HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

Proline-rich Akt substrate of 40 kDa (PRAS40): A new modulator and target of insulin action

Characterization of the function of PRAS40 in Akt- and mammalian target of rapamycin complex 1 (mTORC1)-signaling pathway

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There is a fine line between wrong and visionary. Unfortunately you have to be a visionary to see it.

- Sheldon Cooper –

Zusammenfassung

Nach neusten Schätzungen wird die weltweite Prävalenz von Diabetes von 366 Millionen Betroffenen im Jahr 2011 auf 552 Millionen im Jahr 2030 ansteigen. Diese Zahlen spiegeln die Notwendigkeit wider, dass die molekularen Mechanismen, die zu der Entstehung dieser Erkrankung führen, schnellstmöglich aufgedeckt werden, um neue therapeutische Strategien entwickeln zu können. Sowohl die Entstehung von Insulinresistenz im Skelettmuskel, Leber und Fettgewebe, als auch die Dysfunktion der β-Zellen des Pankreas bestimmen die Pathogenese des Typ 2 Diabetes (T2D). Dabei spielt vor allem die Entstehung einer Insulinresistenz im Skelettmuskel eine entscheidende Rolle. Die Sensitivität dieser Zellen auf Insulin zu reagieren ist dabei gestört, lange bevor klinische Symptome erkennbar sind. Jedoch sind die Ursachen und Faktoren, die zur Entstehung von Insulinresistenz beitragen, nicht zufriedenstellend aufgedeckt und verstanden. Zusätzlich sind noch immer nicht alle Bestandteile des Insulinsignalweges identifiziert, so dass die physiologische sowie auch pathophysiologische Regulation dieses zentralen Signalweges unzureichend verstanden wird. Aus diesem Grund ist ein zentrales Ziel der Diabetesforschung den Insulinsignalweg weiter zu charakterisieren und die Mechanismen aufzudecken, die eine Störung des Insulinsignalwegs im Skelettmuskel bewirken.

Sowohl der Akt- als auch der *mammalian target of rapamycin complex 1* (mTORC1)-Signalweg spielt eine entscheidende Rolle bei der Kontrolle und Regulierung der Insulinwirkung. Eine Dysregulation dieser Signalwege konnte bereits mit der Entstehung von Insulinresistenz in Verbindung gebracht werden. Das Protein *proline-rich Akt substrate of 40 kDA* (PRAS40) ist nicht nur Bestandteil und Substrat des Proteinkomplexes mTORC1 sondern auch eines derjenigen Proteine, die am stärksten durch die Proteinkinase Akt reguliert werden. Obwohl bereits gezeigt werden konnte, dass die Insulin-vermittelte Phosphorylierung von PRAS40 sowohl in Nagern nach einer Hochfett-Diät als auch in Menschen mit T2D reduziert ist, ist die Funktion dieses Proteins im Insulinsignalweg unvollständig aufgedeckt. Aufgrund dieser unzureichenden Kenntnisse über PRAS40 war das Ziel dieser Arbeit die Funktion von PRAS40 auf den Insulinsignalweg sowie auf den mTORC1-Signalweg in Skelettmuskelzellen zu untersuchen. Der Knockdown von PRAS40 führte zu einer verminderten Insulin-vermittelten Phosphorylierung von Akt und seinen Substraten. Zusätzlich war die Insulin-induzierte Aufnahme von Glukose in die Muskelzellen beeinträchtigt. Bei Abwesenheit von PRAS40 war Insulin zudem nicht mehr in der Lage, die mTORC1 Aktivität zu steigern, was auf eine verminderte Aktivität des PI3K/Akt/TSC2 Signalweges zurückzuführen ist. Die Beeinträchtigung der

Insulinwirkung durch den Verlust von PRAS40 konnte durch eine verminderte Proteinexpression von *insulin receptor substrate* (IRS) 1 erklärt werden. Die Inhibierung der Kinase p70S6K, welche bekannt ist, den aktiven Abbau von IRS1 zu begünstigen, konnte weder die verminderte IRS1-Proteinexpression noch die Insulinsensitivität bei Abwesenheit von PRAS40 normalisieren. Im Gegensatz dazu, konnte die Reduktion der IRS1 Proteinabundanz durch den Knockdown von PRAS40 mit einer gesteigerten Aktivität des 26S Proteasoms und durch eine erhöhte Genexpression der E3-Ligasen *MuRF1* und *FBXO32* erklärt werden. Die pharmakologische Inaktivierung des Proteasoms in PRAS40-knockdown Zellen durch MG-132 normalisierte die IRS1 Proteinexpression sowie die Insulinsensitivität. Im Gegensatz zu diesen Ergebnissen bewirkte die Überexpression von PRAS40 eine signifikante Erhöhung der Insulinsensitivität in humanen Skelettmuskelzellen *in vitro* sowie *in vivo* in Mausmuskeln. Diese Effekte konnten erneut durch Veränderungen des IRS1 Proteinlevels erklärt werden. Die Überexpression von PRAS40 bewirkte eine verstärkte Proteinexpression von IRS1, was vornehmlich durch eine verminderte Aktivität des Proteasoms induziert wurde. Es stellte sich heraus, dass die positiven Effekte von PRAS40 auf die Aktivität des Proteasoms unabhängig von posttranslationalen Modifikationen (genauer gesagt Phosphorylierung) sowie von der Bindung von PRAS40 an mTORC1 vermittelt wurden. Im Gegensatz dazu, konnte die Insulinresistenz, die durch eine chronische Behandlung der Skelettmuskelzellen mit Insulin induziert wurde, nur durch Überexpression von PRAS40 aufgehoben werden, wenn Phosphorylierung von PRAS40 und/oder die PRAS40-mTORC1-Bindung ermöglicht werden konnte. Diese mTORC1-Bindung und Phosphorylierungsstellen waren zudem essentiell für die Inhibierung des mTORC1 durch PRAS40-Überexpression. Wir konnten des Weiteren zeigen, dass PRAS40 eine Sequenz besitzt, welche den Export von Proteinen aus dem Kern reguliert. Die Einfügung einer Mutation innerhalb dieser Sequenz führte zu einer verstärkten Akkumulierung von PRAS40 im Zellkern von A14 Fibroblasten und resultierte in einer verminderten Insulin-induzierten Aktivierung des Akt- und mTORC1 Signalweges. Schlussendlich konnten wir bestätigen, dass die Phosphorylierung von PRAS40 an Thr246 ein möglicher Biomarker für Insulinresistenz darstellt. Sfrp5, welches identifiziert wurde die Insulinwirkung in humanen Adipozyten zu modulieren, inhibierte die Insulin-vermittelte Phosphorylierung von Akt. Dieses spiegelte sich zusätzlich in einer verminderten Phosphorylierung von PRAS40 an Thr246 durch Insulin wider.

Zusammenfassend zeigen unsere Daten, dass PRAS40 als ein neuer Modulator der Insulinwirkung identifiziert werden konnte. PRAS40 wirkt regulierend auf die Proteinabundanz von IRS1 und bestimmt dadurch die Insulinsensitivität. Dabei besitzt dieses Protein zwei unabhängige Funktionen in der Zelle: Auf der einen Seite wirkt PRAS40 inhibierend auf den mTORC1 Signalweg, auf der anderen Seite wird die Aktivität des Proteasoms durch PRAS40 moduliert. Zukünftige

Experimente müssen jedoch den Mechanismus aufdecken, wie der Einfluss von PRAS40 auf das Proteasom vermittelt wird.

Summary

According to current estimations, the worldwide prevalence of diabetes will increase from 366 million in 2011 to 552 million in 2030, emphasizing the exigency of understanding the molecular mechanisms underlying the pathophysiology of this disease in order to develop new therapeutic strategies. Both, insulin resistance in skeletal muscle, adipocytes and liver as well as β-cell dysfunction are the core pathophysiological defects in type 2 diabetes (T2D). In this context, insulin resistance in skeletal muscle represents a critical determinant in pathogenesis of T2D and occurs long before clinical symptoms are observed. However, the underlying mechanisms as well as the involved factors contributing to the progression of insulin resistance are not yet fully elucidated. Intermediates involved in insulin signaling are pleiotropic and their role in physiological and pathophysiological insulin action is often undefined. For that reason, one important issue of diabetes research is to understand the normal insulin signaling cascade as well as to identify and characterize the multiple mechanisms involved in the disturbance of insulin action in skeletal muscle.

The Akt and mammalian target of rapamycin complex 1 (mTORC1) signaling pathway play a predominant role in normal control of insulin action as well as in pathophysiology of insulin resistance. Proline-rich Akt substrate of 40kDa (PRAS40), a component and substrate of mTORC1, is one of the most prominent Akt-substrates in skeletal muscle. Although insulin-mediated phosphorylation of PRAS40 is impaired in skeletal muscle of high-fat diet fed rodents and humans with T2D, its function in insulin action is incompletely understood. Due to this inadequately defined function of PRAS40 in insulin signaling, one of the aims of this thesis was to characterize the function of PRAS40 on insulin action as well as on mTORC1 signaling in skeletal muscle. Knockdown of PRAS40 inhibited insulin-mediated phosphorylation of Akt and its downstream targets and importantly, reduced insulin-stimulated uptake of glucose. In addition, activation of the mTORC1 pathway by insulin was decreased, likely due to inhibition of the PI3K/Akt/TSC2 axis. The reduction in insulin sensitivity by PRAS40 knockdown could be ascribed to a marked reduction in insulin receptor substrate (IRS) 1 protein expression. Pharmacological inhibition of p70S6K, which has been linked to IRS1 degradation, did not restore IRS1 expression and insulin sensitivity in cells lacking PRAS40. Rather, knockdown of PRAS40 elevated the activity of the 26S proteasome, and increased the mRNA expression of the E3 ligases *MuRF1* and *FBXO32*. Pharmacological inhibition of the proteasome using MG-132 restored IRS1 abundance and insulin sensitivity in PRAS40-knockdown cells. In contrast to these results, overexpression of PRAS40 significantly increased insulin sensitivity in human skeletal

muscle cells *in vitro*, as well as *in vivo* in mice muscle. Again, these effects were mediated by alterations in IRS1. Protein abundance of IRS1 was increased after PRAS40 overexpression, which was likely induced via down-regulation of the proteasome activity by PRAS40 overexpression. Importantly, these beneficial effects were independent of posttranslation modifications (more precisely phosphorylation) and binding of PRAS40 to mTORC1. In contrast, protection against hyperinsulinemia-induced insulin resistance was only observed after PRAS40 overexpression when phosphorylation of PRAS40 and/or binding to mTORC1 was feasible. These events were also indispensable for inhibition of mTORC1 signaling by PRAS40 overexpression. Furthermore, our data revealed that PRAS40 possesses a functional nuclear export sequence. Enforced nuclear accumulation of PRAS40 via mutation of this sequence resulted in decreased insulin-mediated activation of Akt as well as of mTORC1 signaling pathway in A14 fibroblasts. Finally, we and others identified phosphorylation of PRAS40 at Thr246 as a possible biomarker for insulin resistance. Sfrp5, a newly identified modulator of insulin action in human adipocytes, inhibited insulin-mediated Akt phosphorylation, which was also displayed in a reduction in the ability of insulin to induce PRAS40 phosphorylation.

In summary, we could demonstrate that PRAS40 is a new modulator of insulin action via affecting IRS1 protein abundance. In this context, PRAS40 processes a dual function; on the one hand, PRAS40 regulates mTORC1 function. On the other hand, PRAS40 affects the activity of the proteasome, which is mediated in an mTORC1-independet manner. However, the underlying mechanism of PRAS40 action on the proteasomal machinery has to be elucidated in the future.

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Abbreviations

4E-BP1	Eukaryotic initiation factor 4E binding protein 1
AAA-PRAS40	Mutant form of PRAS40 with Thr246Ala, Ser183Ala, Phe129Ala
ADA	American Diabetes Association
Akt (PKB)	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AS160	Akt substrate of 160kDa
ASI	Average spot intensity
ATP	Adenosine triphosphate
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
DEPTOR	DEP domain-containing mTOR-interacting protein
ERK	Extracellular-signal-regulated kinase
FBXO32	F-box protein 32
FFA	Free fatty acid
FOXO	Forkhead box transcription factor
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GLUT	Glucose transporter
Grb	Growth factor receptor-bound protein
GSK	Glycogen synthase kinase 3
GSV	GLUT4 storage vesicles
GTP	Guanosine-5'-triphosphate
HbA1c	Glycated haemoglobin A1c
HFD	High-fed diet
HUVEC	Human umbilical vein endothelial cell
IGF	Insulin growth factor
IGFR	Insulin growth factor receptor
IKK	Inhibitor of nuclear factor kappa-B kinase subunit
IL	Interleukin
IMCL	Intramyocellular lipids
IR	Insulin receptor
IRS	Insulin receptor substrate
IR-β	Insulin receptor beta chain
JNK	c-Jun NH_2-terminal kinase
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MetS	Metabolic syndrome
mLST8	Mammalian ortholog of yeast lethal with Sec13 protein 8
mRNA	Messenger RNA
mSIN1	Mammalian stress-activated protein kinase-interacting protein

mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex
MuRF1	Muscle RING-finger protein-1
NES	Nuclear export sequence
NFκB	Nuclear factor kappa-B
NT-siRNA	Non-target siRNA
p70S6K	p70 ribosomal S6 kinase
PDK1	3-Phosphoinositide-dependent protein kinase 1
PH	Pleckstrin homology
Phe	Phenylalanine
PI3K	Phosphatidylinositol 3´ kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PK	Protein kinase
PP2A	Protein phosphatase 2 A
PPAR	Peroxisome proliferator-activated receptor
PRAS40	Proline-rich Akt substrate of 40kDA
PRAS40-KD	PRAS40 knockdown
PROTOR	Protein observed with rictor
PTEN	Protein phosphatase and tensin homolog
PTP	Protein tyrosine phosphatases
raptor	Regulatory associated protein of mTOR
Rheb	Ras homolog enriched in brain
rictor	Rapamycin-insensitive companion of mTOR
ROS	Reactive oxygen species
S6K	Ribosomal S6 kinase
Ser	Serine
Sfrp5	Secreted frizzled-related protein 5
sh-RNA	Small hairpin RNA
siRNA	Small interfering RNA
SNP	Single-nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBC1D1	Tre-2/USP6, BUB2, cdc16 domain family member 1
Thr	Threonine
TK	Tyrosine kinase
TNFα	Tumor necrosis factor-α
TOS	TOR signaling motif
TSC	Tuberous sclerosis complex
Tyr	Tyrosine
VEGF	Vascular Endothelial Growth Factor
Wnt5a	Wingless-type MMTV integration site family member 5a
WT	Wild type

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CHAPTER 1

General Introduction

1.1. Type 2 diabetes mellitus

1.1.1 Definition, classification and prevalence of diabetes

Diabetes mellitus covers a group of metabolic disorders, which are characterized by elevated blood glucose concentrations. Hyperglycemia resulting from diabetes occurs as a consequence of abnormalities in insulin action, which result from inadequate insulin secretion from the β-cells of the pancreas and/or decreased tissue response to insulin. Symptoms of diabetic hyperglycemia are polyuria, polydipsia, weight loss, vision disorders and susceptibility to infections as well as ketoacidosis as an acute consequence (1). However, of more importance is the increased incidence for long-term consequences due to chronic abnormalities in carbohydrate, fat and protein metabolism; diabetes increases the incidence of microvascular complications, including retinopathy, nephropathy, neuropathy and erectile dysfunctions, as well as macrovascular complications such as cardiovascular diseases, in particular ischemic heart diseases, peripheral vascular diseases, and cerebrovascular diseases, resulting in organ damage and failure in up to one half of patients with diabetes (2,3). Taken together this disease is associated with reduced life expectancy and diminished quality of life.

According to guidelines for diagnosis of diabetes defined by the American Diabetes Associations (ADA), one of the following criteria must be fulfill; (1) glycated hemoglobin A1C (HbA1C) levels ≥ 6.5%, or (2) fasting plasma glucose (FPG) levels ≥ 126mg/dl (7.0 mmol/l), or (3) 2h plasma glucose levels during an oral glucose tolerance test (OGTT) ≥ 200mg/dl (11.1 mmol/l), or (4) a random plasma glucose level of ≥ 200mg/dl (11.1 mmol/l) in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis (1). The majority of patients with diabetes can be divided in two subtypes, type 1 diabetes (T1D) and type 2 diabetes (T2D).

T1D accounts for 5-10% of diabetic patients and is caused by an immune-mediated destruction of the β-cells of the pancreas. This disease is characterize by the presence of autoantibodies (e.g. against insulin, glutamate decarboxylase 65 or tyrosine phosphatases IA-2 and IA-2β), which are detectable in up to 90% of these patients and serve as diagnostic markers. Additionally, T1D is characterized by a strong human leukocyte antigen (HLA)-association. Due to an absolute insulin deficiency induced by the destruction of β-cells, patients with T1D have a lifelong need for insulin treatment. Multiple genetic predispositions and environmental factors are linked to the autoimmune destruction of β-cells, but are yet incompletely defined (1,4). Although T2D has been increasingly diagnosed in children and adolescents in the last decades, T1D is the most common type of diabetes in youth. However, one fourth of patients with T1D are diagnosed as adults and in

up to 10% of adults, which were primarily diagnosed with T2D, autoantibodies associated to T1D are detectable. This subtype of T1D is also termed latent autoimmune diabetes of adults (LADA). Finally, a minority of patients with T1D (less than 10%, most of African or Asian ancestry) suffers from episodic ketoacidosis and insulin deficiency but shows no evidence of β-cell autoimmunity, defining the idiopathic or type 1B diabetes (1,3-5).

The non-insulin-dependent T2D or adult-onset diabetes accounts for 90-95% of diabetic cases and is characterized by resistance to insulin action in peripheral tissues, inadequate secretion of insulin as well as impaired suppression of glucagon secretion (1,6). This type of diabetes was long thought to be a metabolic disorder in elderly, however, an alarming increase of newly diagnosed T2D in children and adolescents could be observed in the last two decades. T2D may remain undiagnosed for many years because hyperglycemia develops gradually and symptoms are unnoticed. However, damages of organs and neurons, especially resulting from micro- and macrovascular complication can already progress (6,7). As T2D patients usually display only relative insulin deficiency, no daily doses of insulin are required, whereas changes in dietary and physical activity behavior and/or medication are used as therapeutic options.

Other forms of diabetes are mediated by specific genetic defects, e.g. of β-cell function (maturity-onset of diabetes in the young (MODY)) or mutations in mitochondrial DNA (maternally inherited diabetes and deafness (MIDD)), by diseases of the exocrine pancreas (pancreatitis) or by drugs and chemicals as well as due to infections (3,8,9). Furthermore, pregnant women can develop gestational diabetes, which symptoms resemble T2D very much. Although this type of diabetes mostly disappears after delivery, the incidence for developing T2D later in life is increased in these women (10). During manifestation of T2D, the progression from normal glucose tolerance (NGT) to T2D involves stages of impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), also known as prediabetes. These patients do not meet the criteria for diabetes, yet their glucose levels are higher than those considered normal. Per year 5-10% of the individuals with prediabetes become diabetic, but the same portion returns to normoglycemia by lifestyle- or drug-based interventions. However, the ADA estimates that 70% of prediabetic patients will develop diabetes as well as cardiovascular diseases (11).

According to current estimations the worldwide prevalence of diabetes mellitus will increase from 366 million in 2011 to 552 million in 2030 due to an increase in economic development and urbanization, which will lead to changes in lifestyles characterized by reduced physical activity and increased obesity. Interestingly, the number of patients with diabetes is inversely correlated with the current income status, with the greatest increase being expected in low-income countries (92%) (12). In line with this prevalence, diabetes imposes an increasing economic burden on national health care

systems worldwide. In 2010 the expenditure on diabetes care was estimated to be at least USD 376 billion and will raise to USD 490 billion in 2030 (13). More important, diabetes is ranked as the fourth leading cause of death in most high-income countries. In 2012, 4.8 million deaths were caused by diabetes and its complications, especially due to cardiovascular diseases, emphasizing the importance of discovering new therapeutic options and facilitating early diagnosis (14).

1.1.2 Aetiology of type 2 diabetes

Nowadays, it is well established that environmental factors causing metabolic disorders like obesity, hypertension and dyslipidemia trigger the progression of glucose intolerance. These disorders are collectively described as the metabolic syndrome (MetS, or syndrome X) and together provide an indicator for the risk of diabetes, heart disease, stroke and other cardiovascular complications. Individuals with metabolic syndrome display a five-fold increased risk for progression of T2D compared with those without the syndrome (15). According to a new definition published by the International Diabetes Federation (IDF), the criteria for an individual to be defined as having the metabolic syndrome must fulfill central obesity (for Europeans defined as waist circumference >94cm (males)/80cm (females) or body mass index (BMI) >30kg/m^2) plus any two of the following additional factors: raised triglyceride levels (≥150mg/dl (1.7mmol/l)), reduced high-density lipoprotein (HDL)-cholesterol levels (<40mg/dl in males, <50mg/dl in females), increased blood pressure (systolic ≥130mmHg, diastolic ≥85mmHg) or raised fasting plasma glucose (≥100mg/dl) (16,17). Among these criteria, obesity shows the strongest correlation with the incidence of T2D. Obese individuals, characterized by having a BMI >30kg/m^2, display a 3-7 times higher incidence for T2D compared to normal weight controls. An even up to 20-fold increased risk for T2D was observed in patients with morbid obesity (BMI >35kg/m^2). Interestingly, visceral fat mass rather than subcutaneous fat seems to be strongly associated with an abnormal metabolic profile, indicating that adiposity expressed in terms of waist-hip ratio (WHR) has a greater ability for predicting T2D than BMI (18). However, not all individuals with T2D are obese and not all obese individuals develop T2D. Other obesity-independent risk factors for diabetes are age greater than 45 years, family history, ethnic background (risk is greater in Hispanics, African-Americans, Native Americans and Asians), hypertension, gestational diabetes, physical inactivity, low birth weight, high exposure to certain environmental toxins, depression, smoking, lack of sleep and dietary factors, like fast food intake, whole grain-poor diet as well as vitamin D and calcium deficiency (19-22).

Although these environmental risk factors play a pivotal role in the progression of T2D, unequivocal evidence exists that genetic and epigenetic factors are also highly associated with the

development of this disease. With the introduction of high-throughput technology and genome-wide association studies (GWAS), multiple candidate genes could be identified. To date, a total of 58 loci have been identified to be associated with T2D (23). The majority of these loci are associated with impaired β-cell function like transcription factor 7-like 2 (*TCF7L2)*, which displays the strongest susceptibility locus for T2D. In the short list of loci related to insulin resistance, peroxisome proliferator-activated receptor gamma (*PPARG)* and insulin receptor substrate 1 (*IRS1)* have been well recognized, given their known function in inulin action (23). However, only 10% of the heritability of T2D can be explained by susceptibility loci identified so far, with each locus having a low effect size (24). Combinations of information from multiple genetic variants may be helpful in identifying individuals with a high risk for T2D. Nevertheless, the idea that epigenetic processes are more important for the pathogenesis of T2D is supported by several epidemiological and experimental studies. DNA methylation and histone modifications may affect multiple phenotypes and contribute to the risk of diabetes. Especially the role of epigenetics in fetal and neonatal programming has been a major area of research activity. The idea of a "fetal metabolic programming" hypothesizes that nutritional and other exposures during early life generate long-term changes that later predispose to T2D and cardiovascular diseases. A growing body of data supports the idea that epigenetic changes are responsible for this link between intrauterine environment and predisposition to T2D. However, a lack of studies investigating the role of epigenetics in humans has led to only few reliable estimates regarding the quantitative contribution of epigenetic processes with respect to variance in diabetes risk (25,26).

Altogether, this supports the idea that individual predisposition to T2D reflects a complex mixture of environmental, genetic and epigenetic influences.

1.1.3 Pathophysiology of type 2 diabetes

Both insulin resistance in skeletal muscle, adipocytes and liver as well as β-cell dysfunction are considered as the core pathophysiological defects in T2D. These metabolic alterations start years before diabetes development and proceed in a multistage process (11,27). The first stage is characterized by a compensatory increase of insulin secretion and expansion of β-cell mass as a response to insulin resistance in the periphery. In the second stage normal glucose levels can no longer be maintained because compensation by β-cells begins to fail. Changes and loss of β-cell function start to arise in this stage. Importantly, mainly due to high concentrations of glucose creating a detrimental environment (also termed glucotoxicity), the glucose-stimulated insulin secretion (GSIS) is inhibited and changes in β-cell phenotype are induced. Individuals in stage two

usually evade progression to diabetes for years, but when β-cell mass becomes insufficient at some critical point, glucose concentrations rise over a relatively short period of time (stage 3). For the first time, noticeable symptoms like weight loss and polyuria can occur. The majority of patients with T2D remain a lifetime in the fourth stage, where they secrete enough insulin to remain in this stage and not to progress ketoacidosis. However, β-cell mass is already reduced by 50% compared to healthy individuals. Very few patients develop such as serve loss of β-cell mass that they become ketotic and dependent on exogenous insulin supply (stage 5) (11,27,28). Impaired β-cell function can also be induced by reduced secretion of incretin hormones, such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP). In diabetic and obese individuals incretin secretion is blunted and therefore contributed to impaired β-cell function (29,30).

The initial event inducing the changes in β-cell function is the reduction of insulin sensitivity in the periphery. Several studies showed that insulin resistance occurs 10-20 years before the onset of the disease and serves as the best marker of whether or not an individual will later develop diabetes (31,32). Insulin resistance strongly correlates with obesity and ectopic fat accumulation and results in abrogation of insulin action mainly in skeletal muscle, adipocytes and liver.

Insulin resistance in skeletal muscle represents a critical determinant in pathogenesis of T2D. Therefore, further characterization and understanding of skeletal muscle insulin action and mechanism leading to muscle insulin resistance will provide new strategies to prevent whole-body insulin resistance and improve insulin sensitivity.

1.2. Insulin action in skeletal muscle

The highly conserved hormone insulin contains 51 amino acids (5.8 kDa) and is produced by the pancreatic β-cells of the islets of Langerhans. The main function of insulin is to act on carbohydrate, fat and protein metabolism. This includes the decrease in hepatic glucose production, glucose uptake by muscle and adipose tissue, increased glycogen synthesis, inhibition of glycogen breakdown, stimulation of fatty acid synthesis in adipose tissue and liver, suppression of lipolysis, increased formation and storage of triglycerides, suppression of fat oxidation, induction of protein synthesis, increased amino acid uptake, stimulation of growth as well as differentiation and vasodilation (33). All these effects are mediated by binding of insulin to the insulin receptor located at the cell surface.

1.2.1 Insulin receptor signaling

The function of insulin receptor (IR) signaling in skeletal muscle can be divided in three primary areas: metabolism/glucose uptake, mitogenesis/growth and aging/longevity (34). Insulin receptors consist of two extracellular α subunits and two transmembrane β subunits, which are linked by disulfide bonds and form a heterotetramer (150kDa). Insulin binds to two sites located on each α-monomer and induces a conformational change of the β-subunits and concomitant activation of the intracellular tyrosine kinase activity resulting in autophosphorylation of the juxtamembrane tyrosine residues. These residues then act as docking sites for a variety of substrates. After insulin binding, the IR is internalized and insulin is released from IR. While insulin is degraded in the lysosomes, the IR is recycled to the cell membrane. Due to alternative splicing two variants of IR exist (IR-A and IR-B), which differ in 12 additional amino acids resulting in differences in tissue distribution, ligand specificities, kinetics and internalization rates (34,35). Whereas IR-A is ubiquitously expressed, IR-B is expressed largely in the classically insulin sensitive tissues of liver, skeletal muscle and adipose tissue (36).

1.2.1.1 Function of IR in metabolism

The primary pathway regulated by insulin is the stimulation of the translocation of the insulin-responsive glucose transporter type 4 (GLUT4)-containing vesicles to the plasma membrane enabling glucose uptake and decreasing blood glucose level. This aim is achieved via activation of three independent signaling cascades; (i) the insulin receptor substrates (IRS)/phosphatidylinositol 3´ kinase (PI3K)/protein kinase B (PKB or Akt) pathway plays a pivotal role in insulin-mediated glucose uptake and progression of insulin resistance and will be discussed in detail in the following sections (see 1.2.2). (ii) Activation of the adapter protein with pleckstrin homology and Src homology domain (APS)/ casitas B-lineage lymphoma (Cbl)/c-Cbl-associated protein (CAP) complex is a PI3K-independent mechanism by which GLUT4 translocation is evoked in skeletal muscle (34). APS binds to activated IR and recruits Cbl together with CAP to the IR, where Cbl gets phosphorylated on multiple tyrosine residues. This induces the disassociation and translocation of the CAP/Cbl complex to specialized microdomains of the plasma membrane, the caveolar lipid rafts. Here, small guanosine triphosphate-binding proteins (TC10) get activated and facilitate GLUT4 translocation (Fig. 1.1). (iii) The third way by which insulin increases the amount of GLUT4 on the plasma membrane is via activation of mammalian uncoordinated-18 (Munc18) proteins. In 2011 Jewell et al. could demonstrate for the first time that Munc18c is phosphorylated at Tyr521 by the IR in skeletal muscle and adipocytes (37). Phosphorylation of Munc18 releases the syntaxin 4/synaptosomal-associated

protein 23 (SNAP23) complex and enables its binding to the v-SNARE protein vesicle-associated membrane protein 2 (VAMP2). This in turn activates the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE)-machinery involved in coordination of GLUT4 exocytosis (Fig. 1.1) (34,37).

