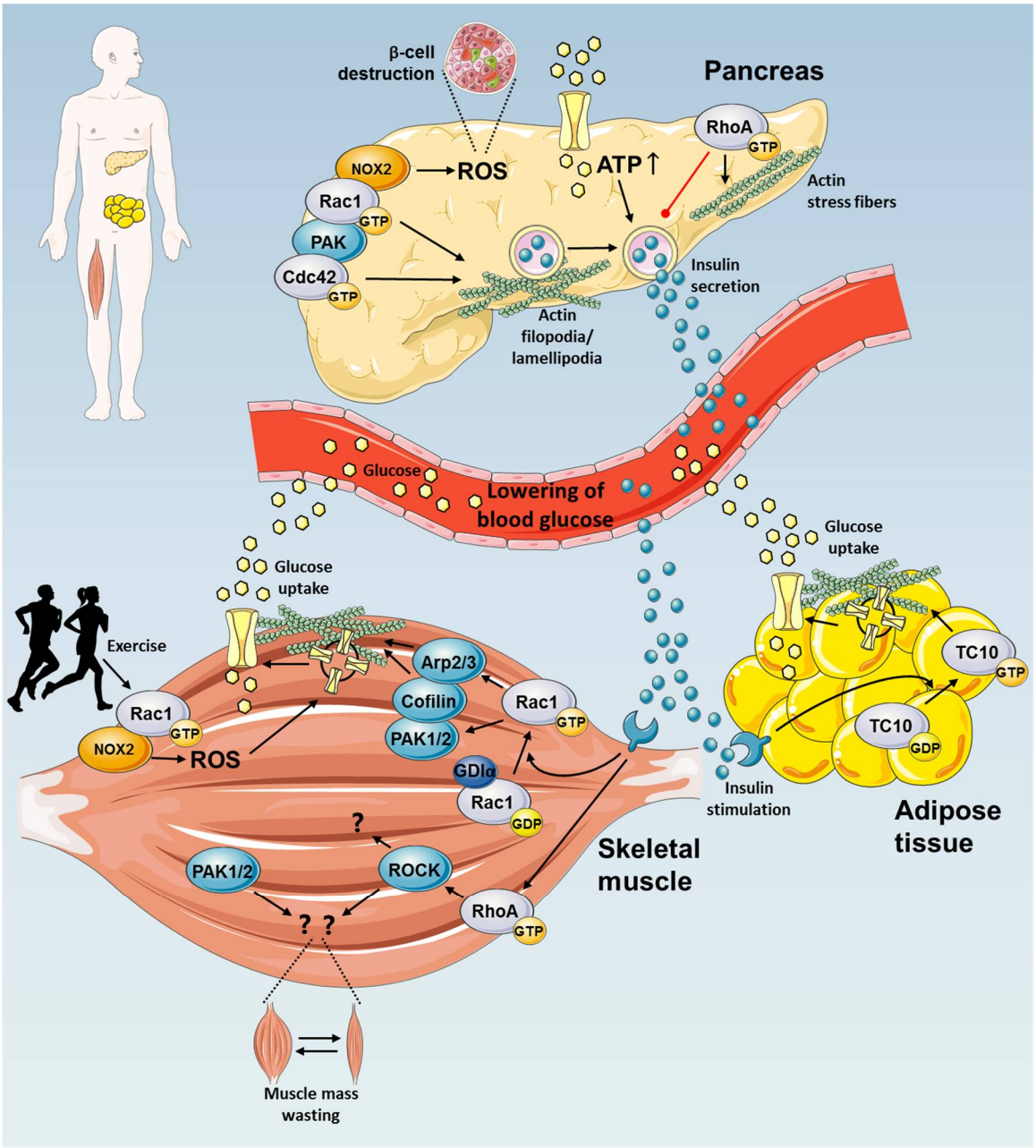

Figure 2. Rho GTPases play tissue-specific crucial roles in fully differentiated tissues, including the pancreas, skeletal muscle, and adipose tissue with the main function of lowering blood glucose.