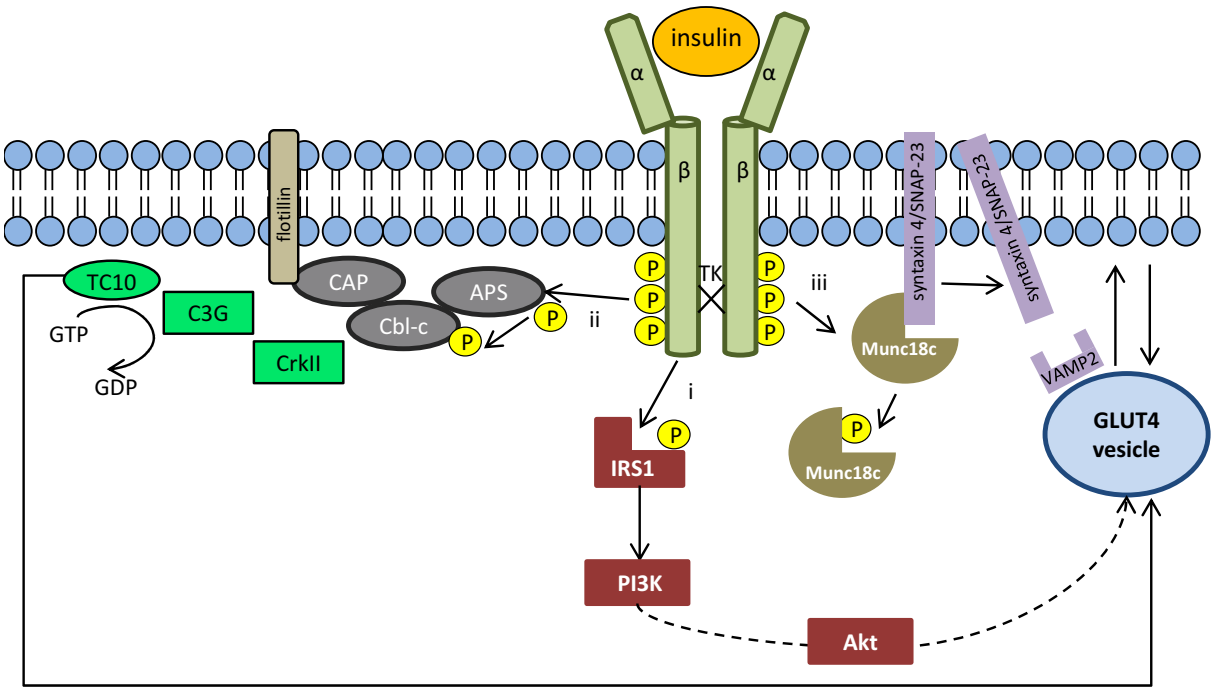

Figure 1.1 IR-mediated GLUT4 translocation. Binding of insulin to insulin receptors results in conformational change and activation of intrinsic tyrosine kinases (TK) leading to autophosphorylation of the β-subunits. Exocytosis of GLUT4 vesicles can be induced via three independent mechanisms; (i) IRS1 binds to activated IR and induces PI3K/Akt-mediated GLUT4 translocation. (ii) Cbl is recruited to the plasma membrane and is phosphorylated by APS, which results in localization to lipid rafts via binding to CAP and flotillin. Here, phosphorylated Cbl recruits CrkII and the nucleotide exchange factor C3G, which acts on TC10 and results in GLUT4 translocation. (iii) IR phosphorylates Munc18c, which leads to dissociation of Munc18c from syntaxin 4. Syntaxin4 forms together with SNAP23 the only known active t-SNARE protein in skeletal muscle for GLUT4 exocytosis. Free syntaxin 4 functions in glucose uptake by interacting with the v-SNARE VAMP-2, localized at the GLUT4 vesicles. Under basal conditions Munc18c prevents syntaxin 4/VAMP-2 interaction and must undergo conformational change by insulin-mediated phosphorylation to allow vesicle fusion.

1.2.1.2 Function of IR in growth and aging

Like many growth factor receptors the IR regulates mitogenesis via activation of the RAS/mitogen-activated protein (MAP) kinase pathway. Hereby, the adaptor protein Grb2, which is constitutively linked to the son of sevenless (SOS) protein, binds to phosphorylated IRS or Shc proteins, causing the recruitment of Grb2-SOS complex to the plasma membrane. Although IRS also binds Grb2, it is believed that the Shc-Grb2-SOS complex is predominantly involved in insulin-mediated stimulation of growth. Once the Grb2-SOS complex is formed, p21ras gets activated via

GTP loading. This leads to phosphorylation and activation of serine/threonine kinases, including rapidly accelerated fibrosarcoma (Raf) and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK), resulting in activation of extracellular-signal-regulated kinase (ERK). Once ERK gets phosphorylated, it is translocated from the cytoplasm into the nucleus, where it phosphorylates a number of substrates involved in activation of transcription and cell-cycle progression (34,38,39). Besides activation of the RAS/MAPK pathway, IR can directly induce gene transcription via activation of the transcription factor signal transducer and activator of transcription (STAT) 5b. Upon phosphorylation, STAT-5b homodimerizes through SH2 domains and translocates to the nucleus (Fig. 1.2) (34,40). Importantly, the IR subunits α and β can form heterodimers with the insulin growth factor receptor (IGFR) to form IR-IGFR hybrids. These hybrids bind insulin growth factor (IGF) with the same affinity as IGFR homodimer, however, insulin binds with significantly lower affinity to hybrid receptors (41). Therefore, IR-IGFR hybrids more likely function in transduction of mitogenic signaling rather than metabolic downstream signaling.

Genetic suppression of insulin and IGF signaling can extend longevity in worms, insects, and mammals. In line with this, both obesity and diabetes are associated with shortened life expectancy. Furthermore, caloric restriction, resulting in low insulin and IGF-1 levels, is the most effective method to increase lifespan in model systems. This effect is mediated by IR signaling cascades, which are also involved in regulation of metabolism and growth. A pivotal role for forkhead box transcription factor (FOXO), sirtuins (Sir) and mammalian target of rapamycin (mTOR) complex 1 (mTORC1) could be demonstrated in this scenario (Fig. 1.2) (42). Preliminary evidence indicates that it is relevant for regulation of longevity in human as well.

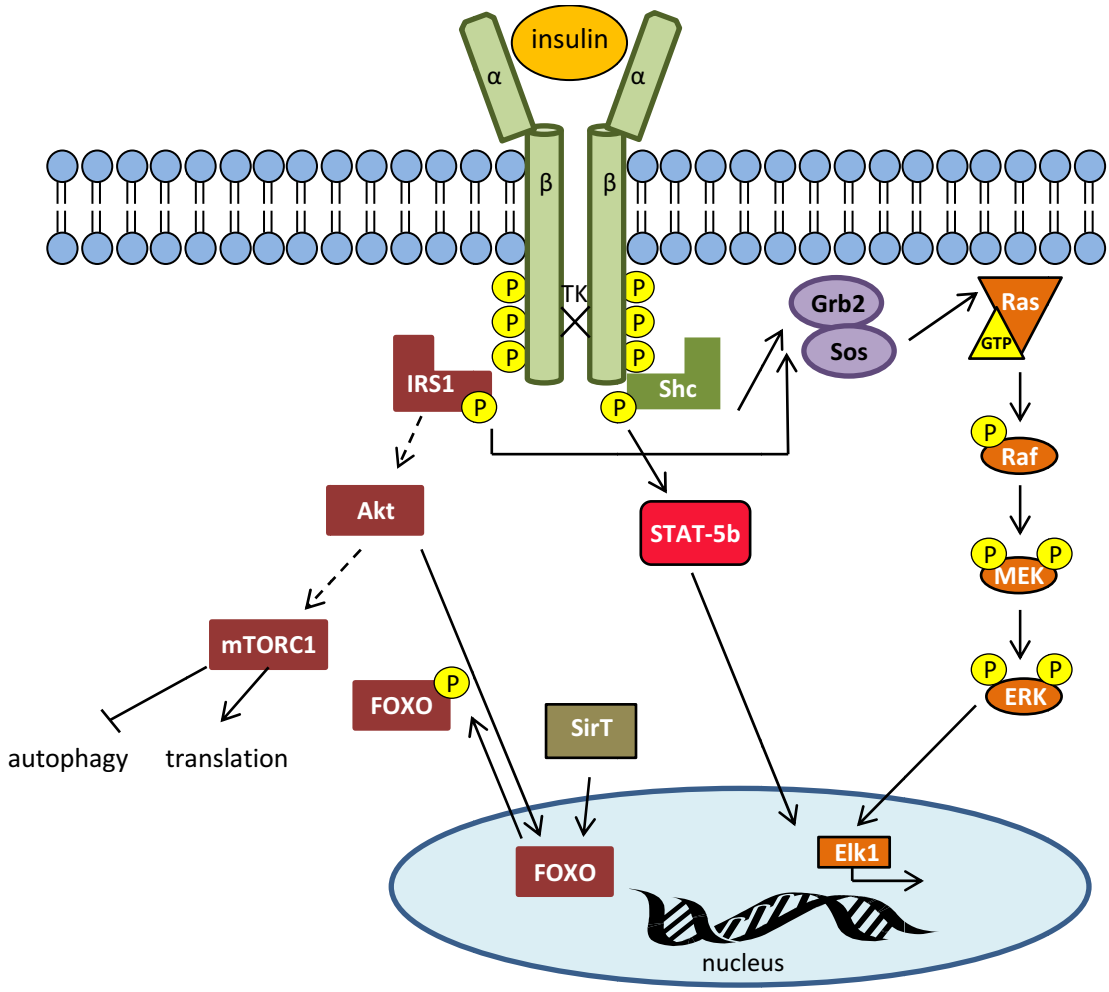

Figure 1.2 Role of IR signaling in mitogenesis and longevity. Autophosphorylation of IR leads to activation of IRS1 and Shc. These substrates provide docking areas for molecules containing SH2- domains, such as Grb2. The Grb2-SOS complex activates Ras, leading to sequential activation of the kinases Raf, MEK-1/2 and ERK-1/2. These MAP kinases stimulate various genes regulating growth and differentiation via activation of transcriptions factors, like ETS domain-containing protein (ELK-1). Furthermore, IR can directly activate translocation of the transcription factor STAT-5b into the nucleus, where it induces transcription of mitogenic genes. Signaling via the IR also regulates processes involved in aging and longevity: IR-mediated activation of Akt leads to activation of mTORC1 and phosphorylation of FOXO resulting in cytosolic localization of this transcription factor. Under conditions of insulin or serum withdrawal, FOXO is not phosphorylated and translocates into the nucleus, where it activates longevity genes and suppresses pro-aging genes. FOXO proteins can also be directly regulated by sirtuins (SirT).

1.2.2 IRS/PI3K/Akt-signaling pathway

Induction of the PI3K/Akt pathway is responsible for most of the metabolic effects of insulin and is initiated by tyrosine phosphorylation of the IRS proteins. Among the hundreds of molecules that participate in insulin signaling, IR/IRS, PI3K and Akt are the three best-defined critical nodes of

insulin action (43). This pathway is highly conserved in metazoan organisms and tightly controlled via a multistep process, which should be presented in the following sections.

1.2.2.1 IRS proteins

Tyrosine phosphorylation of IRS proteins constitutes the first event after activation of the IR that mediates intracellular insulin action. Importantly, IRS proteins do not possess an intrinsic catalytic activity but contain several functional domains which mediate interaction with the IR and several substrates. A phosphotyrosine-binding (PTB) domain, located at the amino terminus, is responsible for binding to the IR or IGFR. A pleckstrin homology (PH) domain, also located at the amino terminus, is required to elicit an optimal signal response. Although the underlying mechanism is unknown, deletion of this domain results in significantly decreased insulin-stimulated IRS1 tyrosine phosphorylation. The C terminus of IRS proteins contains numerous tyrosine residues that, after phosphorylation by the IR, act as on/off switches to recruit and regulate various substrates that contain Src-homogy-2 (SH2) domains.

The family of IRS proteins consists of 4 members, IRS1-4 respectively. IRS1 and IRS2 are widely distributed, including tissues thought to be most important for glucose and lipid homeostasis, while the other members have restricted distributions. IRS1 and IRS2 are expressed in muscle, liver, fat and pancreatic islets, whereby IRS-1 appears to be more important in muscle metabolism and IRS-2 may play greater roles in liver, adipose tissue and islet β cells. IRS3 is expressed in murine adipocytes and brain, but has so far not been detected in humans, whereas IRS4 is limited to embryonic tissue, brain, thymus or cell lines. Recently, two potential new members, IRS5 and IRS6, have been identified (43). The function of these proteins in signaling and if they really can be classified as IRS proteins need to be elucidated in the future (43,44).

IRS1 and IRS2 are large proteins (160-185kDa), which are homologous in their PH and PTB domain and possess many similar tyrosine phosphorylation motifs. However, in contrast to IRS1, IRS2 contains the kinase regulatory loop binding (KRLB) domain, which interacts with the IR, but not with IGFIR, and may function to limit IRS2 tyrosine phosphorylation. Furthermore, knockout studies in mice provide strong evidence that the IRS proteins have non-redundant functions in insulin signaling. *IRS1* $^{-/-}$ mice show a 70% reduction in body size mainly due to IGF1 resistance, whereas *IRS2* $^{-/-}$ mice show altered growth in only a few tissues, like brain, ovaries and pancreatic β-cells. Interestingly, in both models peripheral insulin resistance could be observed, but only *IRS2* $^{-/-}$ mice develop diabetes due to loss of β-cell function (45,46). *In vitro* knockdown studies using IRS1- or IRS2-specific siRNAs in L6 myotubes implicate that IRS1 seems to be more closely linked to glucose uptake, whereas IRS2 is found to be important for regulation of MAPK activity (47). In line with this, a large body of human

studies link the occurrences of IRS1 polymorphisms to insulin resistance and diabetes, whereas no consistent association between IRS2 polymorphism and neither insulin resistance nor diabetes could be observed (48). Until now the function of IRS3 and IRS4 is not investigated in detail. *IRS4* $^{-/-}$mice only show a mild reduction in growth, reproduction and insulin sensitivity, indicating that IRS4 cannot activate MAPK and PI3K to the same degree as IRS1 or IRS2 (49). Finally, the ability and affinity to bind and activate various SH2-substrates as well as their cellular compartmentalization differ between the members of the IRS family (43). Downstream substrates of IRS proteins in response to insulin include the p85 regulatory subunit of PI3K, Grb2, and SH2 domain-containing protein tyrosine phosphatase-2 (SHP2). Although binding of other SH2-domain containing adaptor proteins to IRS has been reported, the dominant function for IRS proteins in IR-mediated regulation of metabolism is the amplification of PI3K signaling to activate the serine/threonine kinase Akt (43) (Fig.1.3).

1.2.2.2 PI3K

PI3K belongs to a family of lipid kinases that form different isoform and have been divided into different classes (class Ia, Ib, II, III) based on their structure, substrate specificity and mechanism of activation (50). Class Ia PI3K consists of a regulatory and a catalytic subunit, each of which occurs in several isoforms (regulatory: p85α, p55α, p50α, p85β; catalytic: p110α, p110β, p110δ). Inhibition or transgenic deletion of class Ia PI3K abrogates nearly all effects of insulin´s metabolic actions, highlighting the essential role of these proteins in insulin signaling. The p85α protein is best known as the regulatory subunit of PI3K. As such, it has major well-described functions in the stabilization and regulation of the p110 catalytic subunit of PI3K (51). In unstimulated cells the complex of p85 and p110 is located in the cytosol and PI3K activity is repressed. In response to insulin or other growth factors IRS proteins get activated, enabling binding of p85 through the SH2-binding domains, leading to recruitment of p85-p110 complexes to the plasma membrane where termination of the repression of p110 catalytic activity is induced. Active PI3K phosphorylates the inositol ring of phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) on the 3-position, resulting in generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3s are important lipid messengers, which recruit a number of downstream signaling proteins containing pleckstrin homology (PH) and PH-like domains to the plasma membrane thereby transmitting the signal elicited by PI3K activation. Two of the best-characterized substrates are the serine/threonine kinase Akt and the AGC kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Fig.1.3) (52).

1.2.2.3 Serine/threonine kinase Akt

Akt (or protein kinase B (PKB)), a 56kDa member of the ACC serine/threonine kinase family, represents a pivotal node in many signaling pathways downstream of G-protein-coupled receptors and growth factor receptors, such as IR and IGFR. As such, Akt plays a crucial role in cell survival, proliferation, growth, migration, polarity, insulin-mediated glucose uptake, glucose and lipid metabolism, contractility in skeletal muscle and cardiomyocytes, angiogenesis and self-renewal of stem cells. In line with this, alterations in Akt activity are linked to diseases such as cancer, T2D, cardiovascular, neurodegenerative disorders and muscle hypertrophy (53). Three isoforms, namely Akt1, Akt2 and Akt3 are encoded from three different genes on different chromosomes in mammalian cells. While Akt3 is predominantly found in testis, brain, kidney and heart, Akt1 and Akt2 are expressed ubiquitously. All three members of this family consist of a highly conserved domain structure with an N-terminal PH-domain, a kinase domain and a C-terminal regulatory tail containing a hydrophobic motif (53). However, several studies using either siRNA–mediated knockdown or knockout mice demonstrate that the different Akt isoforms regulate distinct biological processes. While Akt1 seems to be more important for insulin-mediated regulation of cell growth and survival, Akt2 contributes to insulin-induced control of glucose metabolism. Mice with a global knockout of *Akt1* are viable but smaller compared to wild type littermates and display a reduced life span in response to genotoxic stress. However, these mice do not develop insulin resistance or a diabetic phenotype (54). In contrast, *Akt2* knockout results in impairment of the ability of insulin to lower blood glucose (55). Furthermore, a study in humans could demonstrate that a mutation in the kinase domain of Akt2 is associated with insulin resistance and diabetes (56). Due to the fact that Akt3 is predominantly expressed in brain, *Akt3* $^{-/-}$ mice display impaired brain development but do not show any impairment of growth or glucose homeostasis (57). Studies investigating the specific function of different Akt isoforms in muscle show that Akt1 is important for myoblast differentiation via regulation of the transcription factor myogenic factor 3 (MyoD) (58). Furthermore, deletion of Akt1 is associated with increased basal fatty acid uptake and β-oxidation in myotubes, which is linked to decreased glucose uptake (59,60). The importance of Akt2 for insulin-induced glucose uptake could be confirmed by the observations that glucose uptake and glycogen synthesis is reduced after siRNA-silencing of Akt2 in muscle cells (59). In contrast, overexpression of constitutively active Akt1 or Akt2 results in muscle hypertrophy consistent with increased mTORC1 activation, and a 60% increase in glycogen accumulation. However, only Akt2 overexpression results in increased glucose uptake *in vivo* (61). Interestingly, studies with double Akt isoform knockout mice reveal some overlap or compensation among the isoforms, indicating that both, overlapping as well as specific roles for Akt family members exist (62).

All three members of the Akt family are recruited to the plasma membrane upon growth factor and insulin stimulation. Here, binding of the N-terminal PH domain to PIP3 leads to conformational changes within the protein and enables phosphorylation and activation of Akt kinase activity. PDK1, which is co-recruited to the membrane after PI3K activation, phosphorylates Akt at Thr308 within the catalytic motif (Thr309 in Akt2/Thr305 in Akt3) (53). Phosphorylation of this residue increases Akt activity by about 100-fold. However, maximal activation of Akt kinase requires the additional phosphorylation of Ser473 (Ser474 in Akt2/Ser472 in Akt3) within the hydrophobic domain (63) (Fig.1.3). The kinase responsible for Ser473 phosphorylation within Akt was unknown for a long time. Today, mammalian target of rapamycin complex 2 (mTORC2) is believed to be the predominant kinase involved in Ser473 phosphorylation of Akt. However, skeletal muscle-specific deletion of rictor, the key regulator of mTORC2, revealed that other kinases are also involved in Ser473 phosphorylation, such as mitogen-activated protein kinase 2, integrin-linked kinase (ILK), protein kinase C isoforms or DNA-dependent protein kinase (DNA-PK) (64). It is currently believed that Ser473 phosphorylation can facilitate Thr308 phosphorylation, however, studies using alanine mutations show that Akt Thr308 and Ser473 can be phosphorylated independently from each other (63). Further studies demonstrated that a deletion of regulatory subunits of the mTORC2, such as rictor, mSIN1 or mLST8, on the one hand selectively inhibit Ser473 phosphorylation and phosphorylation of the Akt substrate FOXO3, but on the other hand do not affect phosphorylation of other Akt targets such as glycogen synthase kinase 3 (GSK3)-β or tuberous sclerosis complex (TSC)-2, demonstrating that Ser473 may determine Akt specificity rather than Akt activity (64,65). After activation of Akt at the plasma membrane, the kinase dissociates and translocates to the cytosol and various subcellular compartments, including the endoplasmic reticulum, mitochondria, Golgi and nucleus, where it activates a plethora of substrates by serine and/or threonine phosphorylation. Akt substrates consist of a consensus motif (R-X-R-X-X-S/T) (66), which results in thousands of potential Akt substrates. However, until today 60-70 of these substrates have been characterized (53). Akt either regulates the function of these substrates, alters their localization or modifies their stability. GSK3 was the first physiological target of Akt to be identified (67,68). Insulin-mediated phosphorylation of GSK3 by Akt results in inhibition of its function and increased glycogen synthesis. A number of Akt substrates, such as Bcl-2-associated death promoter (Bad), Bcl-2-associated X-protein (Bax) and apoptosis signaling kinase 1 (ASK-1) are involved in the regulation of apoptosis, cell cycle progression and cell survival. In general, activation of Akt results in induction of anti-apoptotic signaling events, thus pointing out the pivotal relevance of Akt in cancer development. In the nucleus Akt regulates the gene expression of diverse gluconeogenic and lipogenic enzymes by controlling the activity of the transcription factor family FOXO (Fig. 1.3). Another important substrate is TSC2, which

controls mTORC1, an essential regulator of cell growth and insulin signaling (see 1.2.3 and Fig.1.4). Furthermore, in muscle Akt seems to regulate the composition of the myofribrillar-cytoskeletal system (60).

1.2.2.4 Akt-mediated Glut4 translocation

One of the key roles of Akt in skeletal muscle is to mediate the translocation of the insulin- and contraction-responsive glucose transporter GLUT4 from intracellular compartments to the plasma membrane. This process involves multiple signaling molecules and phosphorylation events and finally results in the induction of glucose uptake into the cell. GLUT4 molecules reside in specialized intracellular vesicles, termed GLUT4 storage vesicles (GSVs) which, in the basal state, slowly recycle between different intracellular compartments and the plasma membrane (69). Following insulin stimulation, these GSVs are mobilized within minutes and transferred to distinct docking positions at the plasma membrane. This complex mechanism is executed by a multiplicity of effector molecules starting from the insulin receptor itself via PI3K and Akt and finally resulting in the phosphorylation of RabGTPase-activating protein (GAP)-domain containing proteins (70). One of these proteins was first described in 2002 as Akt substrate of 160 kDa (AS160, TBC1D4) (71) and since then represents a major focus of research on insulin action (72-74). The direct phosphorylation by Akt leads to an inactivation of the GTP-hydrolysis activity of AS160 and subsequently to an accumulation of small GTP-bound Rab GTPases present in GLUT4-containing vesicles. Various Rab proteins (Rab 8, Rab 10, Rab 14) have been found in *in vitro* approaches as targets for AS160 in GSVs (69,72). In the basal state, AS160 is bound to GSVs, accelerating their intrinsic GTP hydrolysis activity and leading to the retention of GLUT4-containing vesicles in the cytosol. Upon stimulation, AS160 GAP activity is inactivated and GSVs are translocated to the cell surface, resulting in enhanced glucose uptake (Fig. 1.3) (69,72). Two models propose how AS160 phosphorylation can lead to GLUT4 translocation: i) It could be shown that 14-3-3 adaptor proteins bind to phosphorylated AS160 resulting in an inhibition of its GAP activity (75). ii) Alternatively, phosphorylation of AS160 could lead to translocation of AS160 from GSVs to the cytosol, thus enabling conversion of Rab proteins to their active (GTP-bound) form and mediating GSV trafficking to the cell membrane (75). Importantly, contractile activity in muscle can also stimulate GLUT4 translocation independently of insulin (76,77). Exercise stimulates glucose uptake via activation of AMP-activated protein kinase (AMPK) and calmodulin, which results in phosphorylation of AS160. A close homologue of AS160 TBC1D1 (tre-2/USP6, BUB2, cdc16 domain family, member 1) was recently described to act via the same Rab GTPases and therefore conducting a similar function in the retention of GSVs (77-79). While AS160 is expressed in both, adipose tissue and skeletal muscle, TBC1D1 expression is mostly restricted to the

muscle. That leads to the assumption that TBC1D1 represents the major target for contraction-induced glucose uptake while AS160 might be more relevant for insulin-mediated processes (77).

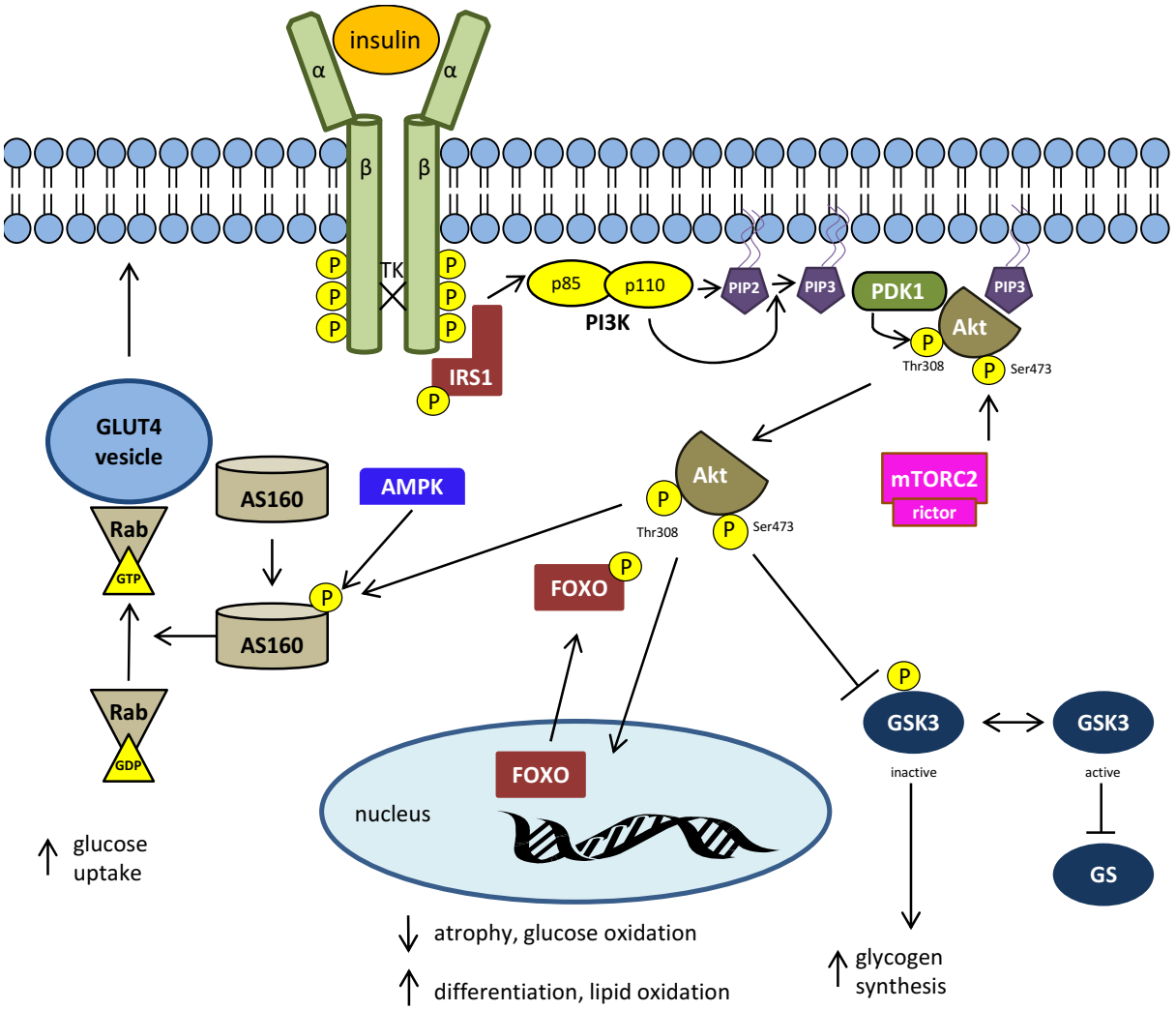

Figure 1.3 Simplified overview of the IRS/PI3K/Akt signaling in muscle. Activating tyrosine-phosphorylation of IRS1 by IR recruits PI3K to the plasma membrane by binding the regulatory subunit p85. The catalytic subunit p110 converts PIP2 to PIP3, which binds to the PH domains of PDK1 and Akt. When located at the plasma membrane, Akt becomes phosphorylated by PDK1 at Thr308 and by mTORC2 at Ser473. Activated Akt translocates to the cytosol or nucleus, where it phosphorylates a plethora of substrates involved in glucose homeostasis: GSK3 activity is inhibited by Akt-mediated phosphorylation releasing the inhibitory function of GSK3 on glycogen synthesis (GS). FOXO transcription factors, which in unstimulated cells are located in the nucleus, trigger the transcription of genes involved in regulation of glucose and lipid metabolism as well as muscle atrophy. Akt phosphorylates FOXO transcription factors on multiple sites, leading to the export of phosphorylated FOXO proteins from the nucleus and inhibition of their transcriptional functions. In skeletal muscle and adipose tissue insulin-stimulated GLUT4 translocation is induced through Akt-mediated phosphorylation of AS160 (or TBC1D1) resulting in inhibition of their GAP activity. This leads to GTP loading of Rab proteins on GLUT4-containing vesicle and to the translocation of GLUT4 to the plasma membrane, enhancing glucose uptake. In skeletal muscle, contraction-mediated glucose uptake is induced via activation of AMPK, which in turn phosphorylates AS160.

1.2.3 mTORC1 signaling

The mammalian target of rapamycin (mTOR) kinase plays a crucial role in nutrient sensing signaling pathways and in maintaining metabolic homeostasis. The control of muscle mass by mTOR relies on a balance between anabolic processes, like protein synthesis and nutrient storage, and catabolic processes, such as utilization of energy stores (80). mTOR is ubiquitously expressed with the highest abundance in brain and skeletal muscle and is activated under nutrient-rich conditions, particularly by amino acids, glucose and insulin (80). The kinase mTOR is found in two distinct multimeric protein complexes, mTORC1 and mTORC2, which differ in their composition, function and regulation. In addition to mTOR, the proteins mammalian ortholog of yeast lethal with Sec13 protein 8 (mLST8) and DEP domain-containing mTOR-interacting protein (deptor) are shared between both complexes, whereas proline-rich Akt substrate of 40kDa (PRAS40) and regulatory associated protein of mTOR (raptor) are only found in mTORC1. The mTOR complex 2 further consists of the unique components rapamycin-insensitive companion of mTOR (rictor), mammalian stress-activated protein kinase-interacting protein (mSIN1) and protein observed with rictor (PROTOR, also known as PRR5) (81). Importantly, only mTORC1 is susceptible for acute inhibition through the drug rapamycin. As mentioned before mTORC2 was found to be responsible for Akt-Ser473 phosphorylation, but has further been identified to regulate cytoskeleton organization (82). So far little is known about the mechanism of how mTORC2 is activated and regulated. In contrast, mTORC1 function and regulation is much better characterized. Insulin-mediated activation of mTORC1 is mainly regulated through the tumor suppressor complex TSC1/2. The TSC2 component has a GAP activity toward the GTPase Ras homolog enriched in brain (Rheb). Akt-mediated phosphorylation of the TSC-complex results in inactivation of the GAP-activity and conversion of Rheb-GDP to the active Rheb-GTP form. In turn, Rheb-GTP binds and activates mTORC1. In contrast, the major pathway by which amino acids control mTORC1 activity is distinct from that of insulin. Instead of signaling through the class I PI3K/Akt/TSC2 pathway, amino acids mediate mTORC1 activation by induction of class III PI3K hVps34 (83). Another current model how amino acids increase mTORC1 activity includes the participation of Rag subfamily of Ras small GTPases (RagA, RagB, RagC, RagD). This class of proteins is believed to induce mTORC1 translocation to lysosomal membranes, where Rheb is located. Furthermore, the recruitment of mTORC1 to lysosomal membranes seems to be dependent on another protein complex, called ragulator. The model by Sancak et al (84) implies that in the presence of amino acids, the Rag GTPases, which are tethered to the lysosomal surface by the ragulator, serve as a docking site for mTORC1, allowing mTORC1 to associate with endomembranes and become activated by Rheb (Fig. 1.4). However, the exact mechanism by which amino acids promote mTORC1 activation requires

additional investigations. Besides insulin and amino acids, the energy status of the cell regulates mTORC1 activation. During energy deprivation by exercise, hypoxia or nutrient deprivation, the adenosine monophosphate (AMP) level increases and promotes activation of AMPK. AMPK-mediated phosphorylation and activation of the TSC1/2 complex results in repression of mTORC1 signaling (Fig.1.4) (85). Furthermore, AMPK can directly phosphorylate the regulatory mTORC1 component raptor and thereby inactivate mTORC1 (86). An additional regulator of mTORC1 is phosphatidic acid (PA), which directly binds mTOR and activates mTORC1 signaling (87).

The two best characterized substrates of mTORC1 signaling are ribosomal S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (Fig.1.4). S6K, which is activated by phosphorylation through mTOR (Thr389 on the p70S6K isoform), regulates a number of downstream targets resulting in increased mRNA translation and elongation as well as ribosome biogenesis (81). Phosphorylation of 4E-BP1 results in dissociation of 4E-BP1 from eukaryotic translation initiation factor 4E (eIF4E), enabling binding of translation initiation factors and induction of mRNA translation. Together, activation of 4E-BP1 and p70S6K results in increased protein synthesis. In parallel, mTORC1 is also important for suppression of autophagy, a nutrient-recycling process in which cellular macromolecules and organelles are degraded into their constituent components (88), and induction of mitochondria biogenesis (89). As discussed in the following section, mTORC1 furthermore plays an essential role in regulation of insulin signaling (see 1.2.4 and 1.3.1.2). However, despite these diverse processes controlled by mTOR, only few mTORC1 substrates are known. Analysis of the mTOR-regulated phosphoproteome by quantitative mass spectrometry revealed up to hundred phosphopeptides as regulated by mTOR. These may display new starting points for investigations of mTOR biology (90).

Null alleles of the components of mTORC1, such as mTOR or raptor, or of its upstream regulators, like TSC2, result in very early embryonic lethality, due to the defined role of mTORC1 in growth and cellular physiology (91). In contrast, muscle-specific deletion of raptor or mTOR results in viable animals, which display decreased muscle mass, increased glycogen content and decreased oxidant capacity, predominantly due to decreased mitochondrial biogenesis compared to control animals (92,93).

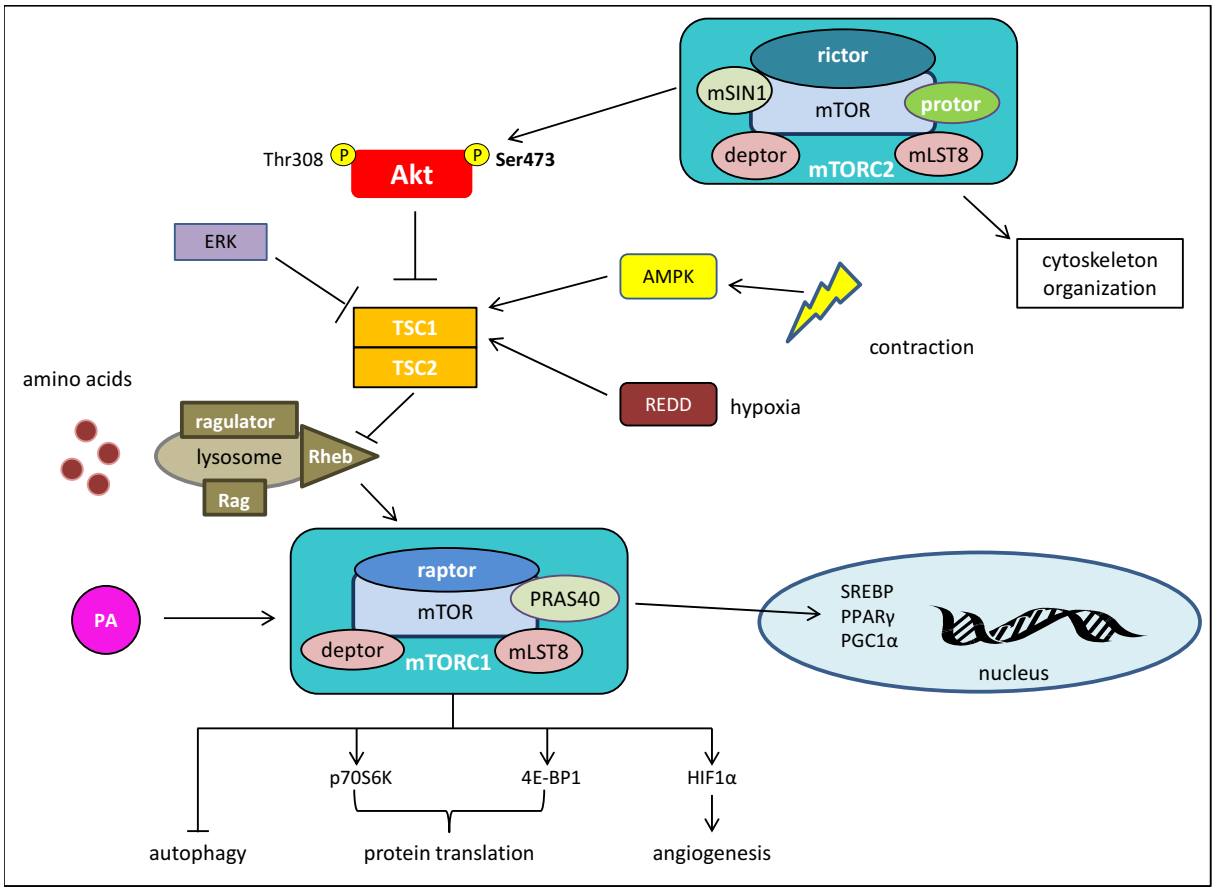

Figure 1.4 Simplified overview of the mTORC1 signaling. The kinase mTOR is a component of two distinct protein complexes; growth factor receptors activate mTORC2, which consists of its regulatory subunit rictor, deptor, mSIN, mLST8 and protor proteins and participate in cytoskeletal remodeling and Akt activation by mediating Ser473 phosphorylation. In contrast to mTORC2, mTORC1 consists of the regulatory subunit raptor, deptor, mLST8 and PRAS40. mTORC1 activity is regulated by many different mechanisms; activated Akt inhibits the GAP-activity of TSC1/TSC2 complex, resulting in increased level of GTP-bound Rheb and activation of mTORC1. TSC2 can further be regulated by ERK kinases, AMPK or REDD proteins, which are activated by growth factors, contraction or hypoxia, respectively. Amino acids enhance mTORC1 activity via recruitment of mTORC1 to the membrane of lysosomes, which is realized by the action of Rag GTPases and ragulator proteins. Once translocated to the lysosomes, mTORC1 interacts with GTP-bound Rheb. An additional regulatory mechanism of mTORC1 signaling involves the lipid second messenger phosphatidic acid (PA). Activated mTORC1 regulates multiple protein substrates, which are for example involved in autophagy and angiogenesis. Two of the best characterized substrates are p70S6K and 4E-BP1, which both regulate translation and protein synthesis. Furthermore, mTORC1 regulates gene expression via activating several different transcription factors, such as sterol regulatory element-binding protein (SREBP), peroxisome proliferator-activated receptor (PPAR)-γ (among others important for regulation of genes involved in lipid synthesis) and PPARγ co-activator 1α (PGC1α) (which increases mitochondrial biogenesis).

1.2.4 Negative regulation of insulin signaling

Several mechanisms have been described for negative regulation of the insulin signaling pathway. These include negative regulation by inhibitory serine/threonine phosphorylation and dephosphorylation of IR by protein tyrosine phosphatases (PTPs). For example, knockout of PTP1B in mice results in increased insulin sensitivity and resistance to diet-induced obesity (94). Another regulator of IR activity is the plasma cell membrane glycoprotein PC-1, which binds the IR α-subunit and inhibits insulin-mediated tyrosine kinase activity (38). Suppressor of cytokine signaling (SOCS) proteins abrogate IR signaling via competition with IRS at the docking sites on the IR (95). Finally, growth factor receptor bound (Grb) proteins 10 and 14 can directly bind IR and inhibit tyrosine activity, resulting in reduced IRS phosphorylation. Grb10- and Grb14-deficient mice both exhibit improved whole-body glucose homeostasis as a consequence of enhanced insulin signaling (96). Additionally, Grb10 was found to induce degradation of the IR via recruitment of ubiquitin ligases (97) and was identified as an mTORC1 substrate (98,99).

IRS activity is highly regulated by serine/threonine phosphorylation at the C terminus of these proteins. This region encodes for more than 70 regulatory residues, which undergo phosphorylation by insulin and other stimuli, like cytokines and free fatty acids. In general, phosphorylation of these serine residues is believed to negatively regulate IRS signaling and is increased in terms of insulin resistance. Again, many of these IRS kinases are downstream elements of insulin signaling, such as mTORC1. Importantly, some serine phosphorylation events also mediate positive effects on insulin signaling. It is believed that these positive serine phosphorylation events allow a correct tyrosine phosphorylation of IRS1 and the propagation of the insulin signal (100).

Downstream of IRS, insulin signaling can be abrogated by action of the protein phosphatase and tensin homolog (PTEN). This protein inhibits PI3K/Akt signaling by dephosphorylating PIP3 at the 3 position. Other enzymes, which regulate insulin signaling via dephosphorylation of the inositol ring, are the SH2-containing phosphatases (SHIP1 and SHIP2). Akt activity can be directly inhibited by binding partners, such as thioesterase superfamily member 4 (THEM4). Furthermore, Akt signaling is terminated by dephosphorylation of Thr308 and Ser473 through the action of protein phosphatase 2 (PP2) and PH domain leucine-rich repeat phosphatase (PHLPP) (64). Tribbles-3 (TRB3) inhibits activation of Akt by binding unphosphorylated Akt and preventing phosphorylation (101).

Importantly, it is getting evident, that inducers of insulin resistance share similar mechanisms to promote insulin resistance. Especially, the hyperactivation of regulatory feedback mechanism on IRS proteins seems to play a pivotal role in reduction of insulin sensitivity. Some of these mechanisms will be discussed in the next section (see 1.3.1).

1.3. Pathophysiology of muscle insulin resistance

The balance and regulation of insulin-mediated signaling pathways is pivotal for the maintenance of glucose homeostasis and is disturbed in patients with T2D, resulting in insulin resistance. The term insulin resistance encompasses both a defect in the ability of insulin to suppress hepatic glucose production and a defect in insulin-responsive glucose uptake (69). In the past decades great effort has been made to identify the molecular processes and causative agents leading to the progression of insulin resistance. In several *in vitro* and *in vivo* studies the crosstalk between adipose tissue and skeletal muscle has been identified as an important factor contributing to the development of insulin resistance. As mentioned previously, insulin resistance in muscle arises long before hyperglycemia becomes evident. Several studies using the gold standard euglycemic hyperinsulinemic clamp technique to quantify insulin sensitivity demonstrated that 80-90% of the infused glucose is taken up by skeletal muscle and that this uptake is markedly reduced (up to 50%) in patients with T2D (102-104). These studies demonstrate that the primary defect with regard to insulin sensitivity in patients with T2D occurs in skeletal muscle. Identifying the molecular mechanism of insulin resistance in muscle as one of the essential defects in T2D will help to prevent and treat T2D.

1.3.1 Mechanism and pathways involved in induction of insulin resistance in skeletal muscle

Defects in insulin signaling, transport and phosphorylation of glucose, glycogen synthesis and glucose oxidation all contribute to muscle insulin resistance (34). Due to the complexity of insulin signaling, molecular mechanisms leading to insulin resistance are pleiotropic, widespread and may occur at any one of the critical nodes of insulin signaling, beginning with activation of the IR and ending with induction of GLUT4 translocation. Considerable evidence has focused on IRS proteins as a major target of insulin resistance under a broad range of conditions. Modification of IRS activity thereby is induced via several mechanisms including protein/protein interactions, inhibitory (hyper)-phosphorylation, tyrosine dephosphorylation, degradation as well as other posttranslational modifications such as O-linked glycosylation (105) and is mainly induced by feedback mechanisms.

1.3.1.1 Inhibitory phosphorylation of IRS1

Phosphorylation of IRS1 has emerged as a key step in the control of insulin signaling both under physiological and pathological conditions. Many inducers of insulin resistance were demonstrated to activate IRS kinases that negatively modulate insulin signaling. In this context, the list of IRS kinases implicated in induction of insulin resistance is increasing rapidly, together with identification of new inhibitory phosphorylation sites within IRS1. As mentioned above, IRS1 kinases frequently are mediators of insulin action, such as p70S6K, MAPK or proteinkinase C-ζ, and induce inhibition of IRS signaling after prolonged insulin stimulation. Another group of IRS kinases are activated via unrelated (predominantly inflammatory) pathways, like c-Jun NH_2-terminal kinase (JNK), inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) or GSK3β (106). Inhibitory serine/threonine (S/T) phosphorylation of IRS can disrupt insulin action at least at three nodes: interaction of IRS with the plasma membrane, interaction of IRS with IR or interaction of IRS with downstream effectors, such as PI3K. Importantly, one stimulus can increase the phosphorylation of many S/T residues resulting in abrogation of signaling at different nodes. The tail of IRS1 includes the majority of the described S/T phosphorylation sites and abrogates interaction of IRS1 with PI3K. However, for example Ser24 phosphorylation within the PH domain, induced by PKC, is linked to a decreased capacity of IRS1 to bind to the plasma membrane (107). Examples for inhibitory phosphorylation residues near the C-terminus of IRS1 are Ser570, induced by PKCζ, or Ser612, Ser632, Ser636, Ser662 and Ser712, which are phosphorylated by MAP kinases (108). In humans, a twofold increase in Ser636 phosphorylation was detected in skeletal muscle cells from patients with T2D compared to lean controls. This increase was accompanied by enhanced ERK activity and inhibition of the MAPK by PD98059 strongly reduced the level of Ser636 phosphorylation (109). However, one of the best-characterized inhibitory phosphorylation sites, Ser307, is located near the PTB domain and can be induced through JNK, IKKβ or mTORC1 (106,110). Phosphorylation of this site disrupts the interaction between IRS1 and IR and is often used as a marker for insulin resistance. Currently, the list of potential IRS kinases includes mTORC1, different isoforms of PKC, JNK, IKKβ, GSK3, MAPK, AMPK, mouse pelle-like kinase (mPLK), G protein-coupled receptor kinases (GRK)-2, salt-inducible kinase (SIK)-2 and Akt (106).

1.3.1.2 mTORC1-mediated feedback mechanism on IRS1

The mTORC1 pathway has long been known to participate in physiological feedback regulation of insulin signaling. However, permanent activation of the mTORC1/S6K signaling in obesity and diabetes has been demonstrated to promote insulin resistance via chronic inhibitory IRS1

phosphorylation. In line with this, rodents fed with a high fat diet (HFD) to induce obesity and insulin resistance demonstrate enhanced basal activity of mTORC1 and S6K, which was accompanied by Ser632 and Ser302 (corresponding to human Ser636 and Ser307) phosphorylation of IRS1 (111). *In vitro* studies further identified Ser270, Ser307, Ser312, Ser636 and Ser1101 as mTORC1/S6K-dependent phosphorylation sites within IRS1 (110). Mice lacking S6K demonstrate reduced basal serine phosphorylation of IRS1 and are protected against diet-induced obesity and insulin resistance (112,113). Conversely, IRS1 is hyperphosphorylated in *TSC2*$^{-/-}$ mouse embryonic fibroblasts (MEFs), which exhibit constitutive mTORC1 and S6K activation (114). Nevertheless, mTORC1 itself can also affect IRS1 independent of S6K. Tzatos et al. demonstrated that raptor directly binds IRS1 and induces mTOR-mediated IRS1 Ser636/Ser639 phosphorylation (115). Given this important role of mTORC1/S6K signaling in insulin resistance, inhibitors of mTORC1 could represent suitable therapeutic options. However, the use of rapamycin to treat insulin resistance revealed controversial results. In a study with 11 healthy men, performed by Krebs et al., rapamycin partially inhibited hyperinsulinemia- and hyperaminoacidemia-mediated increase in mTOR-stimulated S6K phosphorylation and IRS-1 Ser312/Ser636 phosphorylation (116). Conversely, in other studies rapamycin treatment did not improve either glucose tolerance or insulin tolerance in *ob/ob* mice (117) and in muscle of *P. obesus* (118). Further investigations seem to be required to clarify if targeting mTORC1 signaling is appropriate to treat insulin resistance.

One could argue that hyperactivity of mTORC1 in an insulin resistant state, where PI3K/Akt/TSC2-mediated activation of mTORC1 is abrogated, appears paradoxical. However, excess availability of nutrients could explain this paradox. High blood levels of amino acids, as seen in obesity, were shown to induce the hyperactivity of mTORC1, driving the mTORC1/S6K/IRS1 feedback loop and resulting in insulin resistance (119). In addition, amino acid infusion leads to hyperactivation of S6K and is tightly linked to decreased insulin-stimulated glucose metabolism in human skeletal muscle (120). Nevertheless, the mechanism by which mTOR harmonizes signals from nutrients such as amino acids and insulin to affect metabolism in health and disease remains unclear.

1.3.1.3 Impact of the proteasome

Hyperphosphorylation of IRS1 was found to affect its sub-cellular localization and to induce its proteasome-mediated degradation. Predominantly, these pathways are activated in response to prolonged exposure to insulin or specific activation of the mTORC1 feedback loop. Especially, Ser612 and Ser307 phosphorylation of IRS1 was demonstrated to be involved in PI3K signaling-mediated degradation of IRS1 (106,121). H4IIE rat hepatoma cells expressing a IRS-1 mutant in which Ser312 was changed to alanine were found to be resistant to insulin-mediated IRS1 degradation compared

cells expressing wild type IRS1 (122). In line with these results, reduced IRS1 protein abundance is found in animal models of insulin resistance and in adipocytes isolated from T2D patients (122,123). The importance of the PI3K/Akt/mTOR pathway in mediating IRS1 degradation is further demonstrated by using specific inhibitors of this pathway, thus rescuing insulin-stimulated IRS-1 degradation (124,125). Additionally, inhibitors of the 26S proteasome abrogate IRS1 degradation but do not affect inhibitory phosphorylation of IRS1 (125-127). Degradation of IRS1 is believed to be mediated by binding of phosphorylated IRS1 to 14-3-3 adaptor proteins, which relocate IRS1 from low-density microsomes to the cytosol, where the proteasome machinery carries out its function (126,128).

The 26S proteasome is a multicatalytic enzyme complex present in the nucleus and cytoplasm of all eukaryotic cells. Importantly, the proteasome can only target proteins for degradation, which are attached to small peptides, namely ubiquitin. An ATP-dependent cascade of enzymes, called E1, E2 and E3, induces labeling of target proteins with ubiquitin. To this end, ubiquitin molecules are activated by E1 enzymes and transferred to the ubiquitin-conjugating enzymes E2, which in turn presents ubiquitin to E3 enzymes. E3 are ubiquitin-protein ligases, which bind to the target protein and finally transfer ubiquitin to the target protein. Repeating this process several time, results in creating a polyubiquitin chain that labels the protein for degradation (129). Interestingly, while about 20 E2 and more than hundred different E3 enzymes have been identified so far, only one E1 enzyme is known (130). The 26S proteasome consists of a catalytic 20S core element, which is linked to two 19S regulatory subunits. The 20S is a barrel-like particle formed by four rings made up of two outer α-rings and two inner β-rings. The inner β-rings contain three active sites, which differ in their substrate specificity and activity, termed caspase-like, trypsin-like and chymotrypsin-like activity. These active sites are capable of cleaving peptide bonds at the C-terminal side of acidic, basic, and hydrophobic amino acid residues, respectively (131), generating peptides of 3-25 amino acids (129). Each of the two 19S subunits binds polyubiquitin and cleaves it from the target protein. In turn, the target protein is denaturated and inserted into the proteolytic chamber. Thereby these subunits generate ATP to supply energy continuously for the degradation (132).

Even though a direct link between activated proteasome and insulin resistance has been shown in some studies, the involvement of proteolytic protein breakdown in the pathogenesis of diabetes remains unclear. Importantly, not exclusively IRS1 but also other central members of the insulin signaling, such as the IR and Akt, are targeted for proteasomal degradation (133). Investigations of enzymes involved in protein degradation may open up new option for treatment of insulin resistance and metabolic disorders.

As mentioned above, one of the core functions of insulin in skeletal muscle is to promote protein synthesis via mTORC1 and MAPK activation, but also to inhibit protein degradation. FOXO transcription factors have a central role in the regulation of these processes. In conditions of insulin deprivation or insulin resistance, nuclear localization of FOXO activates the transcription of two important muscle specific E3 ligases, muscle RING-finger protein-1 (MuRF1, also known as tripartite motif containing 63 (TRIM63)) and F-box protein 32 (FBXO32, also known as atrogin-1). These two proteins are tightly linked to protein catabolism in skeletal muscle and muscle atrophy. Importantly, excessive loss of skeletal muscle mass was found in older patients with T2D (134). Therefore, the proteasome may not only participate in induction of insulin resistance but also in progression of late consequences in T2D.

1.3.2 Inducers of insulin resistance in skeletal muscle

It is becoming evident that obesity promotes muscle insulin resistance. During high fat feeding, immune cells infiltrate adipose tissue and initiate a proinflammatory cross-talk cycle with adipocytes, resulting in a low-grade inflammation and insulin resistance in adipose tissue. As a result, excess amounts of hormones, proinflammatory cytokines, chemokines and free fatty acids (FFA) are released, which target skeletal muscle in an endocrine manner (135). Some modulators of insulin action are briefly reviewed in terms of their capacity to induce insulin resistance in muscle in this chapter. Importantly, these effectors act simultaneously and synergistically to abrogate insulin action and glucose uptake in skeletal muscle, which results in whole-body insulin resistance.

1.3.2.1 Hyperinsulinemia

Acute insulin stimulation activates a cascade of downstream signaling pathways, whereas persistently elevated insulin levels causes desensitization against insulin in target cells. As mentioned above the initial stage of progression to T2D is characterized by a compensatory increase of insulin secretion from the pancreatic β-cells, resulting in hyperinsulinemia. *In vitro* studies have demonstrated that chronic exposure (24h) to insulin (mimicking hyperinsulinemia) results in down-regulation of insulin-mediated PI3K/Akt and MAPK signaling as well as glucose uptake via degradation of IRS1/IRS2 in 3T3L1 adipocytes and L6 myotubes (136,137). Haruta et al. demonstrated that insulin-induced inhibitory phosphorylation and degradation of IRS-1 are mediated by a rapamycin-sensitive pathway (138), implicating participation of mTOR in hyperinsulinemia-induced insulin resistance. Besides these effects on IRS degradation, continuous exposure to insulin causes

increased internalization and degradation of IR, resulting in reduction in number of receptors exposed on the cell surface. Furthermore, IR tyrosine activity is diminished after chronic insulin stimulation, mainly induced by inhibitory phosphorylation, dephosphorylation by phosphatases or binding of inhibitory molecules (139). It remains unclear whether hyperinsulinemia is a result or a cause of insulin resistance; insulin resistance induces hyperinsulinemia via increasing blood glucose levels due to defects in glucose uptake, thus hyperinsulinemia in turn exacerbates insulin resistance in peripheral tissues. However, basal hyperinsulinemia has also been shown to induce insulin resistance (139).

1.3.2.2 Adipocyte-derived factors

I. Proinflammatory cytokines

In the last decades it has become evident that adipocytes do not only function in the storage of energy but also secrete a variety of hormones, cytokines and chemokines. Several of these factors, also termed adipokines, have been identified to influence insulin action in skeletal muscle.

Tumor necrosis factor-α (TNFα) is produced mainly by macrophages and adipocytes of fat tissue and is an important regulator of immune response, inflammation, cell apoptosis and survival as well as synthesis of other cytokines, like IL-1 and IL-6. Furthermore, it has been identified as an important mediator of obesity-induced insulin resistance (140). While the role of TNFα in humans is still unclear, this adipokine is highly up-regulated in animal models of obesity. Serum concentrations of TNFα were increased in obese women, with reduction of circulating TNFα levels after weight loss (141). Increased TNFα serum levels were also correlated to low insulin sensitivity, independent of BMI (142). Nevertheless, no beneficial effect regarding insulin sensitivity was observed in rheumatoid arthritis patients after long-term inhibition of TNFα (143). However, in rodent and cell culture models the mechanisms by which TNFα induces insulin resistance in muscle were identified in detail and include activation of several different signaling pathways. TNFα-induced signaling involves activation of MAPK, like ERK1/2, p38, and JNK, but also NFκB signaling. In line with this, silencing of IKKβ, using small interfering RNA, prevents TNFα-induced insulin resistance in human skeletal muscle (144). Furthermore, IKKβ was demonstrated to destabilize the TSC1/TSC2 complex, resulting in the activation of mTORC1 (145). As mentioned above all these signaling pathways are involved in inhibitory phosphorylation and degradation of IRS1, resulting in abrogation of insulin action in skeletal muscle. TNFα was also shown to induce caspase-dependent ubiquitination of Akt1 in 3T3L1 adipocytes (146). Moreover, PTP1B$^{-/-}$ mice showed complete protection against TNF-α-induced insulin resistance, indicating that the phosphatase PTP1B is crucial for TNFα signaling (147).

Due to its high abundance and ubiquitous production, monocyte chemoattractant protein-1 (MCP-1) has been the first discovered and most extensively studied human CC-chemokine (148). Adipocytes have been recognized as an important source of MCP-1 and expression of MCP-1 in visceral and subcutaneous adipose tissue as well as circulating levels are increased in obese patients compared to lean controls (149,150). In adipose tissue, amongst others MCP-1 recruits immune cells and regulates adipogenesis (148), but it is also described to induce insulin resistance in adipocytes and peripheral tissues. Interestingly, in a large cohort of Caucasians, a common A/G polymorphism within MCP-1 negatively correlates with MCP-1 plasma levels and decreases the prevalence of T2D (151). In line with this, overexpression of MCP-1 in adipose tissue mimics effects of HFD-induced metabolic disorders, including insulin resistance, whereas MCP-1 deficiency ameliorates insulin resistance in *db/db* mice and in mice fed a HFD (152). The molecular mechanism by which MCP-1 induces insulin resistance in skeletal muscle remains uninvestigated. However, Sell et al. demonstrated that human skeletal muscle cells express the MCP-1 receptor CCR2 and treatment with recombinant MCP-1 increases ERK1/2 activation but does not activate the NFκB pathway (153), indicating that activation of MAPK may contribute to MCP-1-mediated signaling. It remains speculative if activation of MAPK by MCP-1 participates in induction of insulin resistance or if other signaling pathways are involved.

Chemerin is expressed at the highest levels in adipose tissue and liver and functions as a ligand activator for the G-protein coupled receptor chemokine-like receptor 1 (CMKLR1) (154). The highest expression levels of CMKLR1 have been found in macrophages, immature dendritic cells and white adipocytes, but it is also detectable in human skeletal muscle cells (155,156). Besides its function in recruitment of immune cells, chemerin has been identified as a regulator of adipogenesis and adipose metabolism, indicated by experimental data showing that loss of chemerin or CMKLR1 abrogates adipocyte differentiation and modifies expression of metabolic genes (157). In obese and diabetic mice, the expression of chemerin and its receptors are altered in white adipose, skeletal muscle, and liver tissue. Interestingly, administration of exogenous chemerin in these mice exacerbates glucose intolerance, lowers serum insulin levels and decreases tissue glucose uptake (158,159). In humans, several studies have revealed a positive correlation of chemerin serum levels with BMI as well as markers of the metabolic syndrome (155). Elevated chemerin serum levels have been found in patients with T2D (160). Weight loss due to exercise intervention, caloric restriction or bariatric surgery in obese and diabetic subjects resulted in improved glucose tolerance, which was associated with a reduction in chemerin serum levels (161). Additionally, chemerin has been found to be a strong marker for insulin sensitivity in healthy young men (162). *In vitro* chemerin has been shown to induce insulin resistance via activation of p38MAPK, NFκB and ERK1/2, which results in

decreased insulin-stimulated Akt and GSK3 phosphorylation as well as glucose uptake due to increased inhibitory IRS-1 serine phosphorylation (156).