2.1.1. Insulin secretion by pancreatic β cells

The secretion of insulin is an essential everyday event that occurs following a meal to promote glucose disposition by the muscle and curb hepatic glucose output. Several lines of evidence place Rho GTPases, in particular Cdc42 and Rac1, as important players in glucose-stimulated insulin secretion via their roles in actin reorganization [59–62] as depicted in Figure 2. Glucose is a hydrophilic molecule that cannot cross the lipid bilayer membrane. High blood glucose is sensed pancreatic β cells through GLUcose Transporters

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(GLUTs; predominantly GLUT1 and GLUT3 in human islets, GLUT2 in rodents). In general terms, this rapidly translates into elevated intracellular ATP, which in turn closes the ATP-sensitive potassium ion channel, leading to depolarization of the cell membrane. Depolarization causes the opening of voltage-gated calcium channels, and insulin vesicles fuse with the cell membrane to release insulin (reviewed in [63]). During the fasting state when levels of blood glucose are low, the actin cytoskeleton restricts the access of secretory insulin-containing vesicles to their release sites. In response to a postprandial increase of blood glucose, the β cells actin cytoskeleton is reorganized, enabling insulin-containing vesicle translocation to the plasma membrane. Under normal circumstances, Cdc42 and Rac1activation is needed for this process [55,60,64]. Thus, isolated islets from pancreatic β cell-specific Rac1 knockout mice showed decreased glucose-stimulated insulin secretion in response to high glucose concentrations [62]. On the other hand, *in vitro* studies show that expression of constitutively active Cdc42 interferes with β cell differentiation leading to hyperglycemia [60]. Thus tight regulation of the Rho GTPases must be in place. *In vivo* work from Thurmond's group shows that a downstream effector of Cdc42 and Rac1, PAK1, is involved in insulin secretion using whole body PAK1 knockout mice. The mice were rendered glucose intolerant due to reduced insulin production [65].

Overall, it is well established that Rho GTPases play essential roles in normal β cell function and insulin secretion, primarily via the essential role of Cdc42 and Rac1 in insulin-vesicle translocation in response to increased glucose concentration in the circulation, although downstream mechanisms remain to be clearly defined.

2.1.2. RhoGTPases are implicaed in β-cell dysfunction in metabolic disease

Type 2 diabetes is associated with defects in insulin secretion whereby pancreatic β cells fail to compensate for peripheral insulin resistance (described below) so that hyperglycemia ensues [66]. Before the onset of type 2 diabetes, insulin resistance causes hyperproduction of insulin to overcome the insulin resistance of muscle, adipose tissue, and the liver. Accelerated insulin production is only tolerated for a limited period of time, after which the insulin producing β cells dedifferentiate and insulin production is impaired or even stopped [67]. The stage of insulin hyperproduction has been associated with conditions of glucotoxicity and accelerated production of reactive oxygen species (ROS) in the islets. Rac1 is an important regulator of the assembly of the NADPH oxidase (NOX2) complex at the plasma membrane [48] that is important for glucose-stimulated insulin secretion [61]. Uncontrolled Rac1-mediated superoxide hyperproduction is associated with β cell failure (reviewed in [68]). For example, hyperactivation of Rac1 has been demonstrable in human islets exposed to glucotoxic conditions and in islets derived from the Zucker diabetic fatty (ZDF) rat [69], a model of type 2 diabetes. Indeed, suppression of Rac1 activation protects β cells against noxious effects of glucolipotoxicity and cytokines [69,70]. Rac1 also exerts damaging roles under pathological conditions by inducing NOX2 activity to create excessive oxidative stress, mitochondrial damage and cell death [68,71]. In support of that,

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the expression and activation of Rac1 in pancreatic tissue from ob/ob mice, a model for obesity, is increased [72]. Long-term hyperglycemia causing glucotoxicity has also been linked to RhoA-dependent stress fiber formation and diminished glucose-stimulated insulin secretion [73]. Interestingly, islets from donors with type 2 diabetes display an average PAK1 protein loss of 80% compared with non-diabetics [65] and recent work show that islets from donors with type 2 diabetes had profound defects in glucose-stimulated Cdc42 and PAK1 activation together with impaired glucose-stimulated insulin secretion [74].