These are three examples of how cytokines and chemokines, secreted by adipose tissue, negatively affect skeletal muscle insulin sensitivity and whole-body glucose metabolism. However, current research has identified over 50 adipocyte-secreted factors, and more are yet to be discovered (163).

II. Anti-inflammatory cytokines

Adiponectin is one of the few adipokines that is down-regulated in obesity and that positively influences insulin sensitivity. Interestingly, adiponectin is the most abundant adipokine in serum, accounting for 0.01% of all serum protein (164) and is involved in a wide variety of physiological processes, including energy metabolism, inflammation and vascular physiology, by acting directly in the liver, skeletal muscle and vascular endothelium (165). Plasma levels of adiponectin are higher in women than in men and are decreased in pathologies such as the metabolic syndrome, insulin resistance and obesity (166). This protein is exclusively expressed in adipocytes and secretion is inversely correlated to adipocyte size. Signaling induced by binding of adiponectin to its receptors AdipoR1, AdipoR2 and T-cadherin, which are expressed in diverse peripheral tissues and organs, leads to activation of AMPK, p38MAPK and PPARα, resulting in increased GLUT4 translocation, inhibition of proinflammatory cytokine production, increased fatty acid oxidation and reduction of reactive oxygen species (ROS) production (165). In line with this pivotal role of adiponectin in regulation of metabolism and whole-body insulin sensitivity, common SNP within the adiponectin or AdipoR1 gene is highly associated with insulin resistance and T2D (167,168).

Recently, secreted frizzled-related protein 5 (Sfrp5) has been described as an anti-inflammatory adipokine that antagonizes the proinflammatory action of the adipokine wingless-type MMTV integration site family member (Wnt) 5a (186). Nevertheless, the role of this protein remains unclear because several controversial data have been published regarding the function and expression of this protein in obesity and metabolic syndrome. While a study in Chinese obese and T2D patients demonstrated reduced Sfrp5 plasma levels, no differences between lean and obese subjects regarding Sfrp5 serum concentration was detectable in other studies (169,170). Furthermore, only a very low gene expression of *Sfrp5* was detectable in human white adipose tissue, doubting that Sfrp5 can really be classified as a human adipokine. However, both up-regulated (171) and down-regulated (172) expression of Sfrp5 has been found in adipose tissue of obese animal models. Ouchi et al. demonstrated that Sfrp5 deficiency in mice resulted in deterioration of high-calorie diet- induced glucose intolerance, hepatic steatosis and macrophage infiltration in adipose

tissue. In contrast, Sfrp5 expression was significantly increased in adipose tissue of obese mice compared to lean controls (171). Conversely, acute administration of Sfrp5 to obese and diabetic mice improved glucose tolerance and adipose tissue inflammation (172), demonstrating an anti-inflammatory and anti-diabetic function of Sfrp5 in mice. In contrast, Carstensen et al. reported a positive association of Sfrp5 with insulin resistance and markers of oxidative stress in subjects with a high risk for T2D (170), indicating that the function of Sfrp5 in humans may differ from findings in mice. These contradictions have to be elucidated in future studies.

Additionally the role of other anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1RA), macrophage inhibitory cytokine-1 (MIC-1) or transforming growth factor-β1 in the context of T2D in human remains unclear. However, the balance between proinflammatory and anti-inflammatory cytokines may be fundamental in the development of insulin resistance.

III. Free fatty acids

Fatty acids are mandatory to store and supply energy for the whole body. However, increased availability of FFA has been demonstrated to enhance the amount of ectopic lipid stores in non-adipose tissues, such as skeletal muscle, heart, liver or pancreas and are believed to play an essential role in the induction of insulin resistance (173). In insulin-sensitive conditions, insulin inhibits lipolysis in adipose tissue after food intake, resulting in decreased FFA levels. This suppression is impaired in insulin resistant adipose tissue, leading to excess release of FFA, which accumulate in skeletal muscle and induce lipotoxicity. Intramyocellular lipids (IMCL) are directly linked to insulin resistance by generating active lipid metabolites, like diacylglycerol (DAG) and ceramides. DAG and ceramides activate different isoforms of PKC, JNK and IKKβ, resulting in inhibitory phosphorylation of IRS1 and IR as well as abrogation of insulin signaling. Thus, insulin-stimulated glucose uptake in skeletal muscle is impaired and, due to IKKβ-mediated NFκB activation, proinflammatory pathways are induced (174). Furthermore, ceramides can activate phosphateses such as PP2A, resulting in dephosphorylation of Akt-Thr308 (175). While diet-induced weight loss has been shown to decrease IMCL content (176), exercise has been reported to increase IMCL content (“athlete´s paradox”) (177), indicating that there might be no direct link between the amount of IMCL and insulin resistance (173). However, FFA can also directly induce insulin resistance. In general, FFA can be classified into different subgroups, such as saturated or unsaturated, short or long-chain as well as essential or nonessential FA. Among these subtypes, saturated long-chain FAs such as palmitate or stearic acids have been reported to be the most potent inducers of insulin resistance (178). This effect is directly induced by binding of saturated FA to Toll-like receptor (TLR)-4 in skeletal muscle, resulting in JNK and IKKβ activation (179). In addition, saturated FA increase ROS production

in skeletal muscle and affect mitochondrial function (178). Lipid infusion in healthy humans results in impaired mitochondrial function, demonstrated by decreased ATP synthesis, oxygen consumption and oxidative phosphorylation (180,181), mainly due to decreased expression of genes involved in mitochondrial biogenesis (like PGC-1) and increased ROS production.

1.4. Proline-rich Akt substrate of 40kDa function in skeletal muscle

The list of identified Akt substrates continues to increase. One of the most recently identified substrates is the proline-rich Akt substrate of 40kDa (PRAS40), which is one of the most prominent proteins in muscle that is recognized by the Akt-substrate antibody (182). PRAS40 is ubiquitously expressed but has first been identified in rat H4IIE hepatoma cells, 3T3L1 adipocytes and HeLa cell nuclear extracts (183-185). Insulin stimulates Akt-mediated phosphorylation of Thr246 within PRAS40 in skeletal muscle, which is reduced in conditions of insulin resistance (182). Furthermore, PRAS40 has been shown to modulate protein synthesis and cell cycle progression in skeletal muscle via affecting mTORC1 activity. PRAS40 knockdown in C2C12 mouse myoblast resulted in decreased protein synthesis and proliferation rate. Furthermore, in the absence of PRAS40, myoblasts were lager in diameter and had a reduced rate of myotube formation (186). However, the impact of PRAS40 on insulin action in skeletal muscle has not been investigated so far.

1.5. Objectives

Although insulin resistance is a key component of several chronic syndromes associated with obesity, such as T2D and metabolic syndrome, the involved factors and their underlying mechanisms are not yet fully elucidated. Therefore, a deeper understanding of signaling pathways involved in the transmission of insulin action could result in a better understanding of the pathophysiology of insulin resistance. Furthermore, identification of new key molecules and processes could reveal new therapeutic targets for the treatment of metabolic disorders. As described in the previous sections insulin action in skeletal muscle includes the activation of a plethora of molecules, which are strictly regulated by several regulatory (feedback) mechanisms. The mTORC1 signaling pathway seems to play a predominant role in normal control of insulin action as well as in pathophysiology of insulin resistance. PRAS40 could be a new mediator of signal transduction from Akt to mTORC1 and may participate the in regulation of insulin signaling. Up to now, the function of PRAS40 is incompletely understood because of conflicting results published so far. However, as PRAS40 is one of the most prominent substrates of Akt in skeletal muscle, characterization of this protein may reveal new

insights into insulin signaling and progression of insulin resistance. Therefore, the aim of this thesis was to specify the function of PRAS40 in insulin action and mTORC1 signaling.

- Current literature about PRAS40 is reviewed to obtain an overview over its protein structure, phosphorylation sites and potential function in health as well as in pathogenesis of metabolic disorders and cancer (**chapter 2**). Special attention was paid on controversial data concerning PRAS40´s role in regulation of mTORC1 activity.

- Due to the fact that the function of PRAS40 in insulin signaling is inadequately defined, one of the aims of this thesis was to investigate whether changes in PRAS40 protein expression impact insulin action and mTORC1 signaling in skeletal muscle. For this purpose, PRAS40 was first silenced using siRNAs (**chapter 3)**. Insulin signaling pathway, glucose uptake as well as mTORC1 signaling was analyzed in control and PRAS40 knockdown cells in normal conditions and following MCP-1- and chemerin-induced insulin resistance. To further characterize the role of PRAS40 in insulin action, PRAS40 wild type and a mutant form of PRAS40, where two phosphorylation sites and the mTORC1 binding site have been mutated, were overexpressed *in vitro* in primary human skeletal muscle cells as well as *in vivo* in *tibialis anterior* of C57BL/6J mice (**chapter 4)**.

- Analysis of the PRAS40 protein sequence yielded that this protein consists of a nuclear export sequence (NES), resulting in cytosolic and nuclear localization of PRAS40. The impact of sub-cellular localization of PRAS40 on insulin and mTORC1 signaling has been completely unknown so far and was therefore analyzed in study 4 (**chapter 5**).

- In the last study (**chapter 6**), the potentially new adipokine Sfrp5 was analyzed regarding its effect on insulin and inflammatory signaling in primary human adipocytes and primary human skeletal muscle cells. Special attention was paid on the phosphorylation status of PRAS40 in these conditions.

CHAPTER 2

Study 1:

Role of PRAS40 in Akt and mTOR signaling in health and disease

Role of PRAS40 in Akt and mTOR signaling in health and disease†

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Abstract

The proline-rich Akt substrate of 40-kDa (PRAS40) acts at the intersection of the Akt- and mammalian target of rapamycin (mTOR) mediated signaling pathways. The protein kinase mTOR is the catalytic subunit of two distinct signaling complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2), which link energy and nutrients to the regulation of cellular growth and energy metabolism. Activation of mTOR in response to nutrients and growth factors results in the phosphorylation of numerous substrates, including the phosphorylation of S6 kinase by mTORC1 and of Akt by mTORC2. Alterations in Akt- and mTOR-activity have been linked to the progression of multiple diseases such as cancer and type 2 diabetes. Although PRAS40 was first reported as substrate for Akt, investigations towards mTOR binding partners subsequently identified PRAS40 both as component and substrate of mTORC1. Phosphorylation of PRAS40 by Akt and by mTORC1 itself results in dissociation of PRAS40 from mTORC1, and may relieve an inhibitory constraint on mTORC1 activity. Adding to the complexity is that gene silencing studies indicate that PRAS40 is also necessary for the activity of the mTORC1 complex. This review summarizes the regulation and potential function(s) of PRAS40 in the complex Akt- and mTOR-signaling network in health and disease.

Introduction

Proline-rich Akt substrate of 40-kDa (PRAS40) was first identified as a 14-3-3 binding protein in lysates from insulin-treated hepatoma cells (1), and is identical to the p39 protein which is phosphorylated in PC12 cells treated with nerve growth factor or epidermal growth factor (2), and the nuclear phosphoprotein Akt1 substrate 1 (AKT1S1) purified from Hela cells (3). Although originally described as substrate for Akt (1), analysis of mammalian target of rapamycin (mTOR) immunoprecipitates identified PRAS40 as component and substrate of the mammalian target of rapamycin complex 1 (mTORC1) (4-7).

In addition to mTOR and PRAS40, the mTORC1 complex consists of regulatory-associated protein of mTOR (raptor), the mammalian ortholog of yeast lethal with Sec13 protein 8 (mLST8, also known as GβL), and DEP-domain containing mTOR-interacting protein (deptor) (8). Within mTORC1, raptor functions as a scaffold through regulating the assembly of the mTORC1 complex, the recruitment of substrates, and directing the subcellular localization (8). Both PRAS40 and deptor exert an inhibitory action on mTORC1 activity (8). Activation of mTORC1, which occurs in response to nutrients and growth factors, results in phosphorylation of both PRAS40 and deptor by mTORC1 (8,9). This leads to dissociation of PRAS40 and deptor from the complex and relieves the inhibitory constraint on its activity (8,9). Through phosphorylation of a rapidly expanding list of protein substrates (10-12), mTORC1 participates in multiple cellular processes, including the regulation of cell size, mRNA translation, ribosome biogenesis, lipid biogenesis, vesicle-mediated transport, autophagy, and mitochondrial function (8,13).

The catalytic subunit of mTORC1, the serine-threonine kinase mTOR, is shared with another large multimeric protein complex, mTORC2 (8). While PRAS40 is absent in mTORC2, this complex shares mTOR, mLST8 and deptor with mTORC1, and further consists of the unique components rapamycin-insensitive companion of mTOR (rictor), mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with rictor (Protor1, also known as PRR5) (8). Activation of mTORC2 in response to growth factors has been linked to the regulation of cell survival and cytoskeletal organization (8). Based on recent advances in the characterization of mTORC2-regulated protein substrates one may presume that the list of cellular functions that can be ascribed to mTORC2 will expand accordingly (10-12). Among the substrates of mTORC2 is the serine-threonine kinase Akt (14). Thus, although PRAS40 is not present in mTORC2, this protein complex participates in the regulation of PRAS40 phosphorylation through its effects on Akt (5,10-12,15).

Alterations in the activity of mTORC1 and mTORC2 mediated signaling contribute to the progression of various diseases, like cancer and type 2 diabetes (8). In the case of type 2 diabetes,

hyperactivation of the mTORC1 substrates S6 kinase and growth factor receptor bound 10 (Grb10) has been linked to abrogation of the insulin signaling pathway regulating glucose metabolism (9-11). Furthermore, glucose-induced hyperphosphorylation of PRAS40 has been implicated in the progression of diabetic nephropathy (16). Deregulated activity of mTOR-signaling further fuels tumorigenesis through the mTORC1-dependent stimulation of cellular growth, cell proliferation, angiogenesis, suppression of autophagy, and the mTORC2-dependent regulation of proliferation, cell survival and nutrient uptake (8,17). These multiple aspects of mTORC1 and mTORC2 signaling pathways have been extensively reviewed by others (8,13,18). Here, we focus on one regulatory component of mTORC1 and downstream target of both mTORC1 and mTORC2, PRAS40. In this review, we summarize the regulation of PRAS40 activity and its potential function(s) in the complex Akt- and mTOR-signaling networks in health and disease.

Expression and structure of PRAS40

The gene for PRAS40, located on human chromosome 19q13.33, encodes 3 transcript variants that differ in their 5'-UTR and result in 256 and 276 amino acid proteins respectively. Although the PRAS40 mRNA and protein show a ubiquitous expression in human, rodent and fly tissues (1,19,20), there is currently no information available related to the expression pattern of the various isoforms. The 276 amino acid protein differs from the 256 amino acid variant in a 20 amino acid extension at the amino terminus. As shown in Figure 2.1, the amino terminal part of PRAS40 contains two proline-enriched stretches with an as yet undefined function as well as sequences that have the potential to bind proteins containing Src homology 3 and/or WW-domains (1). The proline-rich regions are followed by two short motifs implicated in mTORC1-binding, i.e. an mTOR signaling (TOS) motif (amino acids 129-133 of the human 256 amino acid PRAS40 protein) (4,21,22), and a Lys-Ser-Leu-Pro sequence (amino acids 182-185) showing resemblance to the RAIP motif, which has been named after a short amino acid sequence found in 4EBP1 (23). Furthermore, sequence analysis of the protein identifies a 10 amino acid stretch in the carboxy-terminal part of PRAS40 (amino acids 218-227 of the human 256 aa protein) which matches the consensus sequence for a leucine-enriched nuclear export sequence (NES), Leu-xx(x)-[Leu,Ile,Val,Phe,Met]-xx(x)Leu-x-[Leu,Ile] (9). Finally, phosphorylation of PRAS40 on multiple residues, including Ser88, Ser92, Ser116, Ser183, Thr198, Ser202, Ser203, Ser211, Ser212, Ser221, and Thr246, has been reported (1,4,10-12,24).

Conservation of PRAS40

PRAS40 is highly conserved in higher species (9). Homologues almost identical to the human protein have been found amongst other species including Pan troglodytes, Bos taurus, Mus musculus, and Rattus norvegicus (9). In these species, also the longer forms with an extension at the aminoterminus have been found. All phosphorylation sites are fully conserved in the PRAS40 variants in these higher species (Figure 2.1) (9). In Xenopus laevis, Danio rerio, and Drosophila melanogaster proteins which are identical to the carboxyterminal part of human PRAS40, but entirely lack the proline-enriched stretches found in the aminoterminal PRAS40 proteins from higher species, have been found (6,9,20). The proteins found in these species show conservation of the TOS- and RAIP- motifs as well as of the phosphorylation sites equivalent to the human residues Ser183, Ser221, and Thr246 (Figure 2.1) (9). Notably, the Drosophila melanogaster protein dPRAS40 was originally identified as CG10109, a gene associated with the mutant *Lobe* phenotype (6,20,25). However, a loss of the genomic region containing the Lobe-allele (L^{rev6-3}) is embryonic lethal and linked to a disturbed development of the ventral eye (25-27). In contrast, flies deficient in the dPRAS40 gene are viable and show no alterations in eye development compared to controls (20). Furthermore, CG10109/dPRAS40 expression did not rescue viability in the L^{rev6-3} strain (20). This indicates that the phenotypes associated with a loss of the Lobe genomic region can not be ascribed to dPRAS40. Importantly, dPRAS40 is a component of the TORC1 complex in this species (20). Thus, it seems appropriate to consider the proteins lacking the aminoterminal proline-rich part found in lower species as PRAS40 homologues. Finally, one report describes weak similarities between human PRAS40 and the dauer or aging overexpressing protein family member 5 (dao-5) protein from Caenorhabditis elegans (7). However, dao-5 seems to lack preservation of the important regulatory features found in PRAS40 from higher species. Therefore, further studies seem required whether PRAS40 is also found in this species.

Binding partners

PRAS40 interacts with 14-3-3 proteins, and the mTORC1 complex (9,28).

Interaction with 14-3-3 proteins

Using GST-pull down and far Western assays as well as co-immunoprecipitation experiments, PRAS40 has been found to interact with 14-3-3 proteins (1,2,21,24,29). Pretreatment of cells with PI3K inhibitors prevented insulin-induced phosphorylation of PRAS40 on Thr246 and 14-3-3 binding to PRAS40 (1). Accordingly, replacement of Thr246 by alanine resulted in a loss of the ability of

PRAS40 to bind 14-3-3 proteins (21,24). Importantly, the interaction between PRAS40 and 14-3-3 proteins is also dependent on amino acids (2). However, the induction of PRAS40-Thr246 phosphorylation by insulin is not prevented by amino acid deprivation. Therefore, additional mTORC1-mediated phosphorylations have been implicated in the formation of PRAS40/14-3-3 complexes (2,21). Indeed, rapamycin partially blocks the interaction between PRAS40 and 14-3-3 proteins (2,21). Also silencing of raptor substantially impaired the insulin-induced binding of PRAS40 to 14-3-3 proteins (21). Accordingly, insulin failed to promote 14-3-3 binding to PRAS40 mutants in which the mTORC1 phosphorylation sites Ser183, and Ser221 were substituted by alanines (21,24). Except for Ser212, which is not required for 14-3-3 binding (24), the involvement of the additional phosphorylation sites in PRAS40 remains to be investigated. Consistent with the idea that mTORC1-mediated phosphorylation of PRAS40 participates in the regulation of 14-3-3 binding, both mutation of Pro185 in the putative RAIP-motif as well as of Phe129 in the TOS-motif markedly impaired 14-3-3 binding to PRAS40 (21,24).

Because overexpression of 14-3-3 proteins relieves the inhibitory action of PRAS40 on mTORC1 activity, it has been proposed that the binding of 14-3-3 proteins to phosphorylated PRAS40 serves to sequester PRAS40 away from mTORC1 (7). However, activation of mTORC1 by phorbol esters can occur independent of PRAS40 binding to 14-3-3 proteins (29). Therefore, additional studies seem required to assess whether 14-3-3 proteins are necessary for the activation of mTORC1 by other factors, like nutrients and growth factors. Furthermore, at least seven different 14-3-3 proteins have been characterized in humans. It remains to be addressed whether these variants impact on PRAS40 binding and possibly regulation of mTORC1 activity.

Interaction with mTORC1

The recent elucidation of the structure of the entire mTORC1 complex supports previous biochemical observations that PRAS40 interacts with mTORC1 through the binding to substrate binding site of raptor (4-7,21,22,30). A recent report also demonstrated an association between dPRAS40 and raptor in Drosophila melanogaster (20). The interaction between PRAS40 and raptor is weakened by insulin and to a lesser extent by amino acids, indicating that phosphorylation of PRAS40 results in dissociation of PRAS40 from mTORC1 (6,7,21,22,31). The dissociation of PRAS40 would then allow the binding and activation of mTORC1 substrates like S6K1 and 4EBP1 to the substrate binding site of raptor. Critical functions for the interaction between PRAS40 to raptor have been ascribed to the TOS- and RAIP-motif, as well as the region located between amino acids 150 and 234 of PRAS40 (4,7,21,22). It should be noted that some reports also implicate the kinase domain of

mTOR in the binding of PRAS40 to mTORC1 (5,7,31). In cells overexpressing mutant forms of mTOR with a deletion or inactivation of the carboxyterminal kinase domain, the amount of PRAS40 in mTOR immunoprecipitates decreased (5,7). Furthermore, phoshorylation mimicking substitutions of two newly identified phosphorylation sites within the mTOR kinase domain, Ser2159 and Thr2164, reduced, while substitution of these amino acids by alanine enhanced the presence of raptor and PRAS40 in mTOR immunoprecipitates (31). It seems likely that these findings can be explained by the identification of the carboxyterminal domain of mTOR as raptor binding domain (30).

Regulation of PRAS40 phosphorylation

Phosphorylation of PRAS40 on multiple residues critically determines the function of the protein because it promotes the binding to 14-3-3 proteins and weakens the interaction with mTORC1. Stimuli that enhance PRAS40 phosphorylation include growth factors, such as insulin, NGF and PDGF, as well as nutrients, such as glucose and amino acids (9,28). Although PRAS40 is phosphorylated on multiple sites, only the pathways regulating Ser183 and Thr246 phosphorylation are well characterized because of the availability of commercial antibodies recognizing these phosphorylated residues.

Phosphorylation of PRAS40-Thr246 by Akt-dependent and –independent pathways

As shown in Figure 2.2, the major kinase promoting PRAS40-Thr246 phosphorylation is Akt. The activation of Akt in response to insulin involves the binding of insulin to the insulin receptor. This leads to tyrosine phosphorylation and activation of the insulin receptor itself and of multiple substrates, such as the insulin receptor substrate (IRS) proteins (32). The tyrosine phosphorylated IRS-proteins recruit phosphatidylinositol-3'-kinase (PI3-kinase) to the plasma membrane, where activated PI3K phosphorylates phosphatidylinositol-4,5-biphosphate to form phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3) (32). PI-3,4,5-P_3 binds to the pleckstrin homology domains of phosphoinositide-dependent kinase 1 (PDK1) and Akt, and mediates the phosphorylation of Akt on Thr308 by PDK1 (32). Studies in cultured cell lines show that the induction of PRAS40-phosphorylation on Thr246 is prevented by PI3K inhibition (1,19,33). Furthermore, the platelet-derived growth factor-induced phosphorylation of PRAS40 on Thr246 is almost completely abrogated in embryonic fibroblasts lacking both Akt1 and Akt2, while inducible activation of Akt alone is sufficient to promote PRAS40-Thr246 phosphorylation in NIH3T3 fibroblasts (1). In line with the regulation of PRAS40-Thr246 phosphorylation by the PDK1/PI3K/Akt-pathway, tumor cells harboring

constitutively active mutants of PI3K or Akt, or a loss of phosphatase and tensin homolog (PTEN), which dephosphorylates PI-3,4,5-P_3, display elevated levels of Thr246 phosphorylated PRAS40, which can be lowered by PDK1 or Akt inhibitors (34-38). For phosphorylation of most downstream substrates, Akt requires phosphorylation of Ser473 by mTORC2 (Figure 2.2). This also applies to PRAS40, since inhibition of mTORC2, either pharmacologically or through silencing of rictor, reduces the phosphorylation of PRAS40 on Thr246 (5,10,11,15). Other stimuli promoting PRAS40-Thr246 through the Akt-pathway include glucose and ceramide 1-phosphate (39,40).

Although Akt is the major pathway regulating PRAS40-Thr246 phosphorylation, PRAS40 can also be phosphorylated on Thr246 by Akt-independent mechanisms in tumor cells (41). Accordingly, the proto-oncogene PIM1 promotes phosphorylation of Thr246 in *in vitro* kinase assays and following enforced expression of PIM1 in myeloid factor-dependent cell progenitors cells (42). In the heart, leucine was found to promote PRAS40-Thr246 phosphorylation via a pathway that requires PI3K and PDK1, but was independent of Akt (43). PDK1 is a key regulator of the so-called AGC-kinases. Although a role for S6K1, protein kinase A, and protein kinase C in the regulation of leucine-mediated PRAS40-Thr246 was excluded in this report, these findings implicate a role for members of the AGC protein kinase family in the phosphorylation of PRAS40-Thr246. In support of this, protein kinase A has been linked to the induction of PRAS40-Thr246 phosphorylation in thyroid cells response to thyroid hormone treatment and elevation of intracellular cAMP levels (44).

Phosphorylation of PRAS40-Ser183 by mTORC1

Phosphorylation of PRAS40-Ser183 in cultured cells is promoted by insulin and amino acids, and blunted by wortmannin, rapamycin, glucose withdrawal and amino acid starvation (4,33). Furthermore, insulin infusion was found to enhance PRAS40-Ser183 phosphorylation in human skeletal muscle as well as in rat cardiac and skeletal muscle (33). So far, only mTORC1 has been identified as upstream regulator of PRAS40-Ser183 phosphorylation (24). Insulin-induced activation of mTORC1 occurs via binding of the GTP-bound form of Rheb to mTORC1. The intracellular levels of Rheb-GTP are regulated by the Akt-mediated phosphorylation of tuberous sclerosis complex 2 (TSC2) (Figure 2.2). This leads to inactivation of the TSC-complex, which acts as a GTPase activating protein on Rheb, and thus an increase in Rheb-GTP levels. The amino acid mediated activation of mTORC1 may not involve Rheb directly. However, amino acids are indispensable for the activation of mTORC1 by insulin and other growth factors and serve to bring mTORC1 in the vicinity of Rheb-GTP, which is localized in the lysosomal membrane (8,45). The recently identified “Rag-Ragulator” complex plays a key role in recruiting mTORC1 to the lysosomal membrane (45). The “Ragulator”complex, consisting

of MP1, p14 and p18, targets the Rag-GTPase complex, consisting of RagA/B and RagC/D, to the lysosomal membrane (45). Amino acids increase the intracellular levels of RagA/B-GTP thereby facilitating the interaction between RagC/D and raptor and thus the activation of mTORC1 (45) (Figure 2.2).

Phosphorylation of PRAS40 on other residues by mTORC1

Beside inducing PRAS40-Ser183, and -Thr246 phosphorylation, phosphopeptide mapping of HEK293 cells metabolically labeled with [^{32}P]orthophosphate revealed that insulin also promotes the phosphorylation of PRAS40 on Ser183, Ser202, Ser203, Ser212 and Ser221 (24). Phosphorylation of these amino acids was also induced in *in vitro* kinase assays on raptor immunoprecipitates, suggesting that mTORC1 mediates these additional phosphorylations (24). In this report, only the insulin-mediated phosphorylations on Ser183 and Ser221 were found to be sensitive to rapamycin (24). Accordingly, phosphoproteomic screens aimed at characterizing mTORC1- and mTORC2-regulated phosphoproteins on insulin-treated HEK-cells showed that rapamycin downr-egulated the phosphopeptide corresponding to PRAS40-Ser183, and that the dual mTORC1/mTORC2-inhibitor Torin-1 prevented the insulin-mediated phosphorylation of Ser183, and Thr246 (11). In contrast, phosphoproteomic analysis of rat livers following fasting and re-feeding showed that rapamycin down-regulated the phosphorylation of the sites corresponding to Ser183 as well as Ser202, Ser203, Ser211, and Ser212 of human PRAS40 (12). Furthermore, in mouse embryonic fibroblasts lacking an upstream activator of mTORC1, TSC2, the phosphopeptides corresponding to Ser88, Ser92, S202, Ser203, Ser212, Ser183, and Thr198 of human PRAS40 were down-regulated by rapamycin (10). In another report, Torin-1 down-regulated the phosphorylation of Ser183, Thr246, Ser202, and Ser212 in wild type embryonic fibroblasts, but up-regulation of these phosphorylation sites in cells lacking TSC2 could not be confirmed (11). Finally, in wild type embryonic fibroblasts, insulin-induced phosphorylation of Ser88, Ser92, Ser202, Ser203, Ser212 and Thr246 was down-regulated by a dual mTORC1/mTORC2-inhibitor Ku0063794 (10). Thus, although these studies identify mTORC1 as potential regulator of the additional phosphorylation sites on PRAS40, further studies toward their regulation, for example by using antibodies recognizing these phosphorylated residues seem required at least to clarify some of the discrepancies that emerged in the phosphoproteomic studies.

Cellular function of PRAS40

Effects on mTORC1 activity

Knockdown of PRAS40 has been reported to increase the basal phosphorylation of the mTORC1 substrates S6K and 4EBP1 in various cell types, including mouse embryonic fibroblasts, HT-29, HeLa, HepG2, HEK293, mesangial cells, and 3T3L1 adipocytes (4,6,7,39). Similarly, silencing dPRAS40 increased S6K phosphorylation and cell diameter in cultured Drosophila cells (6). In line with these observations, overexpression of PRAS40 blunted the insulin-mediated phosphorylation of the mTORC1 substrates S6K and 4EBP1 in cultured 3T3L1 adipocytes and HEK293 cells (4,6,21,22), and reduced the cell size of HEK293T cells and rat embryonic fibroblasts (7). Furthermore, tissue-specific overexpression of dPRAS40 led to tissue undergrowth which could be ascribed to reduced cell size rather than apoptosis, while ubiquitous overexpression of dPRAS40 reduced the size of the entire animal and caused pupal lethality (20). Collectively, these findings support the idea that PRAS40 acts as a negative regulator of mTORC1 activity and that the phosphorylation-dependent dissociation of PRAS40 from raptor relieves an inhibitory constraint on mTORC1 activity.

Yet, other reports argue against this proposed function of PRAS40 in mTORC1 activation. For example, the dissociation of PRAS40 from mTORC1 has been found to promote 4EBP1 binding to raptor, but not to affect basal or insulin-mediated S6K- and 4EBP1-phosphorylation in 293E cells (46). Also in C2C12 myoblasts, but not in differentiated C2C12 myotubes, knockdown of PRAS40 did not affect basal or insulin-like growth factor 1 induced phosphorylation of the mTORC1 substrates S6K and 4EBP1, despite reducing protein synthesis and increasing cell diameter (47). Finally, there are reports indicating that PRAS40 is essential for mTORC1 activity. In HEK293 cells, silencing PRAS40 was found to impair the amino acid and insulin-mediated phosphorylation of 4EBP1 and the S6K-substrate ribosomal protein S6 (21). In line with these findings, one study demonstrated that silencing PRAS40 led to increased AMP kinase mediated phosphorylation of TSC2, which results in inhibition of mTORC1 (48).

A recent report on Drosophila shed light on these seemingly contrasting findings regarding the function of PRAS40 in mTORC1 signaling (20). Deficiency of dPRAS40 did not impact on TORC1 during the growth of the animal. However, in contrast to the situation in larvae, the absence of dPRAS40 led to a dramatic elevation in basal S6K-phosphorylation in the ovaries of adult flies, but not in the rest of the animal (20). The authors ascribed these tissue-specific effects of dPRAS40 to alterations in the post-translational modification of dPRAS40 in the ovary as compared to the rest of the animal (20). Furthermore, the levels of phosphorylated S6K in the ovaries of dPRAS40-deficient

females could not be further enhanced upon activation of the PI3K pathway (20). These molecular alterations were accompanied by increased fertility since dPRAS40-deficient females had larger ovaries and laid more eggs as compared to controls. Importantly, removing one copy of the S6K gene in dPRAS40-deficient females normalized fertility, thereby indicating that the main physiological function of dPRAS40 is to act as an inhibitor of the TORC1 pathway (20). In addition, these findings suggest that post-translational modification, such as phosphorylation, may be critical for the activity of PRAS40 towards mTORC1. Therefore, to clarify the function of PRAS40 in more detail in mammalian cell types, experiments using mutant forms of PRAS40 in addition to silencing of PRAS40 should be considered.

Effects on insulin signaling

As for the effects on mTORC1 activity, contrasting data have also been reported for a regulatory role of PRAS40 in insulin action. In 3T3L1 adipocytes and HepG2 cells, silencing PRAS40 resulted in a decreased insulin-mediated phosphorylation of Akt (7). These results were ascribed to increased phosphorylation of S6K1 which via the induction of serine phosphorylation of IRS1 resulted in degradation of IRS1 and insulin resistance. Decreased IRS1 expression and reduced basal phosphorylation of Akt were also observed following PRAS40 knockdown in C2C12 myoblasts (48). However, in these studies, these effects were paralleled by a reduced mTORC1 activity. In contrast, in HEK293 cells neither overexpression nor silencing PRAS40 affected the phosphorylation of Akt in response to insulin. Thus, as for the effects of PRAS40 on mTORC1 signaling, also the potential regulatory role in insulin action requires further analysis.

Effects on apoptosis and cell cycle progression

Overexpression of PRAS40 reduced neuronal apoptotic cell death in mice after transient focal cerebral ischemia (49,50), and enhanced the survival of motor neurons after spinal cord injury in rats (51). These neuroprotective effects were accompanied by an increased binding of phosphorylated PRAS40 to 14-3-3 proteins (51). Furthermore, inhibition of PI3-kinase increased apoptosis of motor neurons following spinal cord injury (51). Silencing PRAS40 increased apoptosis and lowered cell viability in melanoma cells, and reduced tumor development in mice by increasing apoptosis levels rather than altering the proliferation rates in melanoma tumors (52). In contrast to these studies, one report ascribes a pro-apoptotic function to PRAS40, as silencing of PRAS40 was found to prevent the induction of apoptosis in HeLa cells in response to tumor necrosis factor α or cyclohexamide

treatment (5). Finally, silencing PRAS40 in C2C12 myoblasts had no effect on apoptosis, but reduced the number of cells and the rate of proliferation due to an increased number of cells remaining in the G1-phase (47).

Dysregulation of PRAS40 phosphorylation in disease

Cancer

Human cancers frequently show a sustained activation of PTEN/PI3-kinase/Akt- and mTORC-mediated signaling pathways (8,34). Consequently, elevated PRAS40-Thr246 phosphorylation has been reported in several cancer cell lines as well as in meningiomas and malignant melanomas (28,41,52-54). Furthermore, phosphorylation of PRAS40-Thr246 has been used as biomarker for evaluating the effects of novel inhibitors targeting components of PTEN/PI3-kinase/Akt- and mTORC-mediated signaling pathways in human cancer. In this respect, multiple studies showed that phosphorylation PRAS40-Thr246 state could predict hyperactivation of the PTEN/PI3-kinase/Akt-pathway in multiple cancer cell types as well as their sensitivity to inhibitors of components of these signaling pathways (17,34,37,55). Furthermore, PIM1-mediated hyperphosphorylation has been reported in radiation-resistant non-small cell lung cancer cells (56).

Insulin resistance

Insulin resistance in rodent models and type 2 diabetes is characterized by a reduced insulin-mediated activation of the PI3-kinase/Akt pathway regulating amongst other glucose metabolism. Accordingly, the induction of PRAS40-Thr246 phosphorylation by insulin in reduced in target tissues for insulin action from rodent models of insulin resistance, such as adipose tissue, skeletal muscle, the liver and the heart (19,57,58). *In vitro*, exposure of rat soleus muscle or fibroblasts to palmitate was found to reduce insulin-mediated PRAS40-Thr246 phosphorylation (33,59). Conversely, enhancing insulin sensitivity by weight loss through a very low calorie diet improved the induction of PRAS40-Thr246 phosphorylation in skeletal muscle following hyperinsulinemia in obese patients with type 2 diabetes (60).

Diabetic nephropathy

Hyperglycemia contributes to the development of diabetic nephropathy amongst others via the induction of hypertrophy of the mesangial cells in the kidney. Increased phosphorylation of PRAS40 was found to associate with renal hypertrophy in streptozotocin-induced diabetes in rats (39). *In vitro* studies showed that high glucose promoted the phosphorylation of PRAS40 in mesangial cell via activation of Akt that could be ascribed to reductions in PTEN expression resulting from the induction of microRNA 21 as well as elevated expression of the proto-oncogene DJ-1 (16,61). Silencing PRAS40 was found to mimic the effects of high glucose on hypertrophy in mesangial cells, suggesting that inactivation of PRAS40 by glucose-mediated phosphorylation could participate in the development of renal cell pathologies in patients with diabetes (39).

Concluding remarks

PRAS40 is among the most prominent Akt- and mTORC1-substrates being phosphorylated in response to nutrient and growth factor stimulation in eukaryotic cells. Consequently, phosphorylated PRAS40 has emerged as a robust biomarker for pathological conditions associated with alterations in Akt- and mTORC1-activity as well as effectiveness for inhibitors of these pathways. Intriguingly, the cellular function of PRAS40 has not been completely elucidated in mammalian cells. Yet, genetic evidence from Drosophila supports the concept that PRAS40 functions as a regulator of TORC1 signaling. Importantly, the observed regulation of TORC1 activity by dPRAS40 was tissue-specific and seemed to be determined by tissue-specific differences in the post-translational modification of dPRAS40. Since most studies toward the function of PRAS40 in mammalian cells have been performed by silencing the PRAS40 gene, studies employing overexpression of mutant forms of the protein could be fruitful to clarify the function of PRAS40 in further detail. Furthermore, the results obtained in flies indicate that the generation of mouse models with a (tissue-specific) PRAS40-deficiency or -overexpression could be a promising tool to gain further insight into the function of this protein acting at the intersection of the Akt- and mTORC1-signaling pathway.

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Figures

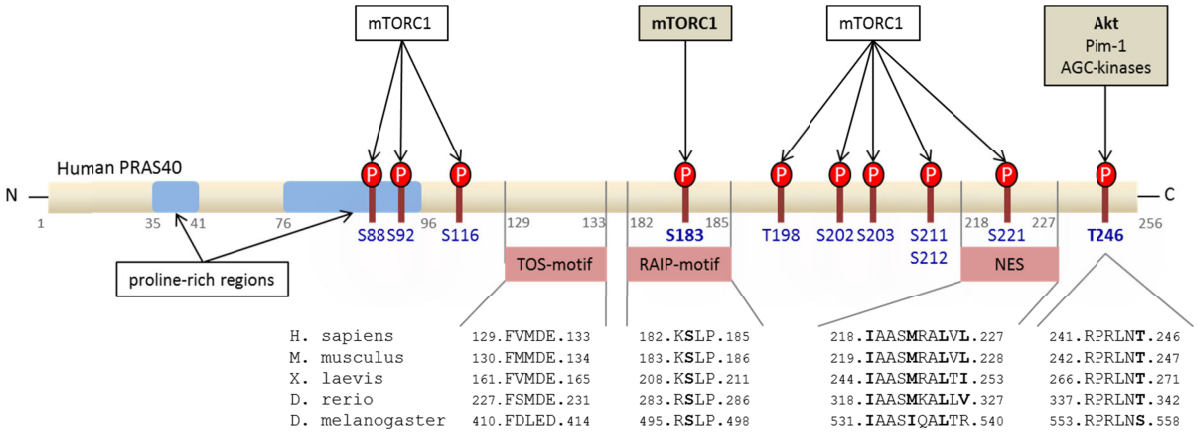

Figure 2.1 Primary structure and conservation of key regulatory regions in PRAS40. The 256 amino acid human PRAS40 protein consists of two proline-rich regions, followed by two short sequences implicated in mTORC1-binding, i.e. the TOS- and the RAIP-motifs, and a stretch that resembles the consensus sequence for a nuclear export signal (NES). The red P's indicate the amino acids that can be modified by phosphorylation by the indicated kinases. The pathways marked in bold have been validated using phospho-specific antibodies. The lower part of the figure shows sequence alignments of the key regulatory regions of the human PRAS40 protein (AKT1S1; NP_001092102) and the AKT1S1/PRAS40 proteins reported in Mus musculus (NP_080546), Xenopus laevis (NP_001084778), Danio rerio (XP_692511) and Drosophila melanogaster (NP_524787).

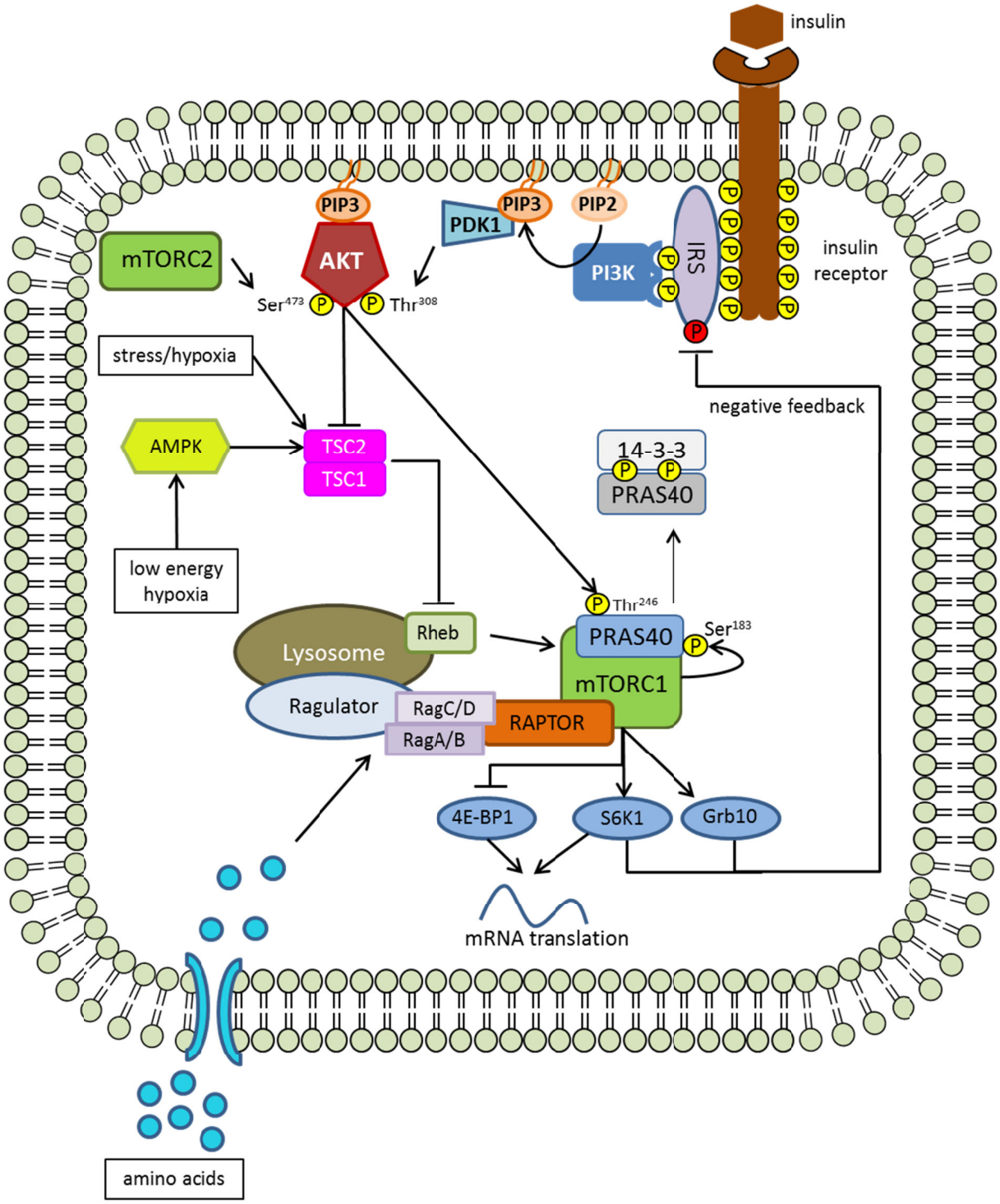

Figure 2.2 Regulation of PRAS40 phosphorylation by Akt and mTORC1. The binding of insulin to the insulin receptor results in activation of the insulin receptor and the induction of tyrosine phosphorylation of the receptor itself and of the insulin receptor substrate (IRS) proteins. The tyrosine phosphorylated IRS-proteins recruit phosphatidylinositol-3'-kinase (PI3K) to the plasma membrane, where activated PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) to form phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 binds to the pleckstrin homology domains of phosphoinositide-dependent kinase 1 (PDK1) and Akt, and mediates the phosphorylation of Akt on Thr308 by PDK1. Phosphorylation of Akt-Ser473 is mediated by mTORC2. Activated Akt then promotes the phosphorylation of Thr246 on PRAS40. In addition, Akt inhibits the activity of the

TSC1/TSC2 complex, which results in increases in the levels of GTP-bound Rheb and activation of mTORC1. Activated mTORC1 phosphorylates multiple protein substrates, including PRAS40, 4EBP1, S6K1 and Grb10. Phosphorylation of PRAS40 results in 14-3-3 binding and dissociation of the mTORC1 complex. Phosphorylation of 4EBP1 and S6K1 regulates amongst others mRNA translation. Furthermore, both activation of S6K1 and Grb10 have been linked to inhibition of the insulin signaling pathway. Other stimuli regulating the activity of the mTORC1 complex and thereby PRAS40 phosphorylation on Ser183 include amino acids. Amino acids act on the Rag proteins, thereby recruiting the Ragulator-Rag-protein complex, which is localized to the lysosome as is Rheb, to raptor, thus allowing the activating of mTORC1. In contrast to growth factors, cellular stresses, like hypoxia and energy deprivation promote the activity of the TSC1/TSC2-complex via AMPK-dependent and –independent phosphorylations of TSC2 (45), thus resulting in inhibition of the mTORC1-pathway.

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CHAPTER 3

Study 2:

Knockdown of PRAS40 inhibits insulin action via proteasome-mediated degradation of IRS1 in primary human skeletal muscle cells

Knockdown of PRAS40 inhibits insulin action via proteasome-mediated degradation of IRS1 in primary human skeletal muscle cells †

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Abstract

AIMS/HYPOTHESIS**:** The proline-rich Akt substrate of 40kDa (PRAS40) is a component of the mammalian target of rapamycin-complex 1 (mTORC1) and among the most prominent Akt-substrates in skeletal muscle. Yet, the cellular functions of PRAS40 are incompletely defined. This study assessed the function of PRAS40 in insulin action in primary human skeletal muscle cells (hSkMC).

METHODS: Insulin action was examined in hSkMC following siRNA-mediated PRAS40 silencing under normal conditions and following chemokine-induced insulin resistance.

RESULTS: Knockdown of PRAS40 (PRAS40-KD) in hSkMC decreased insulin-mediated phosphorylation of Akt by 50% (p<0.05) as well as of the Akt-substrates glycogen synthase kinase 3 (40%) and tuberous sclerosis complex 2 (32%) (both p<0.05). Furthermore, insulin-stimulated glucose uptake was reduced by 20% in PRAS40-KD myotubes (p<0.05). Exposing PRAS40-KD myotubes to chemokines caused no additional deterioration of insulin action. PRAS40-KD further reduced insulin-mediated phosphorylation of the mTORC1-regulated proteins p70S6K (47%), S6 (43%), and 4E-BP1 (30%), as well as expression of growth factor receptor bound protein 10 (Grb10) (35%) (all p<0.05). The inhibition of insulin action in PRAS40-KD myotubes associated with reductions in insulin receptor substrate 1 (IRS1) protein levels (60%) (p<0.05), and was reversed by pharmacological proteasome inhibition. Accordingly, expression of the E3-ligases atrogin-1 and Muscle RING-finger protein-1 (MuRF1) and activity of the proteasome was elevated in PRAS40-KD myotubes.

CONCLUSION: Inhibition of insulin action in PRAS40-KD myotubes was found to associate with IRS1 degradation promoted by increased proteasome activity rather than hyperactivation of the p70S6K negative feedback loop. These findings identify PRAS40 as a modulator of insulin action.

Keywords

chemokines; insulin resistance; IRS1; mTOR; PRAS40; skeletal muscle

Abbreviations

4E-BP1, eukaryotic elongation 4E-binding protein 1; αMEM, α-modified Eagle's medium; Grb10, growth factor receptor binding protein 10; hSkMC, human skeletal muscle cells; IRS1, insulin receptor substrate 1; mTORC1, mammalian target of rapamycin complex 1; MCP-1, monocyte chemoattractant protein 1; MuRF1, Muscle RING-finger protein-1; p70S6K, p70S6 kinase; PI3K**,** phosphatidylinositol-3'-kinase; PRAS40, proline-rich Akt substrate of 40 kDa; TSC2, tuberous sclerosis complex 2

Introduction

The nutrient sensor mammalian target of rapamycin complex 1 (mTORC1) is a key regulator of multiple anabolic responses (1-3). Activation of mTORC1 amongst others enhances the synthesis of proteins and lipids, promotes mitochondrial function, and inhibits autophagy (1-3). In rodent models of obesity and hyperinsulinemia, hyperactivation of mTORC1 signalling in the liver and skeletal muscle has been linked to inhibition of insulin signalling (4-8). This involves the induction of serine phosphorylation of insulin receptor substrate 1 (IRS1) by the mTORC1 substrate p70S6 kinase (p70S6K), which may result in degradation of IRS1 and thereby inhibits the insulin-mediated activation of the phosphatidylinositol-3'-kinase (PI3K)/Akt-pathway (1-3). In addition, stabilization of the expression of growth factor receptor binding protein 10 (Grb10) by mTORC1 mediated phosphorylation has been linked to inhibition of insulin action at the level of the insulin receptor (9-11).

Disturbances in the insulin-mediated activation of the IRS1/PI3K/Akt-pathway, which amongst others facilitates the GLUT4-dependent glucose disposal in skeletal muscle, characterize insulin resistance (12). The impaired activity of the IRS1/PI3K/Akt-pathway also results in reduced insulin-mediated phosphorylation of one of the most prominent Akt-substrates, the proline-rich Akt substrate of 40 kDa (PRAS40), in skeletal muscle of humans with type 2 diabetes and high-fat diet fed rodents (13-15). PRAS40 is a component of the mTORC1 complex (16,17). Yet, studies toward the function of this protein within the mTORC1 complex have yielded conflicting results (18-23). Silencing and overexpression studies mostly in immortalized cultured cell lines have ascribed both inhibitory and stimulatory functions to PRAS40 in the regulation of mTORC1 activity (18-23). Importantly, a recent study conducted in D. melanogaster shed light on these seemingly contrasting findings, and could demonstrate that dPRAS40 acts as an inhibitor of TORC1 signalling in a tissue-specific way and dependent on post-translational modification of the protein (24). Because of the potential modulation of insulin action by the mTORC1 signalling pathway, this study aimed at further detailing the incompletely defined function of PRAS40 in insulin action in primary human skeletal muscle cells (hSkMC). Therefore, PRAS40 was silenced in hSkMC and the effects on insulin action were determined. In addition, we examined whether the chemokines chemerin and monocyte chemotactic protein 1 (MCP-1), which have been implicated in the induction of insulin resistance in hSkMC (25,26), exert an additive effect in hSKMC in which PRAS40 was silenced.

Methods

Culture of human skeletal muscle cells

Primary human skeletal muscle cells isolated from rectus abdominis muscle of six healthy Caucasian donors (3 males of 16, 18, and 21 years of age, and 3 females of 25, 33 and 37 of age) were supplied as proliferating myoblasts from PromoCell (Heidelberg, Germany) or Lonza (Basel, Switzerland) and cultured as described previously (27). Briefly, myoblasts were seeded in six-well culture dishes and were cultured in growth medium containing α-modified Eagle's (αMEM)/Ham's F-12 medium (Gibco, Berlin, Germany) and the supplement pack for skeletal muscle cells from PromoCell (Heidelberg, Germany). After reaching near-confluence, differentiation into myotubes was initiated by replacing the growth medium by αMEM containing 2% horse serum (Gibco, Berlin, Germany). Cells were routinely starved on serum-free αMEM on day 6 of differentiation prior to insulin stimulation on day 7 of differentiation.

Knockdown of PRAS40

On day 3 of differentiation, myotubes cultured as described above were transfected with 75 nmol/l PRAS40-specific or non-target siRNA (Applied Biosysthem, Carlsbad, CA, USA) using the Hiperfect reagent (Qiagen, Hilden, Germany). In all experiments, the effects of two distinct PRAS40 siRNAs were compared. At 24h after transfection, the medium was replaced and differentiation was continued as indicated above.

RNA-isolation and quantitative real-time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and transcribed into cDNA using the Omniscript Reverse Transcription kit (Qiagen, Hilden, Germany). Gene expression levels for F-box protein 32 (*Fbxo32*, also known as atrogin-1), *IRS1*, Tripartite motif containing 63 (*TRIM63*, also known as muscle RING-finger protein-1 (*MuRF1*)), and *PRAS40* was determined by real-time PCR using SYBR green reagents (Promega, Mannheim, Germany) on a Step One Plus Cycler (Applied Biosystems, Carlsbad, CA, USA). Details on the primer assays used are provided in the electronic supplementary material. Expression levels were calculated using the ΔΔCt method using *PPIA* and *RPS28* as housekeeping genes. The expression levels of these genes showed no variations in response to the various treatments applied to the hSkMC in this study.

Analysis of insulin signalling

When indicated, myotubes on day 6 of differentiation were exposed to 2 ng/ml recombinant human monocyte chemoattractant protein 1 (MCP-1) (PeproTech, Hamburg, Germany) or 2 µg/ml recombinant human chemerin (R&D systems, Wiesbaden, Germany) in serum-free αMEM for 24h to induce insulin resistance. For inhibition of protein degradation, myotubes were incubated with 3 µmol/l of the proteasome inhibitor MG-132 (Calbiochem, Darmstadt, Germany) for 24h. When indicated, cells were incubated with 7 µmol/l of the selective p70S6K inhibitor PF-4708671 overnight, or 100 nmol/l rapamycin (Calbiochem, Darmstadt, Germany) for 15 min on day 7 of differentiation, prior to insulin stimulation (porcine insulin, Sigma Aldrich, St Louis, MO, USA) (10 min, 100 nmol/l).

For Western blot analysis, myotubes were lysed in 50 mmol/l HEPES (pH7.4), supplemented with 1% Triton-X100, PhosStop, and Complete Protease Inhibitor cocktails (Roche, Mannheim, Germany). Lysates were tumbled for 2h at 4°C, and cleared by centrifugation for 20 min at 15000rpm at 4°C. Protein concentrations in the lysates were determined using Bradford reagent (Biorad, Munich, Germany). Thereafter, 5 µg of protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore, Schwalbach, Germany) in a semidry blotting apparatus. Blocking of membranes was performed using Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% nonfat dry milk or 5% BSA, respectively, and first antibody was incubated overnight at 4°C. The antibodies used were: Akt, Akt-phospho-Ser473, Akt-phospho-Thr308, glycogen synthase kinase 3β (GSK3β), GSK3α/β-phospho-Ser21/9, mammalian target of rapamycin (mTOR), p70S6K, p70S6K-phospho-Thr389, PRAS40, raptor, ribosomal protein S6, ribosomal protein S6-phospho-Ser240, rictor, tuberous sclerosis complex 2 (TSC2) phospho-Thr1462, and eukaryotic elongation binding protein 4E-BP1 phospho-Thr37/46 from Cell Signaling Technology (Danvers, MA, USA), insulin receptor β-subunit (IRβ), growth factor receptor bound protein 10 (Grb10), and TSC2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), glucose transporter 4 (GLUT4) from Abcam (Cambridge, UK), and polyclonal IRS1 as described (28). After extensive washing membranes were incubated with corresponding secondary horseradish peroxidase-coupled antibody (Promega, Heidelberg, Germany) and protein bands were visualized by enhanced chemiluminescence using Immobilon Western detection reagents (Millipore, Schwalbach, Germany) on a VersaDoc 4000 MP (BioRad, Munich, Germany) work station. Analysis was performed with the quantity one analysis software (Version4.6.7). Signals were normalized by reprobing the membranes with antibodies for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (both from Cell Signaling Technology, (Danvers, MA, USA)).

In one experiment, phosphorylation of p70S6K was assessed using the MILLIPLEX® Map Cell Signalling Buffer and Detection Kit (Millipore, Schwalbach, Germany), a bead-based suspension array using the Luminex xMAP technology. Therefore, myotubes were lysed in 75 µl MILLIPLEX MAP Lysis Buffer and incubated for 20 min at 4°C. Lysates were cleared by centrifugation and protein content was determined by the BCA Protein Assay (Pierce, Rockford, USA). Subsequently, 10 µg of total protein was used for measuring human phospho p70S6K Thr412 MAPmates with the BioPlex 200 System (BioRad, Munich, Germany). Signals were normalized using GAPDH MAPmates and analyzed using Bio-Plex Manager software version 6.0 (Biorad, Munich, Germany). The signals obtained with this method were similar to those obtained by Western blotting with antibodies recognizing phosphorylated p70S6K-Thr389.

Glucose-uptake

Glucose uptake was determined in serum-starved myotubes on day 7 of differentiation. Briefly, cells cultured in 6-well plates were kept untreated or incubated with 100 nmol/l insulin for 30 min prior to the addition of 9.25 kBq per well 2-desoxy-D-^{14}C-glucose (2-DOG) for 2h. Then, cells were washed with ice-cold phosphate-buffered saline containing 0.25 µmol/l cytochalasin B, lysed in 1 mol/l NaOH, whereafter incorporated radioactivity was assessed by liquid scintillation counting.

20S proteasome activity assay

Proteasome activity was determined in serum-starved myotubes grown in 96-well plates. Analysis of the three different proteasome activities (chymotrypsin-like, caspase-like and trypsin-like) was performed using the Proteasome-Glo™ cell-based assay (Promega, Mannheim, Germany) according to the manufacturer´s protocol. Briefly, the luminogenic substrates (Suc-LLVY-Glo™ for the chymotrypsin-like, Z-nLPnLD-Glo™ for the caspase-like and Z-LRR-Glo™ for the trypsin-like activity) were mixed with the Proteasome-Glo™ cell-based buffer and luciferin detection reagent and incubated for 30min. When indicated, cells were incubated with 3µM MG-132 for 15 min, before 100µl of Proteasome-Glo™ reagent was added for additional 10 min to non-target siRNA- or PRAS40 KD-transfected myotubes on day 7 of differentiation. The luminescence was measured using a Tecan Infinite 200 reader (Tecan, Maennersdorf, Germany).

Statistical analysis

Data are presented as means ± SEM. Significant differences were determined by two-way ANOVA (post hoc test: Bonferroni multiple comparison test) or paired two-tailed Student´s t-test using Prism5 (GraphPad, LA Jolla, CA) software. P-values of $p<0.05$ were considered as statistically significant.

Results

Silencing PRAS40 inhibits insulin action in primary human skeletal muscle cells

Knockdown of PRAS40 (PRAS40-KD) in hSkMC reduced PRAS40 mRNA expression by 70% and protein abundance by ~60% as compared to myotubes transfected with non-target (NT) siRNA (supplementary Figure 3.7, Figure 3.1a). PRAS40-KD did not affect protein levels of the insulin receptor β-subunit, Akt, and GLUT4 (Figure 3.1b-d). In contrast, IRS1 protein abundance, but not mRNA expression, was lowered by 60% in cells with PRAS40 KD as compared to NT-siRNA transfected cells (Figure 3.1e, f).