Thus, while the Rho GTPases Cdc42 and Rac1 is critical for insulin secretion, hyperactivation of Rac1 and RhoA negatively affect β cell function during times of stress and may thus be targets for preventing β cell failure in diabetes.

2.2. GTPases regulate glucose uptake

The Rho GTPases Rac1, RhoA and Cdc42 are highly expressed in skeletal muscle and adipose tissue and emerging evidence suggest they have key functions in the regulation of glucose uptake, the maintenance of whole body glucose homeostasis, and metabolic health.

2.2.1. *Rho GTPases in insulin action in skeletal muscle*

In response to insulin, the majority of dietary glucose is taken up by skeletal muscle [75,76]. Comprising 40-50% of the body, skeletal muscle is essential for maintaining whole body glucose homeostasis. Glucose uptake into skeletal muscle is an actively regulated process that necessitates mobilization and insertion of the GLUcose Transporter (GLUT)-4 into the plasma membrane. This mechanism is primarily promoted by insulin and muscle contraction [77,78]. Perhaps not surprisingly due to their major role in vesicle traffick in other cell types, Rho GTPases are involved in the traffick of GLUT4 containing vesicles to the muscle plasma membrane (Figure 2).

Rac1, TC10, and RhoA are activated by insulin in skeletal muscle [79–83]. The first evidence that Rho GTPases were actively involved in the regulation of glucose uptake in skeletal muscle came nearly 20 years ago when the lack of functional Rac1 was observed to attenuate insulin-stimulated GLUT4 translocation in L6 muscle cells *in vitro* [84,85]. Additionally, constitutive active Rac1, but not Cdc42 or TC10, increased the amount of GLUT4 in the plasma membrane [79,86]. Subsequent studies also demonstrated an important role for Rac1 in insulin action in mature skeletal muscle. In mouse skeletal muscle, pharmacological inhibition and genetic ablation of Rac1 abolished insulin-stimulated GLUT4 translocation and partially inhibited insulin-stimulated glucose uptake [82,83,87,88].

The involvement of Rac1 in insulin-stimulated GLUT4 translocation has been ascribed to a Rac1-mediated insulin-stimulated actin remodeling in skeletal muscle cells [79,84,85]. However, the relevance of a mobile actin cytoskeleton in fully mature skeletal muscle was recently questioned as mice that lack either β-actin or γ-actin, the two major actin

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cytoskeleton isoforms in skeletal muscle, displayed normal glucose transport [89]. On the other hand, in mature skeletal muscle, the depolymerizing agent, Latrunculin B reduced insulin-stimulated glucose uptake [83,87], suggesting participation of the actin cytoskeleton.

It is largely unknown how GTPases are activated by insulin but it seems to occur downstream of PI3K and skeletal muscle expresses the following GEFs for Rac1: Tiam1, Trio, Def6, son of sevenless (Sos), Arf6, Vav2, FLJ00068, DBl-I, α- and β-PIX (PAK-interacting exchange factor), and switch-associated protein 70 (SWAP70). Among these, FLJ00068 has been suggested to regulate insulin-stimulated Rac1-dependent GLUT4 translocation [86]. Further research within this area will add to the understanding of the mechanism by which Rac1 regulates GLUT4 translocation.