The effects of PRAS40-KD on IRS1 protein levels were paralleled by a 40-50% inhibition of the insulin-mediated phosphorylation of Akt on Ser473 and Thr308 (Figure 3.2a, b). In line with the reductions in Akt-phosphorylation, insulin-mediated GSK3β-Ser9 and TSC2-Thr1462 phosphorylation were both reduced ~30% in PRAS40-KD versus NT-myotubes (Figure 3.2c, d). The reductions in insulin-mediated Akt, GSK3β, and TSC2-phosphorylation could not be ascribed to alterations in protein levels induced by PRAS40-KD (Figure 3.1, supplementary figure 3.8). Furthermore, two distinct PRAS40 siRNAs yielded similar results as compared to NT-siRNA transfected cells (supplementary Figure 3.9). Insulin-stimulated glucose uptake was blunted by 20% in PRAS40-KD cells versus NT-myotubes with similar data observed for both PRAS40 siRNAs (Figure 3.2f).

This reduction of the insulin-mediated activation of the IRS1/Akt-signalling pathway was comparable to the well-characterized effects observed after chemerin or MCP-1 treatment of hSkMC (25,26) (Figure 3.2). There was no further deterioration of insulin action in PRAS40-KD cells treated with either chemerin or MCP-1 (supplementary Figure 3.10).

Effects of PRAS40 knockdown on mTORC1 signalling

Hyperactivation of the mTORC1 signalling pathway has been associated to inhibition of insulin action amongst others via p70S6K-mediated serine phosphorylation and subsequent degradation of IRS1 (1-3), as well as through the stabilization of the protein levels of an inhibitor of

insulin action, Grb10 (9-11). Because some studies have hinted at an inhibitory role for PRAS40 within mTORC1 (16,17), we next evaluated whether the effects of PRAS40-KD could be ascribed to hyperactivation of the mTORC1-pathway. To monitor activation of the mTORC1-pathway, we first examined the phosphorylation of the mTORC1-substrates p70S6K and 4E-BP1. In NT-siRNA myotubes, phosphorylation of p70S6K-Thr412, its substrate ribosomal protein S6-Ser240, and 4E-BP1-Thr37/46 was increased after insulin stimulation (Figure 3.3a-c). This response was completely prevented in cells pre-treated with rapamycin (Figure 3.3a-c). In PRAS40-KD myotubes, the insulin-induced phosphorylation of p70S6K-Thr412 and S6-Ser240 were both reduced by ~45%, while the induction of 4E-BP1-Thr37/46 phosphorylation by insulin was completely lost (Figure 3.3a-c). The inhibition of the mTORC1 signalling pathway was further substantiated by a reduced abundance of the mTORC1-substrate Grb10 in PRAS40-KD myotubes and in rapamycin-treated cells versus NT-siRNA myotubes (Figure 3.3d). The impaired phosphorylation of p70S6K-Thr412, S6-Ser240, and 4E-BP1-Thr37/46 in insulin-treated PRAS40-KD myotubes could not be ascribed to changes in the protein levels of these targets (supplementary figure 3.8). Importantly, PRAS40-KD neither affected the abundance of mTOR, nor that of the regulatory components of the mTORC1- and mTORC2 complexes, raptor and rictor, respectively (supplementary Figure 3.8). In contrast to PRAS40-KD, exposure of hSkMC to MCP-1 had no effect on insulin mediated p70S6K-Thr412 phosphorylation, S6-Ser240 phosphorylation, or Grb10 abundance, whereas insulin-induced 4E-BP1-Thr37/Thr46 phosphorylation was slightly impaired (Figure 3.3a-d).

Effect of inhibition of p70S6 kinase and the proteasome on protein expression of IRS1

The observed effects on the activity of the mTORC1 pathway seem to exclude an involvement of the p70S6K feedback loop on the inhibition of insulin action observed in PRAS40-KD myotubes. To substantiate this, we examined the effect of PF-4708671, a highly specific inhibitor of p70S6K (29), on IRS1 expression. Treatment with PF-4708671 markedly induced the phosphorylation of p70S6K, but blunted the basal- and insulin-stimulated phosphorylation of the ribosomal protein S6-Ser240 in both NT-siRNA and PRAS40-KD myotubes (Figure 3.4 a, b). Importantly, the decrease in IRS1 abundance in PRAS40-KD cells could not be restored by PF-4708671 (Figure 3.4c).

Since PRAS40-KD inhibits mTORC1-signalling and inhibition of mTORC1 activity has been linked to increased protein degradation amongst others by increased activity of the ubiquitin-proteasome system (30,31), we next examined whether the mRNA expression of the muscle-specific E3-ligases atrogin-1 and MuRF1, which are increased in mice heterozygous for mTOR (30), is affected by PRAS40-KD. As shown in Figure 3.5a, b, the mRNA levels of atrogin-1 and MuRF1 were increased

in PRAS40-KD versus NT-siRNA myotubes. Furthermore, the chymotrypsin- and caspase-like activity of the 20S proteasome was increased in PRAS40-KD versus NT-siRNA myotubes (Figure 3.5 c,d). In contrast, there was no significant difference in the trypsin-like proteolytic activity between PRAS40-KD and NT-siRNA myotubes (Figure 3.5e), although all proteolytic activities examined were sensitive to the proteasome inhibitor MG-132 (Figure 3.5 c-e).

MG-132 was then used to examine whether the reductions in IRS1 protein levels could be ascribed to protein degradation. As shown in Figure 3.6a, MG-132 increased IRS1 protein abundance by 1.3- and 3-fold in NT-siRNA and PRAS40-KD myotubes, respectively. Although protein abundance of PRAS40 was increased by 1.4-fold by MG-132 in both NT-siRNA and PRAS40-KD myotubes, levels of PRAS40 were still reduced by 56% when comparing PRAS40-KD with NT-siRNA myotubes (Figure 3.6b). The increase in IRS1 abundance in MG132-treated PRAS40-KD myotubes was paralleled by a restoration of insulin-mediated phosphorylation of Akt-Ser473 (Figure 3.6c). Treating myotubes with MCP-1, which does not affect mTORC1 signalling, had no effect on atrogin-1 and MuRF1 levels (Figure 3.5a,b). However, chronic exposure of myotubes to MCP-1 also led to a reduction in IRS1 protein abundance (Figure 3.6d), and similar to PRAS40-KD cells, MG-132 fully restored IRS-1 protein levels and insulin action in hSkMC exposed to MCP-1 (Figure 3.6d, e).

Discussion

The present study shows that knockdown of PRAS40 abrogates the insulin-mediated activation of the Akt-pathway regulating glucose uptake in primary human skeletal muscle cells. These effects involve a reduction in the protein levels of IRS1. Importantly, the reduction in IRS1 protein abundance cannot be ascribed to a classical negative regulator of insulin action, the mTORC1/p70S6K-feedback loop. Rather, knockdown of PRAS40 was found to increase the activity of the proteasome, and the reduction in IRS1 levels as well as the associated inhibition of insulin action following PRAS40 knockdown could be restored by inhibition of the proteasome. These findings suggest that PRAS40 can act as a modulator of insulin action which exerts its action by regulating the activity of the proteasome.

Previous studies have reported contrasting findings for the effects of PRAS40 on insulin action (16). In HEK293 cells neither knockdown nor overexpression of PRAS40 affected insulin-mediated Akt-phosphorylation (22). Yet, in line with our findings on primary human skeletal muscle cells, knockdown of PRAS40 resulted in the degradation of IRS1 in 3T3L1 adipocytes, HepG2 cells and C2C12 myoblasts (21,32), and reduction of insulin-mediated Akt-phoshorylation in 3T3L1 adipocytes and HepG2 cells (21). In one report, an increased basal phosphorylation of p70S6K was observed in

PRAS40-KD cells, which may contribute to the degradation of IRS1 through phosphorylation of IRS1 on Ser636 and 639 (21). Accordingly, overexpression of PRAS40 impaired the insulin-mediated phosphorylation of the mTORC1 substrates S6K1 and 4E-BP1 (19,20,22,23). These findings led to the suggestion that the dissociation of phosphorylated PRAS40 from raptor could promote downstream mTORC1 signalling by increased substrate binding to raptor (16,17). However, this proposed function for PRAS40 is in contrast with our findings on primary human skeletal muscle cells and multiple other studies (16,22,33,34). In HEK293E cells, the dissociation of PRAS40 from raptor was found to increase 4E-BP1 binding to raptor, but not to affect the basal or insulin-stimulated phosphorylation of p70S6K and 4E-BP1 (33). In other studies, including the present one, the silencing of PRAS40 impaired both the basal and the insulin-stimulated activation of the mTORC1-pathway (22,34). In PRAS40-KD human primary skeletal muscle cells, this is illustrated by a reduced phosphorylation of the p70S6K-Thr412, ribosomal protein S6-Ser240, and 4E-BP1-Thr37/Thr46, and, for the first time, also by a reduced abundance of Grb10, a newly identified mTORC1 substrate whose levels are stabilized through phosphorylation by mTORC1 (9-11). Although genetic evidence obtained in D. melanogaster hints at a tissue-specific regulation of the dTORC1-pathway by dPRAS40 (24), the impaired activation of the mTORC1 signalling pathway by insulin in the present study most likely results from a reduced activation of the IRS1-Akt-TSC2 axis in PRAS40-KD myotubes rather than from a direct role for PRAS40 in the regulation of mTORC1-signalling (16,17).

A novel finding of the present study is the recognition that PRAS40 participates in the regulation of proteasome activity, as illustrated by elevated mRNA expression of the E3-ligases atrogin-1 and MuRF1, as well as the increased activity of the proteasome in PRAS40-KD myotubes. Importantly, pharmacological inhibition of the proteasome reversed the reduction in IRS1 protein levels and the impaired insulin-mediated phosphorylation of Akt in PRAS40-KD myotubes. This indicates that PRAS40 may affect insulin action by regulating the protein levels of IRS1 through the proteasome rather than the mTORC1/p70S6K feedback loop. Accordingly, inhibition of p70S6K activity did not prevent the reduction in IRS1 protein abundance in PRAS40-KD myotubes. Previous studies already linked the proteasome-mediated degradation of IRS1 to the inhibition of insulin action in response to hyperinsulinemia (35,36) and inflammation (37). In this context, multiple E3-ligases, including F-box only protein 40, Cbl-b, and F-box/WD repeat-containing protein 8, have been linked to IRS1 degradation in response to hyperinsulinemia, inflammation and chronic exposure to insulin-like growth factor 1 (37-41). Furthermore, an increased expression of atrogin-1 and MuRF1 associating with reduced activation of the PI3K/Akt pathway by insulin was described in skeletal muscle of *db/db* mice (42). Interestingly, restoration of insulin sensitivity in skeletal muscle of this animal model by rosiglitazone was accompanied by reductions in the expression of atrogin-1 and

MuRF1 (42). Although these observations are fully in line with our study, further studies are required to assess whether atrogin-1 and MuRF1 indeed function as genuine IRS1-ligases.

Another aspect that remains to be addressed is the molecular mechanism via which PRAS40 regulates the activity of the proteasome. One may speculate that the effects of PRAS40-KD on the proteasome result from inhibition of mTORC1 activity. In primary rat hepatocytes, short-term pharmacological inhibition of mTOR was found to reverse the inhibition of the ubiquitin-proteasome pathway by insulin and amino acids (31). Furthermore, skeletal muscles of mTOR heterozygous mice display elevated expression levels of the E3 ligases atrogin-1 and muscle ring finger -1 (30). However, mTOR heterozygosity was not paralleled by alterations in IRS1 expression in these animals (30). Furthermore, short-term treatment of 3T3L1 adipocytes with rapamycin does not result in abrogation of insulin-stimulated glucose transport (43). Finally, in mice with a muscle-specific deletion of raptor, a critical component of the mTORC1 complex, expression levels of atrogin-1 and MuRF1 were decreased (44). Alternatively, the reduction in Grb10 levels in PRAS40-KD myotubes may participate in the activation of the proteasome. Grb10 has been found to protect the vascular endothelial growth factor receptor from ubiquitin-mediated degradation in HEK293 cells (45). Although Grb10 has been identified as a negative regulator of insulin receptor abundance (10), IRS1 levels are markedly reduced in skeletal muscle from mice with a targeted disruption of either Grb10 alone, or combined with the related Grb14 protein (46,47). Intriguingly, in muscles from Grb10 knock-out mouse, insulin-induced tyrosine phosphorylation of IRS1 was increased, whereas insulin-induced phosphorylation of Akt-Ser473 was decreased (46). In muscles from the combined Grb10/Grb14 knock-out mouse marked decreases were observed in insulin-stimulated IRS1 phosphorylation (47). Although it remains to be clarified whether the reductions in IRS1 protein abundance in the Grb10-deficient animal models indeed result from increased degradation of IRS1, these findings do not exclude the possibility that the reductions in Grb10 protein levels could contribute to the inhibition of insulin action observed in PRAS40-KD myotubes. Collectively these findings clearly indicate that further studies are required toward identification of the signalling pathways linking PRAS40 to the regulation of proteasome activity.

We further observed that exposing PRAS40-KD myotubes to chemokines did not result in an additional deterioration of insulin action. This indicates that PRAS40-KD and chemokines may utilize the same pathway to inhibit insulin action. Indeed, chronic exposure of hSkMC to MCP-1 resulted in degradation of IRS1, which could be reversed by inhibition of the proteasome. However, whereas PRAS40-KD led to inhibition of mTORC1 signalling, MCP-1 treatment had no effect on mTORC1 signalling in myotubes. This suggests that MCP-1 promotes degradation of IRS1 through other mechanisms, such as the induction of serine phosphorylation of IRS1 by members of the MAP kinase

family (48). These inhibitory serine phosphorylations facilitate the binding of 14-3-3 proteins to IRS1 and target the protein for degradation (49). In support of this possibility is the observation that inhibition of the extracellular-signal regulated kinase pathway in hSkMC reverses the impairment of insulin action induced by MCP-1 (25).

Collectively, this study provides novel insights into the function of one of the most prominent substrates for Akt, PRAS40, in skeletal muscle. Rather than the previously suggested p70S6K negative feedback loop, our findings suggest that PRAS40 may modulate insulin action by regulating the protein abundance of IRS1 by affecting the activity of the proteasome.

Source of funding

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Duality of interest

The authors declare that there is no duality of interest associated with this study.

Contribution statement

CW conceived and designed the experiments, researched data, wrote and reviewed/edited the manuscript, DHdW, EBMN, SL, and HAH researched data and reviewed/edited the manuscript, DMO conceived and designed the experiments, researched data, wrote and reviewed/edited the manuscript. DMO is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of the data and the accuracy of the data analysis. All authors have seen and approved the final version of manuscript.

Figures

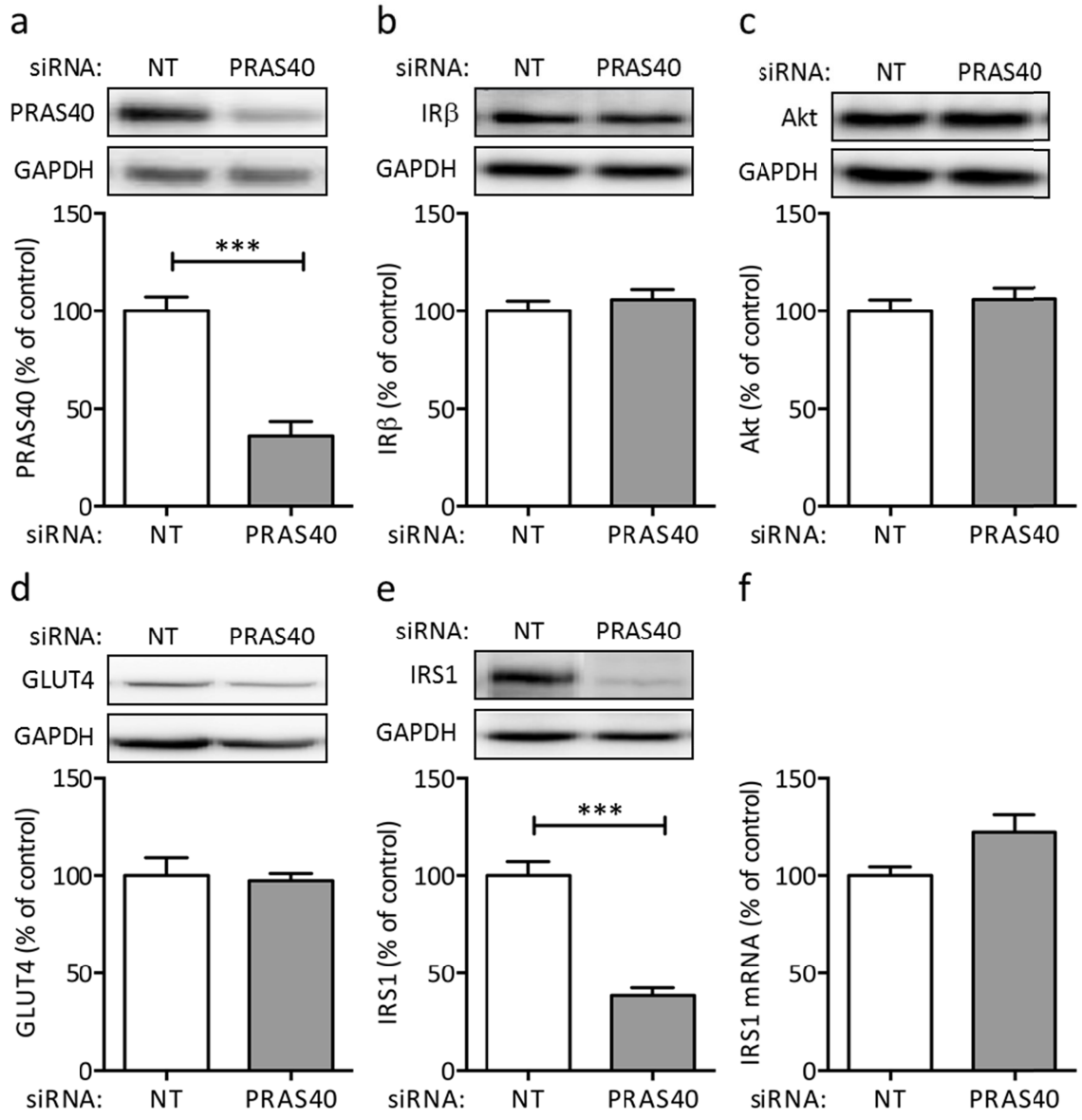


Figure 3.1 Effect of PRAS40 silencing on the expression of key components of the insulin signaling pathway. Lysates from human primary skeletal muscle cells transfected with either a non-target (NT) or PRAS40 siRNA were analyzed for protein levels of PRAS40 (a), the insulin receptor (IR) β-subunit (b), Akt (c), GLUT4 (d), and insulin receptor substrate 1 (IRS1) (e), as well as mRNA levels of IRS1 (f). Protein levels were normalized for GAPDH protein abundance, which was not affected by the experimental conditions used. Data are presented as representative Western blots and bar graphs showing the mean ± standard error of the mean of at least 4 independent experiments using cells from different donors. Values obtained for NT-siRNA transfected cells were considered as control and set at 100%. The effects of silencing PRAS40 on protein levels and mRNA expression were analyzed by a student's t-test. ***, $p<0.001$ versus cells transfected with NT-siRNA.

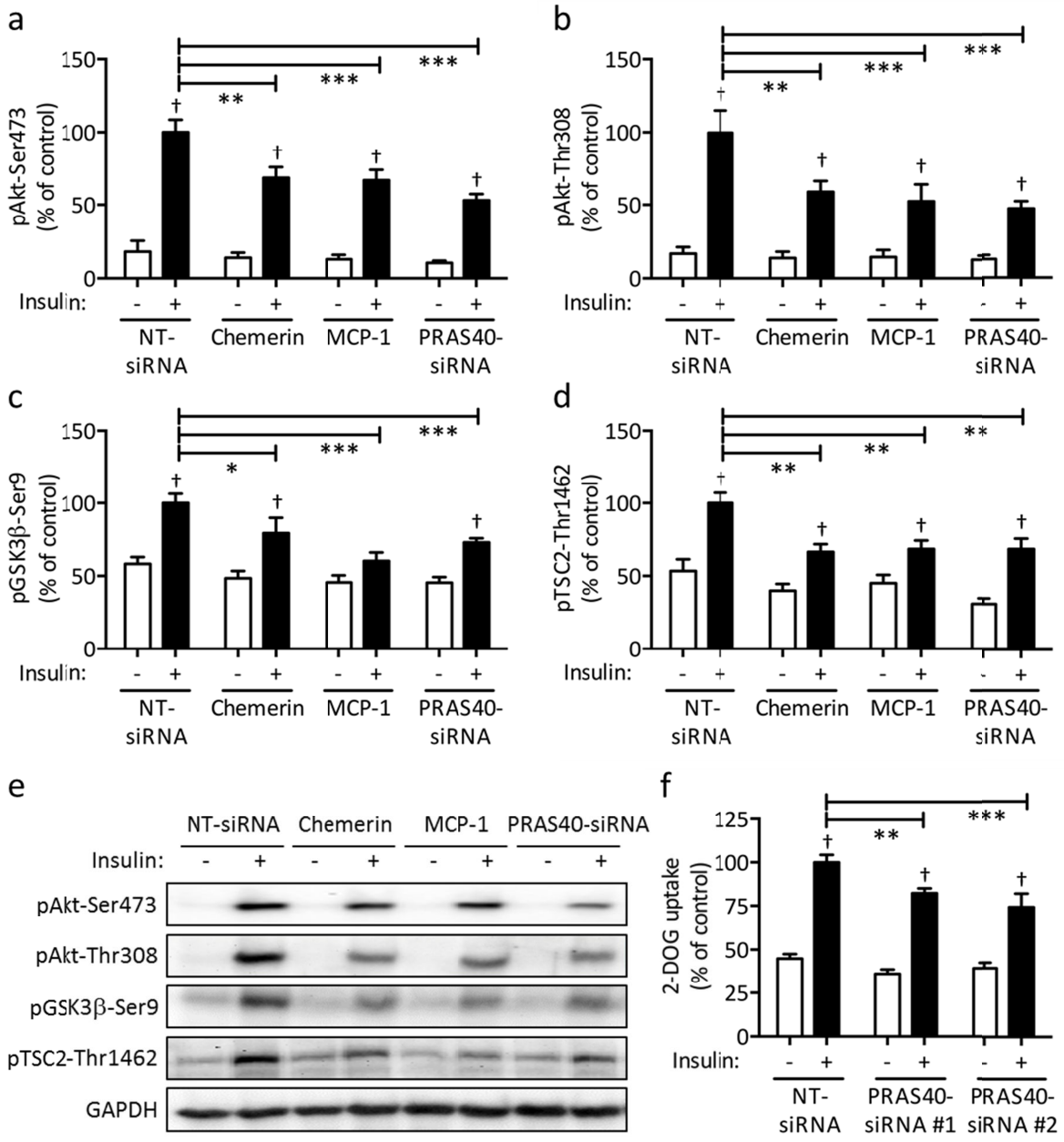


Figure 3.2 Effect of PRAS40 silencing and chemokines on insulin-mediated activation of the Akt signaling pathway and the regulation of glucose uptake. Lysates from human primary skeletal muscle cells transfected with either a non-target (NT) or PRAS40 siRNA, and exposed to chemerin or monocyte chemoattractant protein 1 (MCP-1) prior to insulin stimulation were analyzed for phosphorylation of Akt-Ser473 (a), Akt-Thr308 (b), GSK3β-Ser9 (c), and TSC2-Thr1462 (d). Signals were normalized for GAPDH abundance, which was not affected by the experimental conditions used. Data are presented as representative Western blots (e) and bar graphs showing the mean ± standard error of the mean of the phosphorylation levels obtained in at least 5 independent experiments using cells from different donors. Open bars depict basal conditions, and filled bars represent insulin-treated cells. The values obtained for NT-siRNA transfected insulin-treated cells were considered as control and set at 100%. The effects of PRAS40 silencing on insulin-mediated glucose uptake (f) are the mean ± standard error of the mean of 6 independent experiments using cells from different donors, and using two distinct PRAS40 siRNAs. The effects of the chemokines and silencing PRAS40 on insulin action were analyzed using a two-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, $p<0.001$; **, $p<0.01$, *, $p<0.05$ versus cells transfected with NT-siRNA; †, indicates $p<0.05$ for the effect of insulin- (+) versus untreated-cells (-).

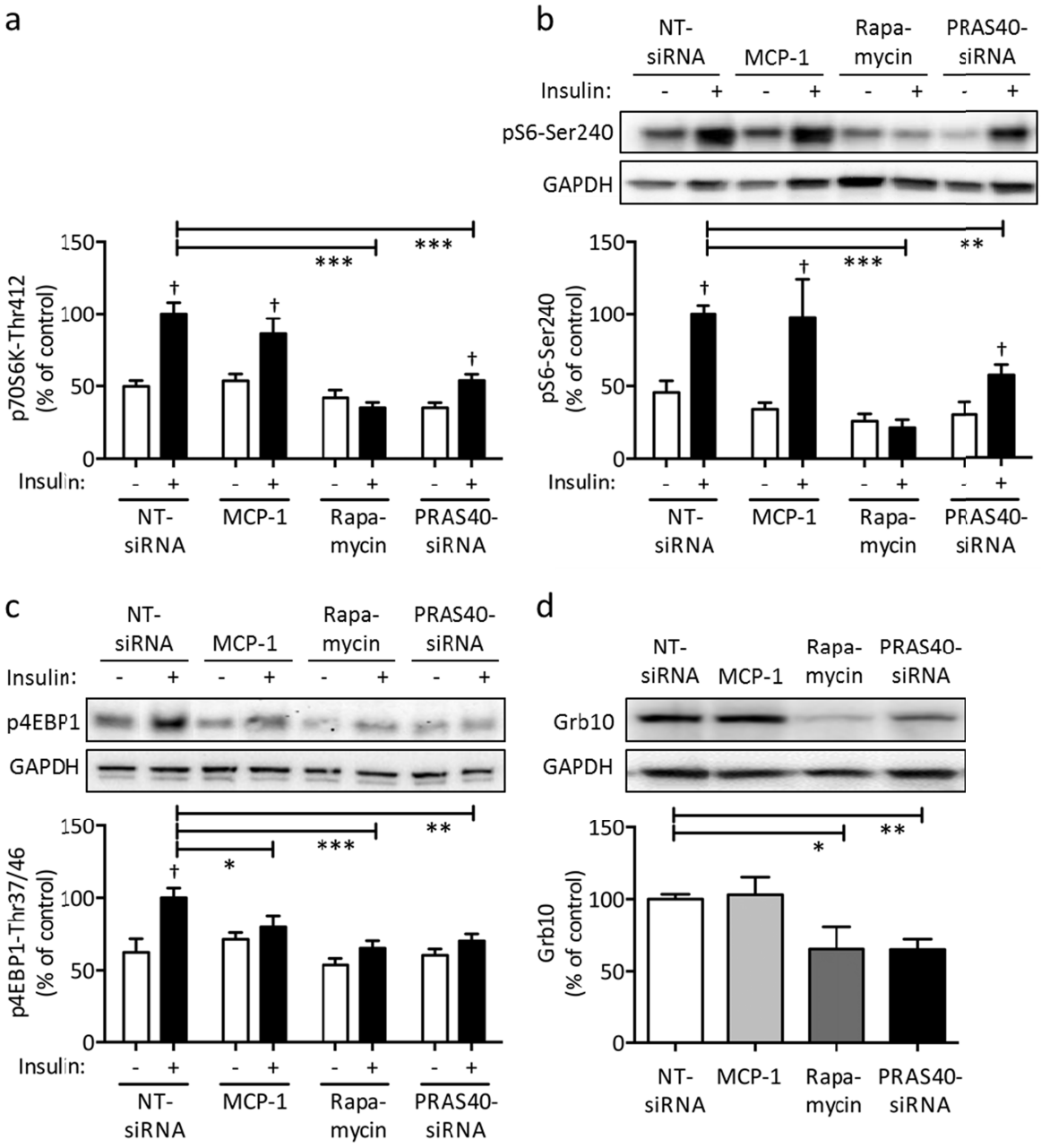
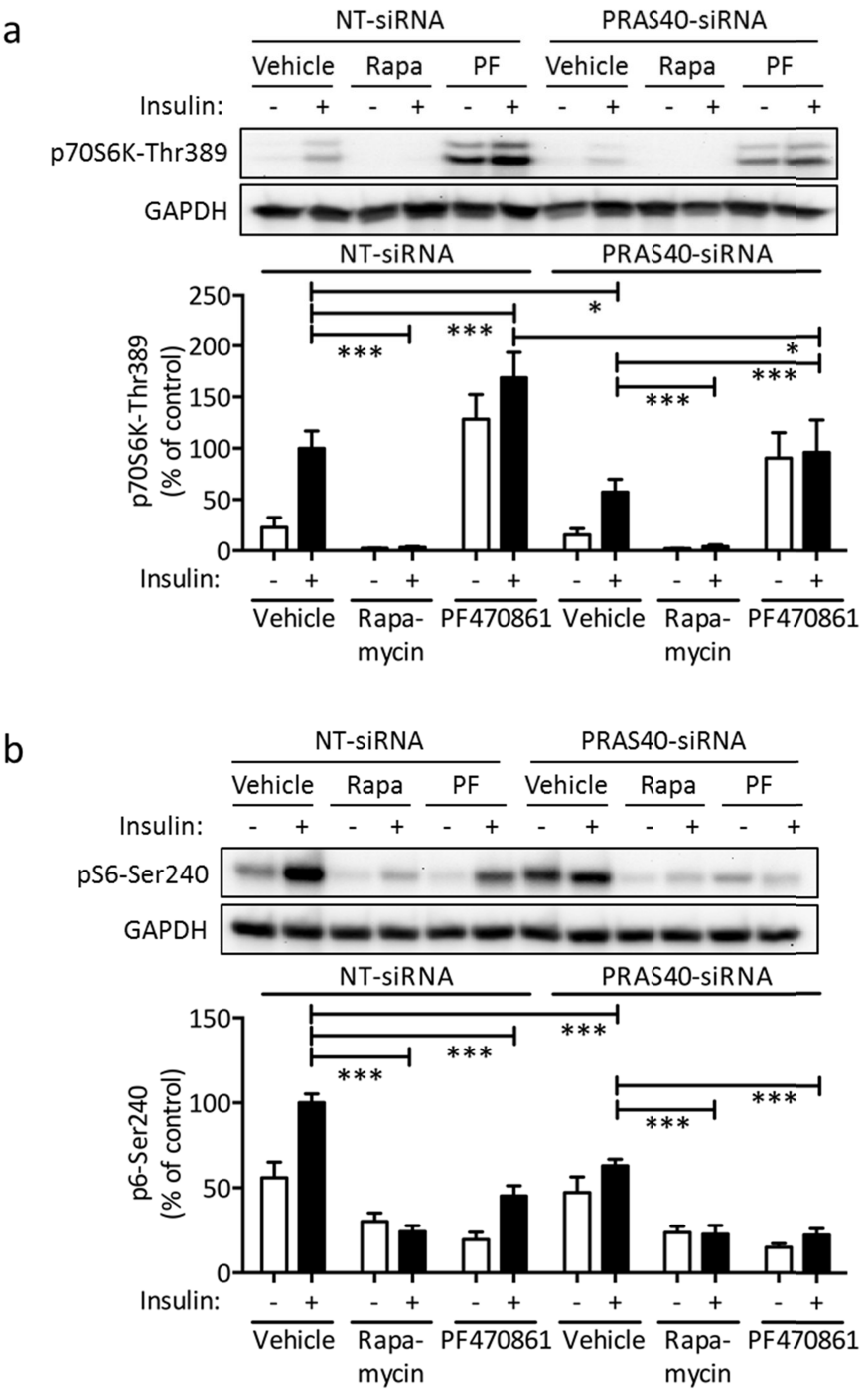


Figure 3.3 Effects of PRAS40 knockdown on mTORC1 signaling. Lysates from human primary skeletal muscle cells transfected with either a non-target (NT) or PRAS40 siRNA, and exposed to monocyte chemoattractant protein 1 (MCP-1) or rapamycin prior to insulin stimulation were analyzed for phosphorylation of p70S6kinase-Thr412 (a) using a Milliplex assay, whereas phosphorylation of ribosomal protein S6-Ser240 (b) and 4EBP1-Th37/46 (c) and levels of Grb10 (d) were determined by Western blotting. Signals were normalized for GAPDH abundance, which was not affected by the experimental conditions used. The bar graphs are the mean ± standard error of the mean of the phosphorylation levels obtained in at least 6 independent experiments using cells from different donors. Open bars depict basal conditions, and filled bars represent insulin-treated cells. The values obtained for NT-siRNA transfected insulin-treated cells were considered as control and set at 100%. The effects of the various conditions on mTORC1 signaling were analyzed using a two-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, p<0.001; **, p<0.01, *, p<0.05 versus cells transfected with NT-siRNA; †, indicates p<0.05 for the effect of insulin- (+) versus untreated-cells (-).


a
NT-siRNA
PRAS40-siRNA
Vehicle
Rapa
PF
Insulin:
p70S6K-Thr389
GAPDH
p70S6K-Thr389 (% of control)
Vehicle
Rapa-mycin
PF470861
b
pS6-Ser240
p6-Ser240 (% of control)

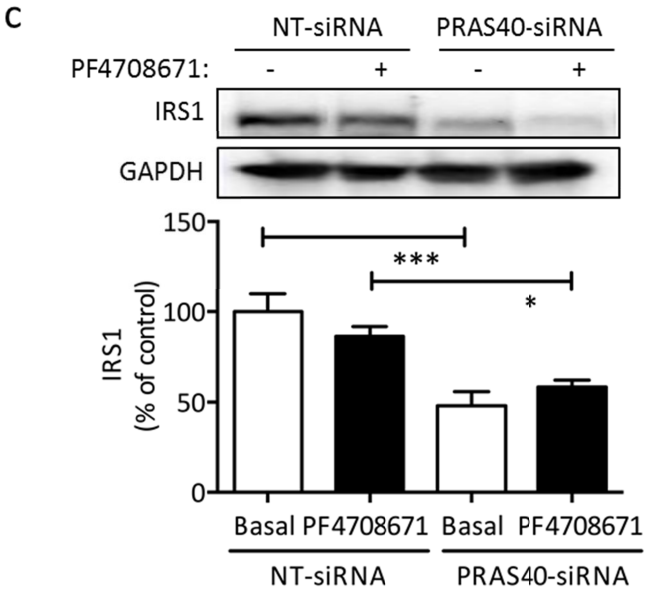


Figure 3.4 Pharmacological inhibition of p70S6 kinase activation does not prevent IRS1 degradation induced by PRAS40 knockdown. Lysates from human primary skeletal muscle cells transfected with either a non-target (NT) or PRAS40 siRNA, and exposed to DMSO (vehicle), rapamycin or PF4708671 prior to insulin stimulation were analyzed for phosphorylation of p70S6kinase-Ser389 (a) and ribosomal protein S6-Ser240 (b) and protein levels of IRS1 (c). Signals were normalized for GAPDH abundance, which was not affected by the experimental conditions used. The bar graphs are the mean ± standard error of the mean of the phosphorylation levels obtained in at least 5 independent experiments using cells from different donors. Open bars depict basal conditions, and filled bars represent insulin-treated cells. The values obtained for NT-siRNA transfected insulin-treated cells were considered as control and set at 100%. The effects of rapamycin, PF-4708671, and PRAS40-KD on p70S6K-Thr389, and ribosomal S6-Ser240 phosphorylation, and IRS1 levels were analyzed using a two-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, $p<0.001$; **, $p<0.01$, *, $p<0.05$ for the comparisons indicated in the bar graphs.

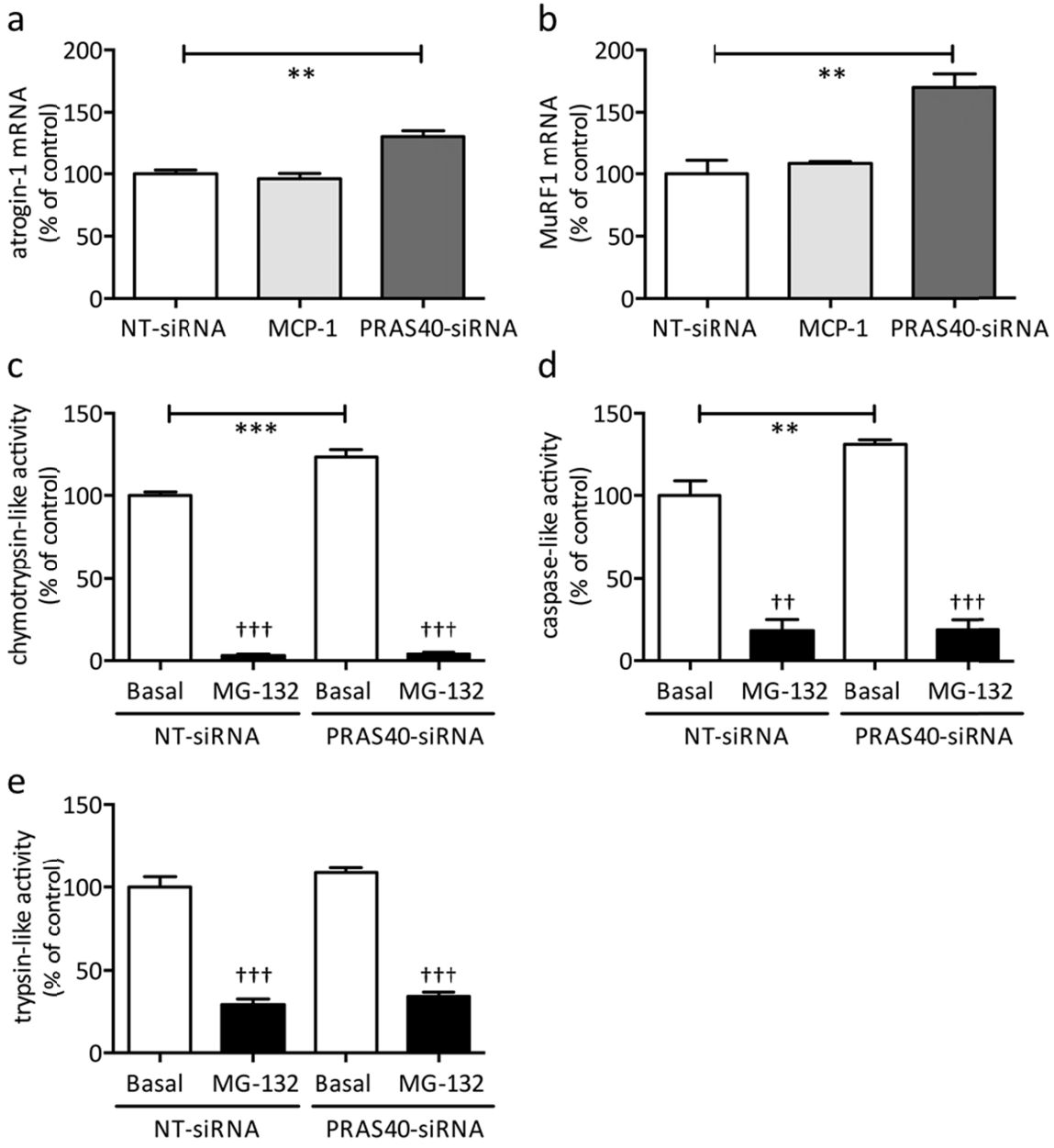
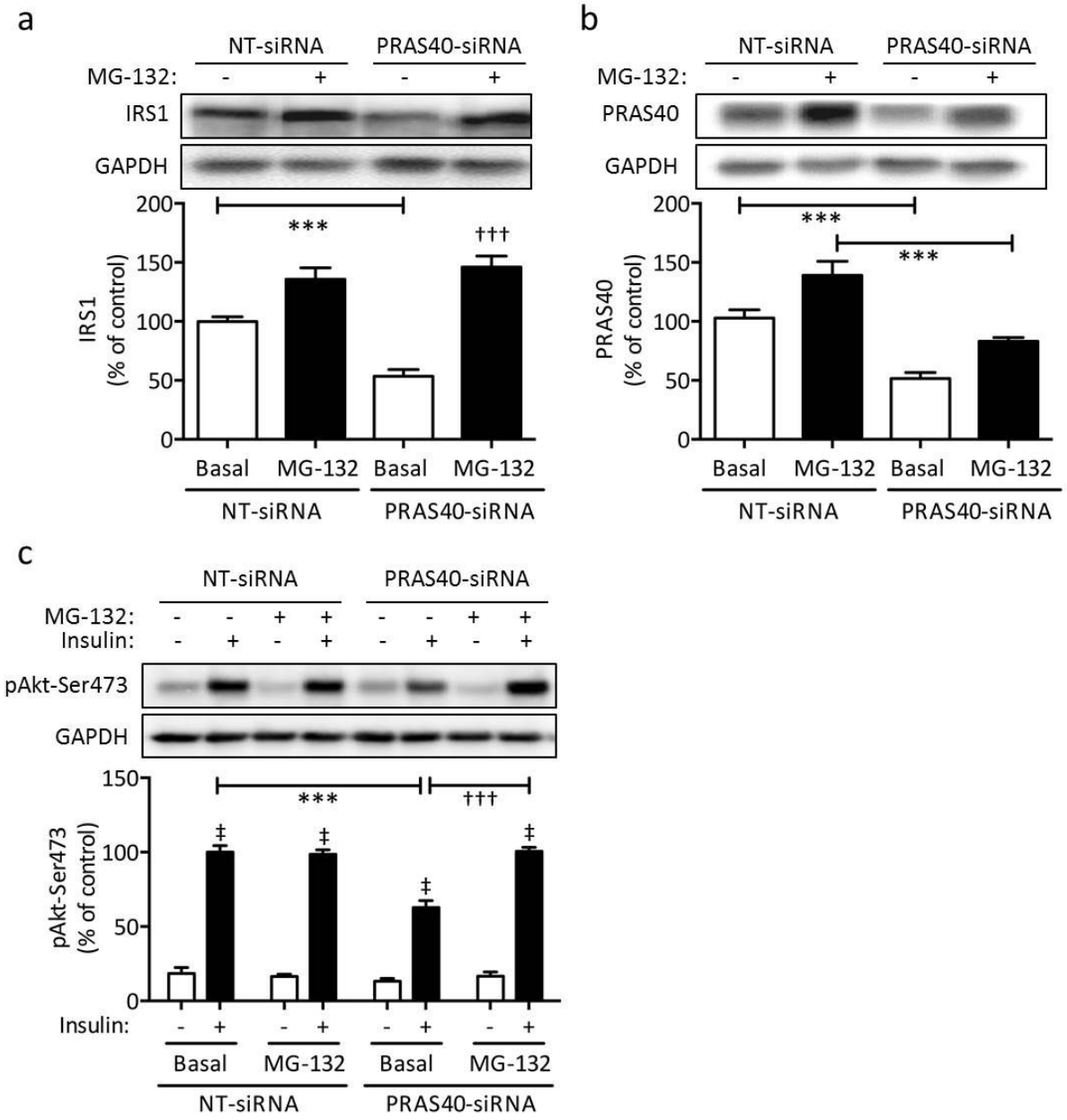


Figure 3.5 Effect of PRAS40 knockdown on determinants of proteasome activity. Human primary skeletal muscle cells transfected with either a non-target (NT) or PRAS40 siRNA were analyzed for mRNA expression of atrogin-1 (a) and MuRF1 (b), and chymotrypsin-like (c), caspase-like (d) and trypsin-like (e) proteasome activity. Data are expressed as mean ± standard error of the mean of at least 5 independent experiments using cells from different donors. The values obtained for NT-siRNA transfected cells were considered as control and set at 100%. The effects of silencing PRAS40 and MCP-1 on gene expression were analyzed by ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. **, $p<0.01$ versus cells transfected with NT-siRNA. The effects of silencing PRAS40 and MG132 on proteasome activity were analyzed by two-way ANOVA. ***, $p<0.001$; ** $p<0.01$ versus cells transfected with NT-siRNA, †††, $p<0.001$; ††, $p<0.01$ for the effect of MG-132 versus basal.


a
NT-siRNA
PRAS40-siRNA
MG-132:
IRS1
GAPDH
IRS1 (% of control)
***
†††
Basal
MG-132
NT-siRNA
PRAS40-siRNA
b
PRAS40
PRAS40 (% of control)
c
Insulin:
pAkt-Ser473
pAkt-Ser473 (% of control)
‡

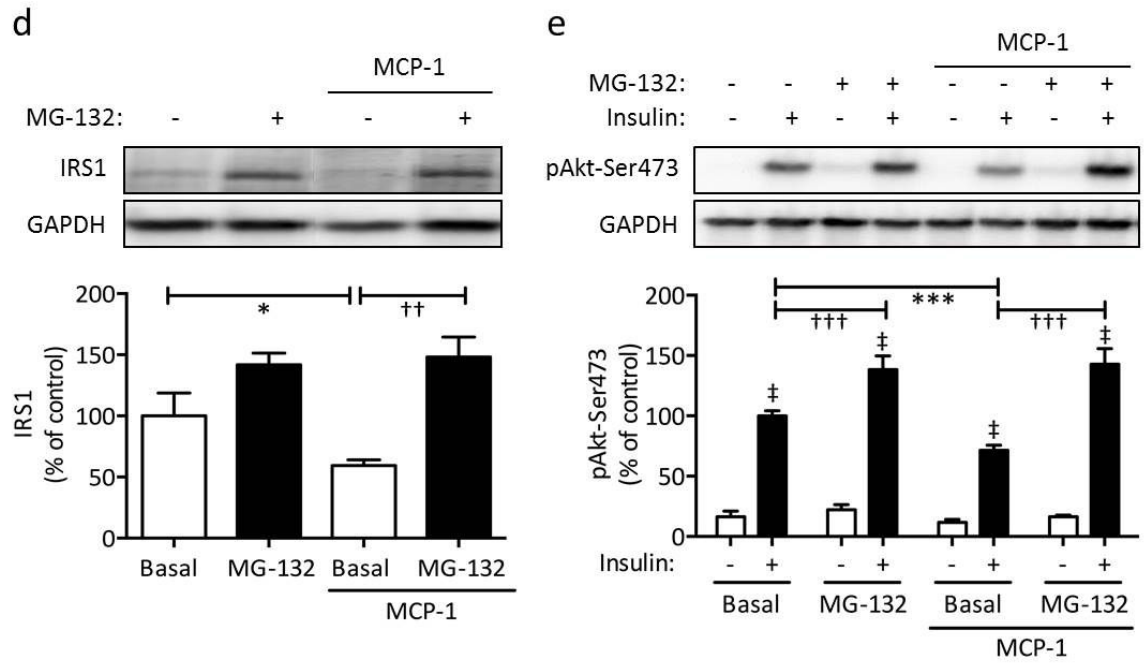


Figure 3.6 Inhibition of the proteasome prevents the induction of IRS1 degradation and insulin resistance by PRAS40 knockdown and monocyte chemoattractant protein 1. Lysates from human primary skeletal muscle cells transfected with either a non-target (NT) or PRAS40 siRNA and incubated with MG-132 or monocyte chemoattractant protein 1 (MCP-1) prior to insulin stimulation were analyzed for protein levels of IRS1 (a/d), and PRAS40 (b), as well as for phosphorylation of Akt-Ser473 (c/e). The protein signals were normalized for GAPDH abundance, which was not affected by the experimental conditions used. Data are presented as representative Western blots and bar graphs showing the mean ± standard error of the mean of the phosphorylation levels obtained in at least 5 independent experiments using cells from different donors. The values obtained for NT-siRNA transfected cells were considered as control and set at 100%. The effects of silencing PRAS40, MCP-1, and MG-132 were analyzed by two-way ANOVA. *, $p<0.05$, ***, $p<0.001$ versus cells transfected with NT-siRNA; †††, $p<0.001$; ††, $p<0.01$ indicates the effect of MG-132 versus basal; ‡, indicates $p<0.05$ for insulin versus basal.

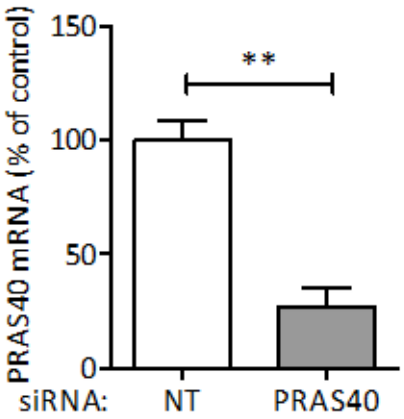


Figure 3.7 (supplementary) Effect of PRAS40 silencing on the mRNA expression of PRAS40. Human primary skeletal muscle cells were transfected with either a non-target (NT) or PRAS40 siRNA at day 3 of differentiation. Differentiated myotubes were harvested at day 7 of differentiation for analysis of mRNA expression of PRAS40 by real-time PCR. Data are presented as the mean ± standard error of the mean of 3 independent experiments using cells from different donors. Values obtained for NT-siRNA transfected cells were considered as control and set at 100%. The effect of silencing PRAS40 on mRNA expression was analyzed by a student's t-test. **, $p<0.01$ versus cells transfected with NT-siRNA.

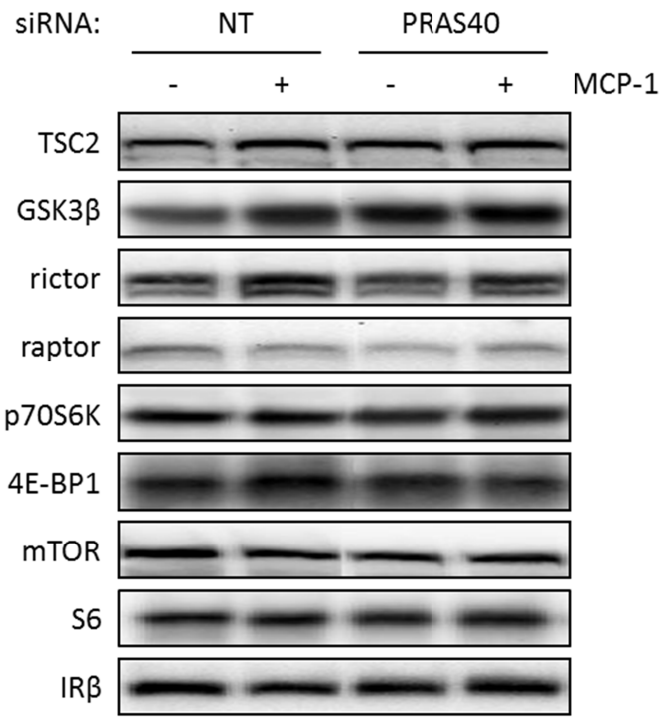


Figure 3.8 (supplementary) PRAS40 silencing and MCP-1 does not affect protein level of mTOR- and insulin signaling mediators. Human primary skeletal muscle cells were transfected with either a non-target (NT) or PRAS40 siRNA on day 3 of differentiation. At day 6 of differentiation, cells were serum-starved or incubated for 24h with 2 ng/ml monocyte chemoattractant protein 1 (MCP-1). Cell lysates were analyzed by Western blotting for total levels of TSC2, GSK3β, rictor, raptor, p70S6K, 4E-BP1, mTOR, and ribosomal protein S6. Membranes were reprobed with antibodies for the insulin receptor-β subunit (IRβ) to verify equal loading.

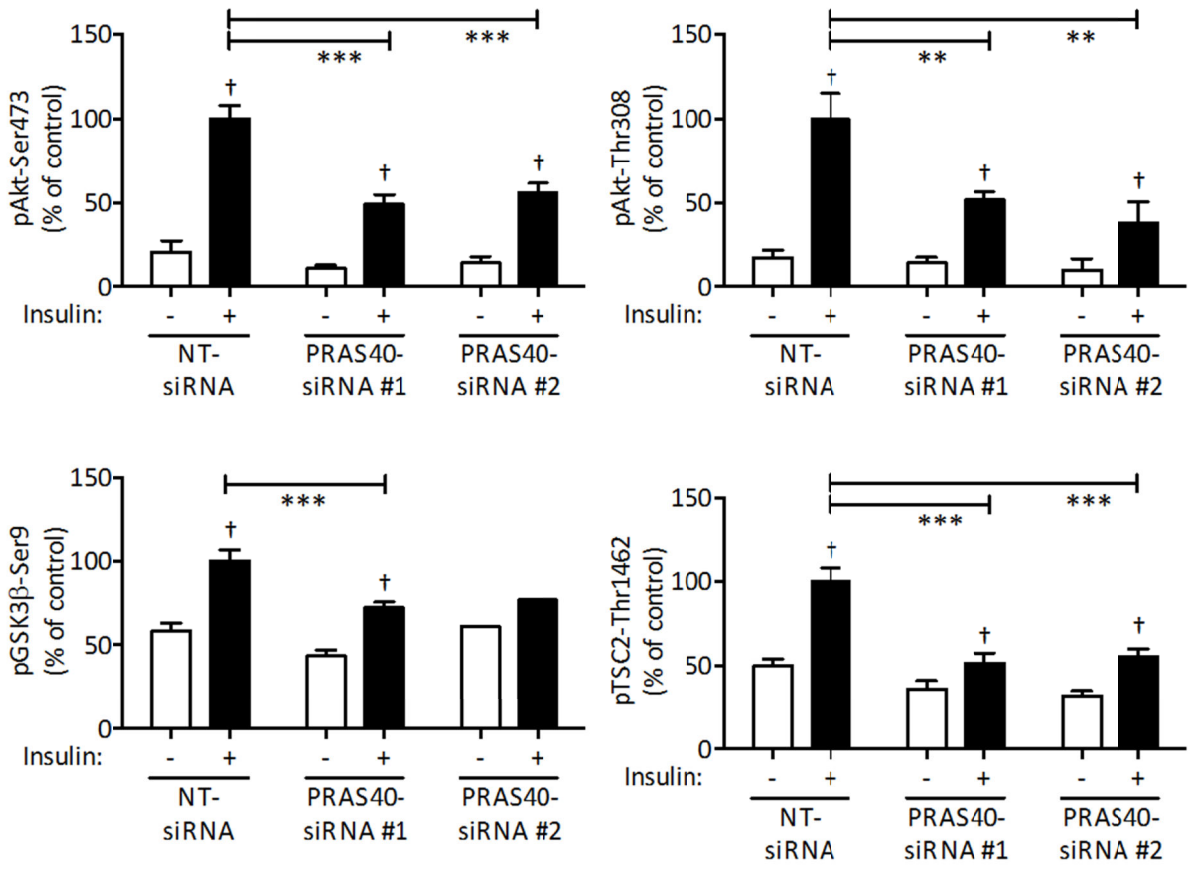


Figure 3.9 (supplementary) Effect of two different PRAS40 siRNAs on insulin-mediated Akt-phosphorylation. Human primary skeletal muscle cells were transfected with either a non-target (NT) or 2 distinct PRAS40 siRNAs on day 3 of differentiation. At day 6 of differentiation, cells were serum-starved for 24h. Then cells were kept untreated (-; open bars) or exposed for 10 min to 100 nmol/l insulin (+; filled bars). Cell lysates were analyzed by Western blotting for phosphorylation of Akt-Ser473, Akt-Thr308, GSK3β-Ser9, and p70S6K-Thr412. Data are presented as bar graphs showing the mean ± standard error of the mean of at least 5 independent experiments using cells from different donors. Values obtained for insulin-stimulated NT-siRNA transfected cells were considered as control and set at 100%. The effects of PRAS40 silencing on insulin action was analyzed using a two-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, $p<0.001$, **, $p<0.01$ versus cells transfected with NT-siRNA; † indicates $p<0.05$ for the effect of insulin- (+) versus untreated-cells (-)

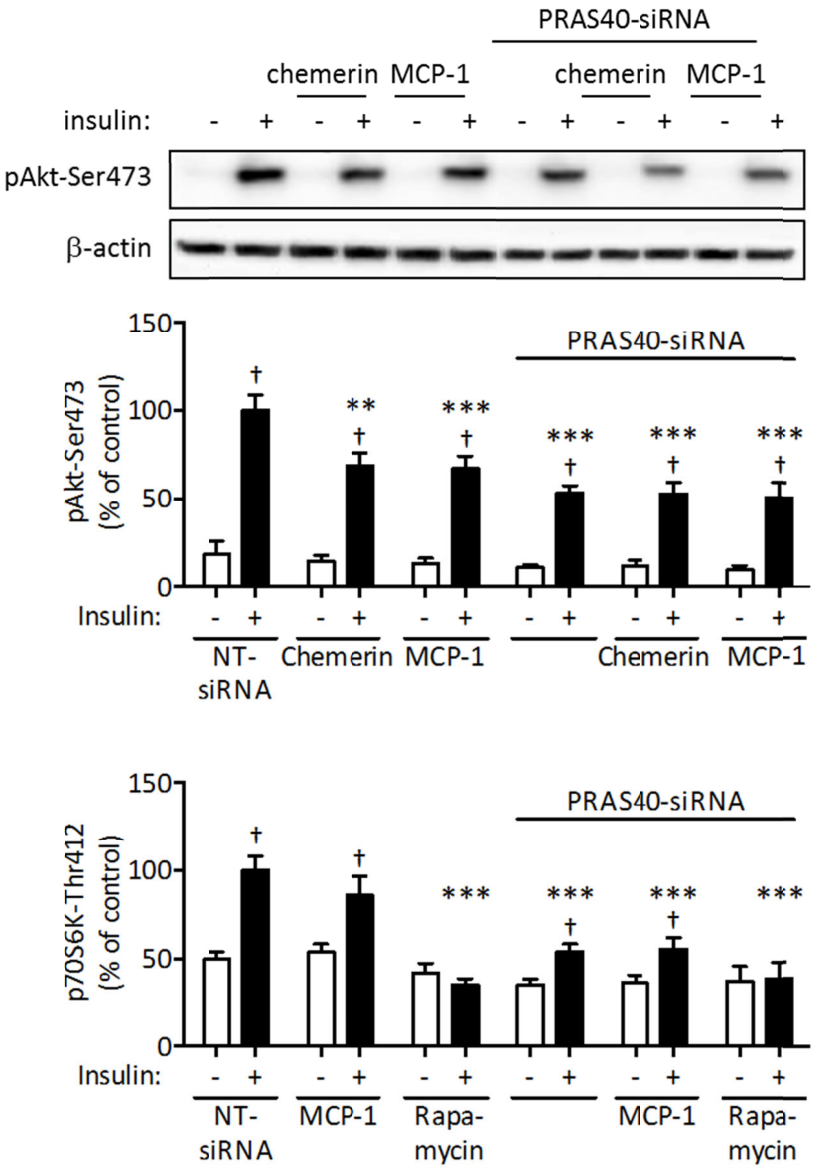


Figure 3.10 (supplementary) Effect of PRAS40 silencing and chemokines on insulin-action. Human primary skeletal muscle cells were transfected with either a non-target (NT) or PRAS40 siRNA on day 3 of differentiation. At day 6 of differentiation, cells were serum-starved or incubated for 24h with 2 µg/ml chemerin, or 2 ng/ml monocyte chemoattractant protein 1 (MCP-1). Then cells were kept untreated (-; open bars) or exposed for 10 min to 100 nmol/l insulin (+; filled bars). Cell lysates were analyzed by Western blotting for phosphorylation of Akt-Ser473, or by magnetic bead based assays for phosphorylation of p70S6kinase-Thr412 (b). Membranes were reprobed with antibodies for β-actin to verify equal loading. Data are presented as representative blots and a bar graph showing the mean ± standard error of the mean of at least 5 independent experiments using cells from different donors. Values obtained for insulin-stimulated NT-siRNA transfected cells were considered as control and set at 100%. The effects of PRAS40 silencing and chemokines were analyzed using a two-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, p<0.001, **, p<0.01, *, p<0.05 versus cells transfected with NT-siRNA; †, indicates P<0.05 for the effect of insulin- (+) versus untreated-cells (-).