RhoGDIα and RhoGDIβ are expressed in skeletal muscle [90,91] and RhoGDIα (but not RhoGDIβ) has a binding site for Rac, RhoA and Cdc42 [15]. Recent data from our laboratory has identified RhoGDIα as a negative regulator of Rac1 and GLUT4 translocation in skeletal muscle. In L6-GLUT4myc myotubes, siRNA-mediated RhoGDIα knockdown augments Rac1 activity and GLUT4 translocation both in the basal state and in response to insulin stimulation. Corroborating those *in vitro* results, RhoGDIα overexpression in mouse skeletal muscle *in vivo* decreased insulin-stimulated glucose uptake and impaired whole-body glucose tolerance (Moller, LLV., Klip, A., Richter EA, and Sylow, L, unpublished data). Thus, RhoGDIα is a novel key regulator of Rac1 important for maintaining the appropriate level of glucose uptake in muscle. The mechanisms by which insulin triggers Rac1 dissociation from RhoGDIα remain to be explored.

Downstream of Rac1, PAK1 and PAK2 (PAK3 is not expressed in skeletal muscle) are activated in response to insulin [65,83,92] and are suggested to mediate Rac1-dependent insulin-stimulated reorganization of the actin cytoskeleton and GLUT4 translocation in skeletal muscle cells [93]. However, the role of group I PAKs in mature mouse skeletal muscle is debated. One study reported markedly impaired insulin-stimulated GLUT4 translocation *in vivo* in skeletal muscle lacking PAK1 [65] while another observed no impairment in insulin-stimulated glucose uptake in these mice [94]. In transgenic mice jointly lacking PAK1 and PAK2 only a mild reduction in insulin-stimulated glucose transport in muscle was evident [94], suggesting that group I PAKs play only a minor role in the regulation of glucose uptake. In support of this, constitutively activated Cdc42, an activator of group I PAKs, did not stimulate GLUT4 translocation in muscle cells *in vitro* [86]. The discrepancy between the PAK studies could perhaps be explained by the age of the mice, as double PAK1/2 knockout mice exhibit other age-related phenotypes [95]. Further downstream of Rac1, signaling through Arp2/3 and cofilin could mediate the cortical actin polymerization evoked by insulin (see Figure 1b). That is suggested because siRNA-mediated silencing of Arp3 abrogates actin remodeling and impairs GLUT4 translocation in L6 myoblasts [46]. Moreover, cofilin knockdown causes overwhelming actin

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polymerization that subsequently inhibits GLUT4 translocation [46]. Those findings suggest that most distal regulators of actin cytoskeleton dynamics evoked by Rac1 are involved in insulin-stimulated glucose uptake, although those findings remain to be confirmed in differentiated muscle and *in vivo*. Thus, while the important role for Rac1 in insulin-stimulated glucose uptake is clear, Rac1 likely mediates glucose uptake via PAK-dependent, but also PAK-independent mechanisms that remains to be elucidated possibly involving Arp2/3 and cofilin.

An input by RhoA in the regulation of insulin-stimulated glucose transport, has also been proposed although the results are few and rather inconsistent [96]. Furthermore, all results are obtained by manipulating pathways downstream of RhoA and in several cases using pharmacological inhibitors reported to have unspecific effects [97]. Among the various effectors of RhoA, the kinase ROCK stands out. Two isoforms, ROCK1 and ROCK2, are expressed in skeletal muscle. ROCK1-deficient mice are insulin resistant [98] and siRNA-mediated silencing of ROCK1 reduced insulin-stimulated GLUT4 translocation and glucose transport in L6 myotubes [99], suggesting that ROCK1 is a positive regulator of insulin signaling. In addition, overexpression of a dominant negative ROCK2 or pharmacological inhibition of ROCKs impairs insulin-stimulated GLUT4 translocation and glucose uptake in muscle *in vivo* [81].

Challenging a positive input of RhoA in the regulation of glucose uptake, membrane-bound (active) RhoA is elevated in skeletal muscles of obese Zucker rats [100]. In that model, overexpressing a dominant-negative ROCK rescued diet-induced obesity and improved glucose tolerance [101], which contrasts with the findings in mice expressing dominant negative ROCK2 described above [81]. Furthermore, knockout of geranylgeranyl diphosphate synthase 1 (GGPPS; a branch point enzyme in the mevalonic acid pathway) specifically in skeletal muscle of mice decreased membrane-associated and prenylated RhoA (=active) and improved whole body glucose tolerance [102]. However, GGPPS might also increase the activity of other GTPases regulated by prenylations, including Rac1, which was not investigated. Thus, the phenotype of these mice might also be due to inhibition of Rac1 and direct evidence for RhoAs role in muscle insulin action is still lacking.

Collectively, there is ample support that Rac1, and possibly RhoA, but seemingly not Cdc42, participate in the insulin-dependent regulation of glucose uptake in skeletal muscle.

2.2.2. Exercise elicits metabolic benefits via regulation of Rho GTPases

It has been convincingly documented that regular exercise improves glycemic control and insulin action among healthy as well as obese and type 2 diabetic subjects [103–105] and that this effect can be superior to those exerted by drugs or insulin therapy [106,107]. Physical activity causes a large increase in energy utilization [108,109]. Glucose is a major fuel source for the contracting muscles and glucose uptake acutely increases more than 50-

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fold during exercise [109]. Because glucose is taken up by the contracting muscles via insulin-independent mechanisms [110,111], it is effective in lowering blood glucose in insulin resistant subjects [112].

Rac1 is activated during exercise in mouse and human skeletal muscle [113] and Rac1 muscle-specific knockout mice exhibit reduced exercise-stimulated glucose uptake in muscle [114,115]. Likewise, GLUT4 translocation in response to electrical pulse stimulation (which mimics, but does not recapitulate muscle contraction) is reduced by inhibition of Rac1 in C2C12 myotubes [116]. Interestingly, exercise results in phosphorylation of RhoGDIα on serine 34 in human skeletal muscle [117]. Whether RhoGDIα serine 34 phosphorylation destabilizes its interaction with Rac1 to permit Rac1 activation during exercise will be interesting to determine.

Very recent results shed light onto the downstream events by which Rac1 may induce glucose uptake during exercise. Henriquez-Olguin et al. showed that Rac1 is implicated in the increase in ROS-production that occurs during exercise in muscle [119]. As described for pancreatic β cells, Rac1 is an integral part of the NOX2 complex in skeletal muscle. However, while Rac1-induced hyperproduction of ROS in β cells is negatively associated with β cell destruction, in skeletal muscle Rac1 seems to yield metabolic benefits. Rac1-mediated production of ROS contributes to increased glucose uptake in mouse skeletal muscle during exercise [119]. Because mice lacking PAK1 displayed normal contraction-stimulated glucose transport in skeletal muscle [94], one can assume that Rac1 does not mediate contraction-stimulated glucose uptake via PAK1. Rac1 would then be available for activation, contributing to NOX2 activation. The resulting ROS production would in turn contribute to the stimulation of muscle glucose uptake by unknown mechanism. Thus, Rac1 helps remove glucose from the blood via insulin independent mechanisms.

In addition to the acute stimulation of glucose uptake by a single bout of exercise, exercise training also induces remarkable skeletal muscle adaptations that benefit metabolic regulation, including the enhancement of insulin sensitivity. Recent work has implicated RhoA in exercise-training adaptations, as seen by the increased expression of ROCK1, ROCK2, and RhoA in rat gastrocnemius muscle following short-term swimming exercise training [120]. ROCK1/2 inhibition by Y-27632 impaired the insulin sensitizing effect of exercise training [120]. Although non-specific effects of pharmacological inhibitors cannot be ruled out, that study suggests that RhoA is involved in the metabolic benefits of exercise training.

In summary, exercise elicits metabolic benefits, several of which involve regulation of Rho GTPases. Future studies should delineate the mechanisms by which Rho GTPases may be influencing health with exercise and importantly if they can be explored pharmaceutically to harness some of the metabolic benefits of exercise in individuals that cannot adhere to an exercise program.

2.3. A possible role for GTPases in adipose tissue glucose uptake

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Although being mainly a fat storage tissue, adipose tissue is also essential for maintaining metabolic homeostasis and health. Especially in conditions of obesity, adipose tissue takes up a substantial amount of glucose following a meal, thereby significantly contributing to the clearance of glucose from the blood. Like in skeletal muscle, adipocyte glucose uptake relies on the translocation of GLUT4 to the plasma membrane for glucose to diffuse across the membrane lipid bilayer. Insulin activates several Rho GTPases in adipocytes, including TC10 [121,122], Cdc42 [123], Rac1 [124], and RhoA [125] but their involvement in adipose tissue glucose uptake is not clear.

An early investigation showed that transfection of a constitutively-active RhoA increased GLUT4 translocation in response to insulin, while dominant-negative RhoA significantly decreased it in 3T3-L1 and rat adipocytes [126]. Another study using siRNA-mediated Cdc42 knockdown reported that Cdc42 could mediate insulin signaling to glucose transport in 3T3-L1 adipocytes [123]. 3T3-L1 adipocytes seemingly respond normally to insulin after transfection of a dominant-negative or constitutively-active Rac1 [127]. However, Marcusohn et al. did not confirm that the mutants, transfected in the fibroblast stage of the culture, persisted in the adipocyte stage where insulin-stimulated glucose uptake was studied. Nevertheless, that study was supported by the finding that pharmacological Rac1 inhibition did not reduce insulin-stimulated glucose uptake in incubated fat pads from mice [83], although actual inhibition of Rac1-signaling was not confirmed in that study. Thus, despite methodological issues, the few studies to date suggest no role for Rac1 in insulin stimulated glucose uptake in adipocytes.

The Rho GTPase TC10 could be the most relevant Rho GTPase to regulate insulin-stimulated glucose uptake in adipose tissue as depicted in Figure 2. Dominant-negative TC10 prevents actin reorganization [128,129], an event that is necessary for adipocytes to translocate GLUT4 to the membrane. Accordingly, dominant-negative TC10 inhibits muscle cell glucose uptake and GLUT4 translocation [121]. That study [121] also reported no effect on insulin-stimulated GLUT4 translocation and glucose uptake of transfecting constitutively-active, or dominant-negative RhoA or Cdc42 in adipocytes (although data was not shown).

Taken together, Rho GTPases could play roles in adipocyte glucose uptake by regulating GLUT4 vesicle dynamics. Indeed, the fusion of GLUT4 vesicles with the adipocyte membrane requires dynamic insulin-induced actin polymerization, evincing the contribution of the actin cytoskeleton at different steps in the process of GLUT4 translocation [130]. However, the studies linking Rho GTPases to this event are few and the results conflicting. Importantly, mechanistic *in vivo* evidence is completely lacking and there is currently no human evidence in the literature. Thus, the involvement of Rho GTPases in adipose tissue function is largely unexplored and exciting discoveries lie ahead.

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2.2.3. Rho GTPases are implicated in insulin resistance –a key contributor to metabolic disease

In insulin resistant human subjects that are obese or have type 2 diabetes, insulin-stimulated glucose uptake by skeletal muscle is a primary site of dysfunction [75]. Due to their key role in skeletal muscle (and perhaps adipocyte glucose) uptake, Rho GTPases are relevant in metabolic diseases, including type 2 diabetes. Decreased skeletal muscle Rac1 signaling has been associated with insulin resistance in insulin resistant obese and type 2 diabetic human subjects [83] as well as in diabetic ob/ob mice and diet-induced obese insulin resistant rodents [87]. This has also been proposed by *in vitro* studies of muscle showing that ceramide-induced insulin resistance was associated with marked impairments in insulin-induced Rac1 activation [85]. However, in another *in vitro* model of insulin resistance, palmitate treatment did not impair Rac1 activity, although the phosphorylation of Rac1's downstream target, PAK1 was reduced [131]. Interestingly, in a recent study from our laboratory, we observed that lack of Rac1 in muscles from diet-induced obese mice exacerbates insulin resistance [132], suggesting a relevance for Rac1 in counteracting metabolic dysfunctions. Those studies highlight the possibility that compromised Rac1 activity and/or downstream signaling contribute to the development of muscular insulin resistance.

RhoA might also regulate glucose homeostasis, as pharmacological inhibition of RhoAs target, ROCK using Fasudil prevented high-fat diet-induced hypercholesterolemia and glucose intolerance in mice [133]. Furthermore, body weight, serum lipid levels and glucose metabolism were improved in mice with whole body overexpression of a dominant-negative ROCK, compared with littermate control mice [101]. The RhoA-ROCK pathway seems to be clinically relevant, since insulin-stimulated muscle ROCK1/2 activity was attenuated in obese and type 2 diabetic subjects during an euglycemic hyperinsulinemic clamp compared to lean control [134]. In muscle cells *in vitro*, palmitic acid-induced insulin resistance was associated with increased expression of ROCK1 [135]. However, that ROCK would negatively affect glucose homeostasis contradict the fact that lean ROCK1-deficient mice are insulin resistant [98] and lean mice with overexpression of a dominant negative ROCK2 display impaired insulin-stimulated GLUT4 translocation and glucose uptake in muscle *in vivo* [81]. Thus the results on RhoA's role in metabolic (dys)regulation warrants clarification.

Taken together, Rho GTPases, in particular Rac1 and RhoA, may be highly implicated in muscular insulin resistance and thereby contribute to metabolic dysregulation in metabolic diseases, such as type 2 diabetes.

3.1. Rho GTPases as hitherto unrecognized regulators of muscle mass

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Muscle mass is important for metabolic health because it increases the amount of tissue available for storing glucose. Although studies are few, there is emerging evidence that Rho GTPases may play hitherto unrecognized roles in muscle mass regulation. Indeed, Rho GTPases are highly regulated during conditions of muscle atrophy. For example, a marked reduction in levels of RhoA was noted in the muscles of unweighted hindlimbs in mice [136] and in dystrophic mice [137] along with rapid atrophy. In male Sprague-Dawley rats, 3 days of hindlimb suspension-induced muscle atrophy decreased RhoA mRNA and protein expression [138]. In contrast, muscle mass loss caused by denervation, increased RhoA expression but returned to baseline when the decline in muscle mass ceased [139]. Conversely, increasing muscle mass using functional overload and anabolic steroid administration in mice [140], or hypertrophy-stimulating resistance training in humans elevated the expression of skeletal muscle RhoA [141]. Thus, RhoA expression is highly regulated in models of muscle mass loss although this remains to be recapitulated in human skeletal muscle [142]. Future studies should investigate muscle mass regulation in muscles that lack RhoA in order to directly determine the implications for RhoA.

Rho GTPases other than RhoA may also be involved in muscle mass regulation as proteomic analysis of mouse muscles following denervation-induced atrophy identified significant changes in RhoGDIα, PAK1-2 and Cdc42 expression [143]. Dominant-negative Cdc42, introduced with a retroviral vector, resulted in fibers that appeared atrophic [144] and blocking Rac1 function in precursors of the indirect flight muscle of *Drosophila* severely disrupts muscle formation. Thus, Rac1 is involved in the regulation of myoblast proliferation and segregation during adult myogenesis [145]. Downstream of Rac1 and Cdc42, PAK1 and PAK2 are activated during mammalian myoblast differentiation. Combined deletion of PAK1 and PAK2 results in reduced muscle mass, a phenotype that is exacerbated after repair to acute injury [95,146]. In support of that, pharmacological inhibition of group I PAKs (with IPA-3) delays skeletal muscle regeneration following cardiotoxin injury *in vivo* [147], suggesting that Rho GTPase-mediated signaling is important for muscle regeneration. Interestingly, insulin-stimulated phosphorylation of PAK1 at threonine 423 [148] and PAK1 protein content [148,149] were markedly increased in follistatin-induced hypertrophic mouse muscle compared to controls.

A role for Rho GTPases in muscle mass regulation is perhaps not surprising given their well-known requirement for tumor growth. Looking ahead, lessons from the tumor literature may help to understand the mechanisms by which Rho GTPases may be involved in muscle mass regulation in connection with metabolic regulation.

3.2. The role of Rho GTPases in muscle wasting conditions

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Skeletal muscle atrophy is a severe consequence of ageing and many chronic diseases, including cancers. Muscle strength is inversely related to death from all causes [150] and is of outmost importance for the preservation of mobility and quality of life, especially in ageing. RhoA and RhoGDIα are both upregulated in mouse skeletal muscle with age-related muscle mass loss [151]. In agreement, single muscle fiber proteomics analysis showed that RhoGDIα protein expression increased with age in both slow and fast muscle fibers from human biopsy samples, while RhoA increased with age predominantly in fast muscle fibers [152]. Importantly, age-related muscle atrophy only occurred in the fast muscle fiber types. However, contradicting those two studies, a recent study found reduced RhoA protein expression in skeletal muscle of middle-aged rats together with diminished levels of ROCK proteins [153]. Thus, Rho GTPases might be differentially regulated at different ages and stages of sarcopenia and this warrants further investigation.

Many cancers are associated with cachexia, a condition of involuntary body weight loss including severe muscle atrophy that is not due to anorexia. Consistent with the role of group I PAKs in muscle mass regulation [95,146], PAK1 mRNA and protein expression are reduced in muscle in cancer-associated cachexia in colon adenocarcinoma C26-bearing mice [147], although PAKs upstream Rho GTPases, Rac1 or Cdc42 was not examined. Interestingly, that study also showed that PAK1 overexpression partly preserved fiber size in cachectic muscles, suggesting that the defect in PAK might be directly involved in the pathogenesis. From these collective studies, RhoA, Rac1 and Cdc42, and PAK emerge as candidate regulators of muscle mass. However, a direct mechanistic role for the Rho GTPases in muscle mass regulation is completely lacking.

As muscle is the largest organ of the body, outmost necessary for mobility and also responsible for the majority of glucose disposal, future studies should investigate the role for Rho GTPases in muscle wasting diseases.

Box 1 | **Unresolved issues**

- There is a lack of *in vivo* experiments to support *in vitro* literature.
- Evidence on Rho GTPases regulatory functions in humans are missing.
- Molecularly, the upstream and downstream regulators of Rho GTPases in different tissues and in response to various stimuli are poorly defined.
- Cross-talk between Rho GTPases is poorly defined but important to delineate, as they challenge all interpretations of data using knockdown or overexpression of a single Rho GTPase.
- Methodological advances to measure GTP binding (fast hydrolysis) *in vivo* is warranted.
- It is unexplored whether Rho GTPases could be targeted pharmacologically in metabolic diseases. This is likely difficult due to their ubiquitous expression and tissue-specific negative (cancer, ROS-production in pancreas) or positive (insulin secretion pancreas, glucose uptake skeletal muscle) effects on health.

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5. Conclusion

In this review we summarize new evidence for the role of Rho GTPases in metabolic regulation in health and disease. We demonstrate that Rho GTPases may be hitherto overlooked players in glucose homeostasis by contributing to metabolically essential functions in skeletal muscle, adipose tissue, and the pancreas. However, this area of research is at its early stages and mechanistic *in vivo* insights are lacking. This will be an exciting area for future discoveries.

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