Electronic supplementary material

Primers used for real-time PCR analysis

For IRS1, muscle RING-finger protein-1 (MuRF1), PRAS40, and peptidylprolyl isomerase A (PPIA), QuantiTect primer assays were purchased from Qiagen (Hilden, Germany). Atrogin-1 primers with the following sequences: forward-ATTAACGAAGCACAAGTCTG, reverse-GAATAAAGATGGCACCAAGG, and ribosomal protein 28 (RPS28) primers with the following sequences: forward-GGTCTGTCACAGTCTGCTCC, reverse-CATCTCAGTTACGTGTGGCG were designed using the NCBI Primerblast tool and ordered from Eurogentec (Seraing, Belgium).

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Wrote the manuscript: 70%
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CHAPTER 4

Study 3:

Overexpression of PRAS40 enhances insulin sensitivity in skeletal muscle

Overexpression of PRAS40 enhances insulin sensitivity in skeletal muscle †

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Abstract

The proline-rich Akt substrate of 40kDa (PRAS40) is a component and substrate of the mammalian target of rapamycin complex 1 (mTORC1), but also a prominent Akt-substrate in skeletal muscle. We recently observed that the knockdown of PRAS40 impaired insulin action by affecting the protein abundance of insulin receptor substrate 1 (IRS1). This study aimed to extend these findings by assessing whether overexpression of wild type PRAS40 (WT-PRAS40) enhances insulin action and protects against the induction of insulin resistance in skeletal muscle. Furthermore, we examined the effects of a mutant form of PRAS40, AAA-PRAS40, in which the two major phosphorylation sites (Ser183 and Thr246) as well as the potential mTORC1-binding site Phe129 were mutated into alanine, on insulin action. In WT-PRAS40, but not in AAA-PRAS40-expressing human skeletal muscle cells (hSkMC), the activation of the mTORC1-pathway by insulin was impaired. However, overexpression of WT-PRAS40 enhanced the insulin-mediated phosphorylation of Akt both in hSkMC and mouse skeletal muscle. Overexpression of mutant AAA-PRAS40 also increased insulin action in hSkMC, although to a lesser extent as compared to WT-PRAS40. The insulin-sensitizing effect of PRAS40 associated with increased IRS1 protein abundance and inhibition of proteasome activity. Finally, overexpression of WT-PRAS40 protected against hyperinsulinemia-induced insulin resistance in hSkMC by preventing the reductions in IRS1 protein levels and Akt-phosphorylation. Collectively, these findings identify PRAS40 as a regulator of insulin sensitivity in hSkMC. In contrast to the regulation of the activity of the mTORC1 pathway, this insulin-sensitizing action occurs independent of binding of PRAS40 to the mTORC1 complex

Abbreviations

4E-BP1, eukaryotic elongation 4E-binding protein 1; AAA-PRAS40??αMEM, α-modified Eagle's medium; FBXO32, F-box protein 32; GAPDH, anti-glyceraldehyde 3-phosphate dehydrogenase; Grb10, growth factor receptor binding protein 10; hSkMC, human skeletal muscle cells; IRβ insulin receptor beta chain IRS1, insulin receptor substrate 1; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; metS, metabolic syndrome; MuRF1, muscle ring finger 1; p70S6K , p70S6 kinase; PI3K**,** phosphatidylinositol-3'-kinase; PRAS40, proline-rich Akt substrate of 40 kDa; TA *Tibialis anterior*, T2D, type 2 diabetes, TSC2, Tuberous sclerosis complex 2; WT, wild type.

Introduction

The regulation of insulin sensitivity is essential for the maintenance of glucose homeostasis and is disturbed in patients with type 2 diabetes (T2D) and the metabolic syndrome (metS) resulting in insulin resistance. Because skeletal muscle is responsible for 80-90% of insulin-stimulated glucose disposal (1,2), insulin resistance in skeletal muscle has an essential impact on the pathogenesis of metS and T2D. Insulin resistance in skeletal muscle is characterized by disturbances in the insulin-mediated tyrosine phosphorylation of the insulin receptor substrate 1 (IRS1), which results in activation of phosphatidylinositol 3'-kinase (PI3K) and phosphorylation of Akt (3). Activation of the IRS1/PI3K/Akt-pathway regulates amongst others the translocation of the facilitative glucose transporter GLUT4 from intracellular compartments to the plasma membrane thereby increasing the rate of glucose transport into the cell (3).

A reduced activity of the IRS1/PI3K/Akt-pathway additionally impairs the insulin-mediated phosphorylation of one of the most prominent Akt-substrates, the proline-rich Akt substrate of 40 kDa (PRAS40), as demonstrated in skeletal muscle of humans with T2D and high-fat diet-fed rodents (4-6). Besides being an Akt-substrate, PRAS40 is also a component and a substrate of the mammalian target of rapamycin complex 1 (mTORC1) (7). This multiprotein complex regulates a variety of anabolic pathways, such as control of cell growth, cell survival, proliferation, metabolism and autophagy (7-9). Previous studies investigating the function of PRAS40 within the mTORC1 complex have yielded conflicting results (10-15). *In vitro* silencing and overexpression approaches mostly in immortalized cultured cell lines have ascribed both inhibitory and stimulatory functions to PRAS40 in the regulation of mTORC1 activity (10-15). Furthermore, a study in *Drosophila melanogaster* proposed that the regulation of mTORC1 activity by PRAS40 is tissue-specific and dependent on posttranslational modification of the protein (16). The function of PRAS40 within the insulin-signaling cascade is also incompletely understood. While multiple studies failed to demonstrate an effect of PRAS40 overexpression or silencing on insulin action in immortalized cell lines (7), we observed that the knockdown of PRAS40 expression in primary human skeletal muscle cells (hSkMC) resulted in the proteasome-mediated degradation of IRS1 (17). This was accompanied by an inhibition of the insulin-mediated activation of the Akt signaling pathway as well as GLUT4-mediated glucose uptake (17). Furthermore, mutation of the nuclear export sequence within PRAS40, resulting in enforced nuclear accumulation and absence of PRAS40 in the cytosol, also impaired insulin action by lowering IRS1 protein levels (18). Because of these modulating effects of PRAS40 on insulin action, the aim of this study was to investigate whether overexpression of wild type PRAS40 (WT-PRAS40) enhances skeletal muscle insulin sensitivity and therefore has a protective effect against the induction of

insulin resistance in this tissue. In addition, we investigated whether the modulating effect of PRAS40 is dependent on its function on mTORC1 signaling by overexpression of a mutant form of PRAS40 in which the two best-characterized phosphorylation sites (Ser183 and Thr246) as well as Phe129 in the potential mTORC1 binding site (TOS-motif) were changed into alanine (AAA-PRAS40) (4,7,15).

Methods

Cultivation and differentiation of primary human skeletal muscle cells

Primary human skeletal muscle cells (hSkMC) isolated from *rectus abdominis* muscle of three healthy Caucasian donors (2 males, 16 and 18 of age; 1 females 33 of age) were supplied as proliferating myoblasts from PromoCell (Heidelberg, Germany) or Lonza (Basel, Switzerland). Myoblasts were seeded in six-well culture dishes and were cultured in growth medium α-modified Eagle's (αMEM)/Ham's F-12 medium (Gibco, Berlin, Germany) containing a supplement pack for skeletal muscle cells from PromoCell (Heidelberg, Germany). When cells reached near-confluence, differentiation of myocytes was induced by replacing the growth medium with differentiation medium, containing αMEM and 2 % horse serum (Gibco). All experiments were performed at day 7 of differentiation, after cells were starved overnight in αMEM without serum. Acute insulin stimulation was performed with 100 nM insulin (porcine insulin, Sigma Aldrich, St Louis, MO, USA) for 10 min. Hyperinsulinemia was mimicked by incubating myotubes at day 6 of differentiation with 50 nM insulin for 24h. Then cells were washed with αMEM and incubated for 1h before acute stimulation with 100 nM insulin and harvesting.

Lentiviral infection of hSkMCs

Empty vector (EV) or constructs expressing human PRAS40 (WT-PRAS40) were generated as described previously (4). Via mutagenesis-PCR Ser at position 183, Thr at position 246, and Phe at position 129 were mutated into Ala (Ser183Ala/Thr246Ala/Phe129Ala; AAA-PRAS40). These constructs were used to produce infectious virus particles (LV). Together with helper plasmids encoding HIV-1 gag-pol, HIV-1 rev, and the VSV-G envelope the respective constructs were cotransfected in virus-producing HEK293t cells as described previously (19). Virus titer was quantified using a HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrixCorp., New York, USA). For overexpression of wild type or mutant PRAS40, cells were infected at day 3 of

differentiation with a MOI of 5. One day after transduction, the medium was replaced by differentiation medium. At day 7 of differentiation, cells were starved overnight in αMEM without serum, and left untreated or incubated with insulin (100 nM; 10 min) before harvesting

Analysis of insulin signaling in hSkMCs

Skeletal muscle cells were lysed and analyzed using Western Blot as described previously (17). Protein bands were visualized by the enhanced chemiluminescence method using Immobilion Western detection reagents (Millipore, Schwalbach, Germany) on a VersaDoc 4000 MP (BioRad, Munich, Germany) work station. Quantification was performed using Quantity one analysis software (Version4.6.7). The following antibodies were purchased from Cell Signaling Technology (Denver, MA, USA): anti-phospho-Akt Ser473 and Thr308, anti-Akt, anti-phosho PRAS40 Thr246, anti-PRAS40, anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), anti-tubulin. Anti-insulin receptor beta chain (IRβ) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti- GLUT4 antibody from R&D Systems (Minneapolis, USA) and anti-IRS1 from Upstate (Millipore, Charlottesville, USA).

Glucose-uptake

Cells were serum-starved for 18h and used for analysis on day 7 of differentiation. After an acute insulin stimulus (100 nM; 30 min) radioactively-labelled 2-desoxy-D-^{14}C-glucose (2-^{14}C-DOG) was added and uptake was assayed for 2h as described previously (20).

RNA-isolation and quantitative real-time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer´s instruction. The RNA was reverse-transcribed into cDNA using the GoScript™ Reverse Transcriptase system (Promega, Mannheim, Germany), and gene expression levels were quantitated by real-time PCR on a Step One Plus Cycler (Applied Biosystems, Carlsbad, CA, USA) using GoTaq® qPCR Master Mix (Promega). For IRS1 and MuRF1, QuantiTect primer assays were purchased from Qiagen (Hilden, Germany). FBXO32 (also known as atrogin-1) primers with the following sequences: forward-ATTAACGAAGCACAAGTCTG, reverse-GAATAAAGATGGCACCAAGG, and ribosomal protein 28 (RPS28) primers with the following sequences: forward-GGTCTGTCACAGTCTGCTCC, reverse-CATCTCAGTTACGTGTGGCG were designed using the NCBI Primerblast tool and ordered from Eurogentec (Seraing, Belgium). The ΔΔCt method was used to normalize the expression data using

RPS28 as housekeeping gene. The Ct values for RPS28 showed no variations in response to the various treatments applied to the hSkMC in this study.

Proteasome activity assay

Proteasome activity was determined in serum-starved myotubes grown in 96-well plates at day 7 of differentiation. Analysis of the caspase-like proteasome activity was performed using the Proteasome-Glo cell-based assay (Promega) according to the manufacturer's protocol as described previously (17). When indicated, cells were incubated with 3µmol/l MG-132 (Calbiochem, Darmstadt, Germany) for 15 min before assay was started. The luminescence was measured using a Tecan Infinite 200 reader (Tecan, Maennersdorf, Germany).

In vivo muscle electroporation (IVE) and ex vivo insulin stimulation in isolated muscles

The animal experiments were performed in accordance with the 'Principle of laboratory animal care' (NIH publication No. 85-23, revised 1996) and the procedures were approved by the local council of animal care in line with the current version of the German Law on the protection of animals. The *in vivo* muscle electroporation procedure was adapted from previous reports (21-23). Briefly, plasmids were purified using an Endotoxin-free Mega-Prep kit (Qiagen, Hilden, Germany), including elution of the DNA in sterile 0.9% NaCl. For electroporation, 12-16 weeks-old C57BL/6J mice were anesthetized using isoflurane and their hindlimbs were shaved. Subsequently, 15 units of hyaluronidase (Sigma, Munich, Germany) dissolved in 30 µl sterile 0.9% NaCl were injected in the *Tibialis anterior* (TA) muscles of both legs. Following a 1 h recovery in their cages, the mice were anesthetized again with isoflurane and 15 µg vector DNA in 30 µl of sterile saline was injected into the TA muscles of both legs. In each animal, one muscle was used as a control and injected with a GFP-expressing control vector. The DNA injection was followed by the application of a pair of tweezer electrodes across the distal limb connected to an ECM-830 electroporator device (BTX, Electro Square Porator ECM 830). The electroporation protocol involved 8 20-ms pulses of 80 V with an interval of 1 sec. After 7 days, mice were anesthetized by an intraperitoneal injection of (500 mg/kg body weight) 2,2,2-Tribromoethanol (Sigma). Then, TA muscles were dissected and subjected to vials containing pre-oxygenated (95% O_2/ 5% CO_2) Krebs-Henseleit buffer (KHB) containing 5 mM HEPES and supplemented with 5 mM glucose and 15 mM mannitol. After muscle dissection, animals were sacrificed by cervical dislocation. All incubation steps were performed under continuous gassing (95% O_2/ 5% CO_2) at 30°C and slight agitation in a shaking waterbath. After recovery, muscles were

transferred to new vials and incubated for 30 min in KHB/ 5 mM HEPES/ 15 mM mannitol/ 5 mM glucose under basal condition or in the presence of 120 nM insulin (Actrapid, Novo Nordisk, Mainz, Germany). Muscles were immediately snap frozen in liquid nitrogen and stored at -80°C for subsequent signal transduction analysis..

Analysis of insulin signaling in electroporated mouse muscle

Muscles were homogenized in buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton-X-100, and a proteinase inhibitor cocktail as well as a Phosphatase inhibitor cocktail (both from Roche, Mannheim, Germany) using a Tissuelyzer (Qiagen). Lysates were centrifuged for 10 min at 16.000 x g at 4°C. Protein content was determined in the supernatant using the BCA Protein Assay Kit (Pierce, Rockford, USA) according to the manufacturer´s instruction. Protein samples (20 µg) were separated by SDS-PAGE and transferred onto polyvinylidine fluoride membranes by immunoblot analysis as described under '*Analysis of insulin signaling in hSkMC'.*

S*tatistical analysis*

Data are presented as means ± SEM. Significant differences were determined by one- or two-way ANOVA followed by Bonferroni multiple comparison analysis as indicated in detail in the legends to the figures. Statistical analysis was performed using Prism 6 for Mac OS X (Graphpad, La Jolla, CA). *P*-values of <0.05 were considered as statistically significant

Results

Overexpression of PRAS40 improved insulin action but decreased mTORC1 signaling in hSkMC

Transduction of hSkMC with lentiviruses encoding WT-PRAS40 or AAA-PRAS40 resulted in a 5.6- and 4.6-fold increases in PRAS40 protein abundance, respectively, versus cells transduced with lentivirus particles containing an empty vector (EV) (Figure 4.1a). Neither overexpression of WT-PRAS40 nor of AAA-PRAS40 affected the protein abundance of IR-β, Akt, or GLUT4 (Figure 4.1b-d). However, IRS1 protein levels were increased by 25% in cells overexpressing WT-PRAS40 versus EV (Figure 4.1e). The elevated IRS1 protein abundance associated with increases in insulin-stimulated Akt phosphorylation at Ser473 and Thr308 (44% and 37%), and elevated insulin-stimulated glucose uptake (23%) in hSkMC expressing WT-PRAS40 versus EV (Figure 4.2a-d). Interestingly, this beneficial

effect of PRAS40 was also observed, although to a lesser extent following expression of AAA-PRAS40. In hSkMC expressing AAA-PRAS40, IRS1 levels were increased by 19% (Figure 4.1e) and insulin-mediated phosphorylation of Akt at both phosphorylation sites examined was enhanced by 15% (Figure 4.2a-c). However, in contrast to WT-PRAS40, expression of mutant AAA-PRAS40 did not result in enhanced insulin-stimulated glucose uptake (Figure 4.2d).

Previous studies have identified PRAS40 as inhibitor of mTORC1-signaling (7). Here, we investigated mTORC1 activity by examining the phosphorylation of p70S6K-Thr389 as well as S6-Ser240. Insulin-stimulated phosphorylation of p70S6K was reduced by nearly 35% (Figure 4.2e) while phosphorylation of S6 was even completely abrogated in WT-PRAS40 expressing mytotubes (Figure 4.2f). In contrast, there were no significant differences in the induction of p70S6K- and S6 phosphorylation by insulin between hSkMC expressing mutant AAA-PRAS40 and EV (Figure 4.2e-f), indicating that phosphorylation and/or mTORC1-binding of PRAS40 is essential for the inhibitory function of PRAS40 on mTORC1.

Overexpression of PRAS40 impairs the activity of the proteasome in hSKMC

We previously showed that silencing PRAS40 in hSkMC decreased the protein abundance of IRS1 through activation of the proteasome, which resulted in impaired insulin action (17). Here, we analysed whether the protective effect of PRAS40 on insulin sensitivity could be ascribed to an inhibition of the proteasome. As shown in Figure 4.3a, both the expression of WT-PRAS40 as well as of AAA-PRAS40 were found to lower the caspase-like activity of the proteasome. This was accompanied by a reduction in the mRNA levels of the muscle-specific E3-ligase *MuRF1,* but not of *FBXO32* (Figure 4.3b-c).

Overexpression of PRAS40 improved insulin action in TA muscle in vivo

The overexpression studies described above suggest a function for PRAS40 as sensitizer of insulin action. To substantiate this notion, we used *in vivo* electroporation to increase PRAS40 levels in *Tibialis anterior* (TA) muscle of C57BL/6J mice. Figure 4.4a shows that protein levels of PRAS40 were increased by nearly 2-fold as compared to TA-muscles expressing the empty control vector (EV). The increase in PRAS40 protein levels was accompanied by a 27% increase in the protein abundance of IRS1 (Figure 4.4b). In contrast, Akt and GLUT4 protein levels were not altered in PRAS40 overexpressing TA muscles (Figure 4.4c-d). In line with our observations in hSkMC, the insulin-

stimulated phosphorylation of Akt at Thr308 and Ser473 were increased by 2.2- and 1.6-fold, respectively in muscles expressing PRAS40 as compared to empty vector (Figure 4.5).

Overexpression of WT-PRAS40 protects against hyperinsulinemia-induced insulin resistance in hSkMCs

To further validate the potential function of PRAS40 as sensitizer of insulin action, we subsequently examined whether overexpression of the protein protects against the induction of insulin resistance. It is well established that chronic exposure to insulin results in a down-regulation of insulin-mediated PI3K/Akt signaling likely via increased internalization and degradation of the insulin receptor as well as degradation of IRS1/IRS2 (24,25). Accordingly, an *in vitro* approach mimicking hyperinsulinemia via long-time (24h) incubation of mytubes with 50nM insulin, significantly impaired the insulin-stimulated phosphorylation of Akt at Thr308 and Ser473 (Figure 4.6a-b) as well the protein abundances of IR-β (32%) (Figure 4.6c) and IRS1 (30%) (Figure 4.6d). Overexpression of WT-PRAS40, but also AAA-PRAS40, improved insulin-stimulated phosphorylation of Akt at Thr308 (Figure 4.6a) in myotubes chronically exposed to insulin as compared to hSkMC transduced with empty vector. The hyperinsulinemia-induced inhibition of insulin-mediated Akt-Ser473 phosphorylation was partially restored by expression of WT-PRAS40, but not by AAA-PRAS40 (Figure 4.6b). Whereas the reduction of IR-β levels by chronic insulin exposure was not rescued by PRAS40 overexpression (Figure 4.6c), overexpression of WT-PRAS40 increased IRS1 protein abundance by 23% (Figure 4.6d). These data further corroborate our findings that PRAS40 functions as a modulator of insulin sensitivity by regulating the protein levels of IRS1.

Discussion

This study extends our previous reports (17,18) demonstrating that PRAS40 is a modulator of insulin action in skeletal muscle. We previously reported that knockdown of PRAS40 as well as enforced nuclear localization of PRAS40 lowered IRS1 protein abundance via activation of the proteasome. This was accompanied by an impaired activation of the IRS/PI3K/Akt pathway by insulin. The present study shows that overexpression of WT-PRAS40 *in vitro* as well as *in vivo* results in increased IRS1 protein abundance, which resulted in enhanced insulin-mediated Akt phosphorylation and increased glucose uptake *in vitro*. This enhanced insulin sensitivity was accompanied by a reduced activity of the proteasome mediating likely the beneficial effect of PRAS40 overexpression on IRS1 stability. Importantly, these beneficial effects of PRAS40 seemed to be exclusively affecting IRS1, since Akt, GLUT4, and IRβ protein abundances were unaffected upon PRAS40 overexpression.

Finally, we could demonstrate that overexpression of PRAS40 partially protects against insulin resistance induced by chronic insulin treatment.

Multiple studies have proposed an important role for PRAS40 in the regulation of mTORC1 activity (12,13,15,26). Specifically, the direct binding of PRAS40 through its TOS motif to raptor, may sequester mTORC1 away from other substrates, such as p70S6K and 4EBP1, and thereby impair their activation (11,12,14,26). Our data confirm this critical role for PRAS40 in the regulation of mTORC1 function. Overexpression of WT-PRAS40 significantly decreased p70S6K and S6 phosphorylation, hinting towards a reduction of the capability of mTORC1 to promote p70S6K activation. While the inhibitory effects of PRAS40 knockdown and enforced nuclear localization of PRAS40 on mTORC1 activity are probably mediated by a secondary effect via inhibition of the IRS1/Akt/TSC2 pathway (17,18), PRAS40 overexpression modulates mTORC1 activity independent of the IRS1/Akt/TSC2 axis. Due to increased activity of IRS1 and Akt in PRAS40 overexpressing muscle cells, the inhibition of mTORC1 activity seemed to be directly mediated by enhanced PRAS40 protein abundance. Furthermore, overexpression of a mutant form of PRAS40, in which both phosphorylation sites (Ser183 and Thr246) as well as the TOS motif (Phe129) were mutated, did not impact mTORC1 activity indicated by comparable insulin-stimulated phosphorylation of p70S6K as well as S6 in AAA-PRAS40-expressing myotubes versus cells transduced with empty vector. This illustrates that full phosphorylation of PRAS40 and/or binding of PRAS40 to mTORC1 is essential for the regulation of mTORC1 activity by PRAS40.

Our data further show that the modulatory effect of PRAS40 on insulin action does not require the binding of PRAS40 to mTORC1 since both the overexpression of WT- and AAA-PRAS40 resulted in increases in IRS1 protein abundance and associated enhanced phosphorylated of Akt after insulin stimulation. This is in contrast to suggestions from previous reports, which proposed that inhibition of p70S6K by PRAS40 overexpression reduced the inhibitory serine phosphorylation on IRS1, and thereby increases phosphorylation of Akt (27-29). However, inhibition of p70S6K does not restore IRS1 protein abundance and insulin sensitivity in hSkMC in which PRAS40 expression was silenced (17). The present data obtained with the AAA-PRAS40 mutant further argue against the importance of the mTORC1/p70S6K-pathway in the regulation of insulin sensitivity by PRAS40. Rather, our data provide further support for the regulation of the activity of the proteasome by PRAS40, as illustrated by the observation that overexpression of PRAS40 reduces the activity of the caspase-like activity of the 26S proteasome, and lowers the mRNA expression of the muscle specific E3 ligase *MuRF1*.

Importantly, WT-PRAS40 and AAA-PRAS40 were equally active in inhibition of the proteasome pathway and stabilization of IRS1 protein levels. However, WT-PRAS40 was more potent

when its considering the 'insulin-sensitizing' potential as compared to AAA-PRAS40. Similar findings were obtained when considering the protective effect of AAA-PRAS40 against hyperinsulinemia-induced insulin resistance. The reason for these discrepancies in activity of regulation of the proteasome/IRS1-axis and insulin-mediated Akt phosphorylation and glucose uptake, especially after hyperinsulinemia, is not completely clear. Yet, these findings are in support with other studies that alterations in IRS1 protein abundance do not necessarily correlate with alterations in Akt signaling and glucose uptake. Comparable observations were made by Hoehn and collegaeus, who reported an uncoupling between IRS1 tyrosine phosphorylation and Akt phosphorylation in an *in vitro* model for chronic hyperinsulinemia (30). These authors suggest that additional aspects could affect IRS1 function, like subcellular localization (30). Also others reported a non-linearity between IRS1 and downstream signaling to Akt. In mouse muscle, shRNA-mediated silencing of IRS1 did not impair glucose disposal (31). Finally, a decrease in IRS1 activity did not affect full Akt phosphorylation by insulin in muscle tissues of insulin-resistant mice (32). In addition to this unanswered question, another limitation of the present study is that we did not further detail the underlying mechanism how PRAS40 interacts and modulates proteasome function. Nevertheless, this study provides further insight into the function of PRAS40 in the insulin signaling pathway in skeletal muscle. While overexpression of WT-PRAS40 impairs mTORC1 activity, the increase in insulin sensitivity by PRAS40 overexpression is independent of mTORC1 activity. Furthermore, PRAS40 modulates IRS1 stabilization through regulation of the proteasome machinery. Finally, while posttranslational modification as well as binding of PRAS40 to mTORC1 seems to be essential for PRAS40 function on mTORC1, inhibition of the proteasome activity is still occurring in cells overexpressing AAA-PRAS40. Collectively, these findings identify PRAS40 as regulator of insulin sensitivity in hSkMC.

Figures

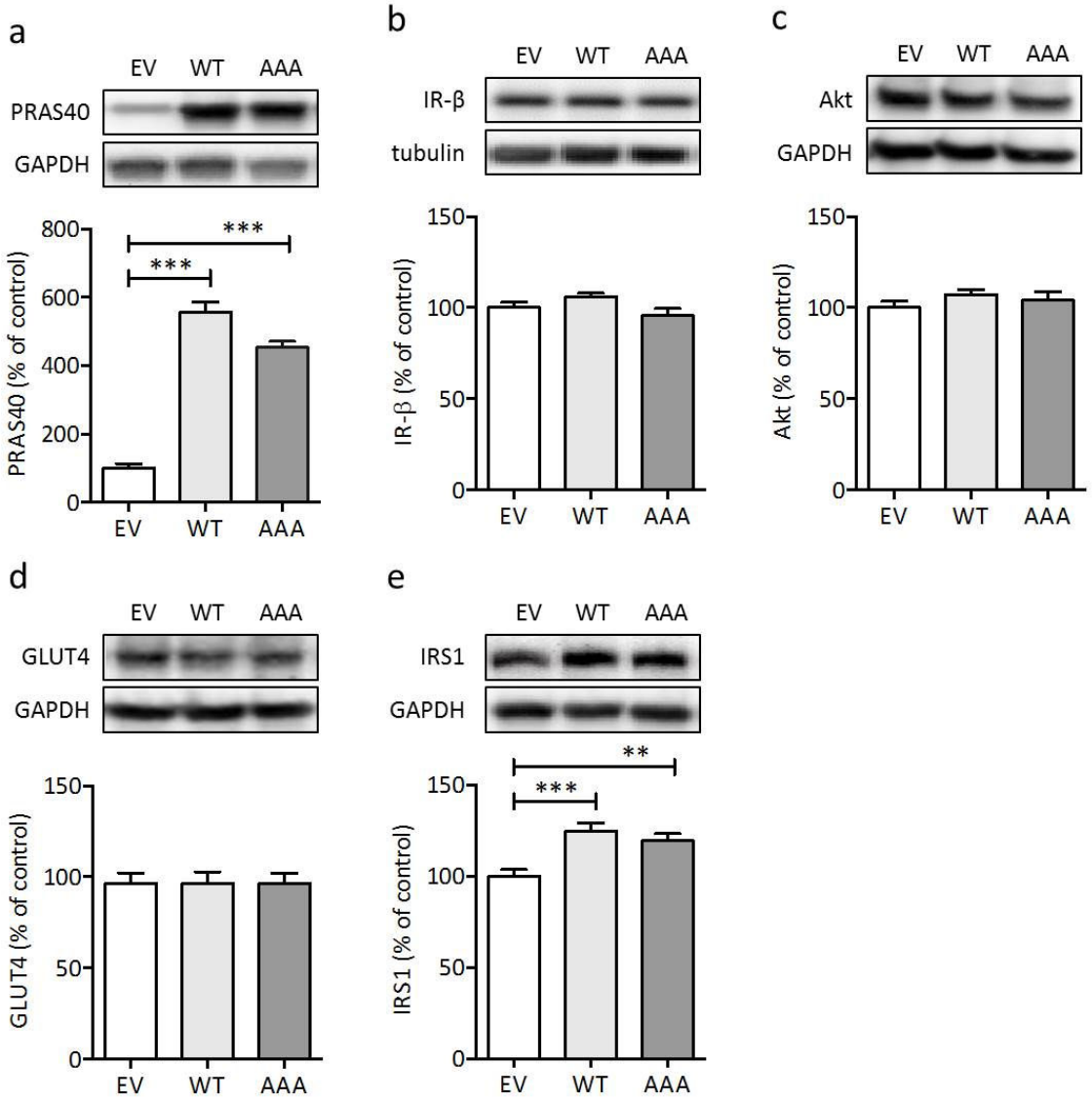

Figure 4.1 Effect of WT- and AAA-PRAS40 overexpression on the protein expression of key components of the insulin signaling pathway. Lysates from human primary skeletal muscle cells transduced with lentiviruses encoding either expressing an empty vector (EV), wild type PRAS40 (WT) or a mutant of PRAS40 (AAA) were analyzed for protein levels of PRAS40 (A), the insulin receptor (IR) β-subunit (B), Akt (C), GLUT4 (D), and insulin receptor substrate 1 (IRS1) (E). Protein levels were normalized for GAPDH or tubulin protein levels, which were not affected by the experimental conditions used in this study. Data are presented as representative Western blots and bar graphs showing the mean ± standard error of the mean of 3-15 independent experiments using cells from different donors. Values obtained for EV-transduced myotubes were considered as control and set at 100%. The effects of WT- or AAA-PRAS40 overexpression on protein levels and mRNA expression were analyzed by one-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$ versus EV-control cells.

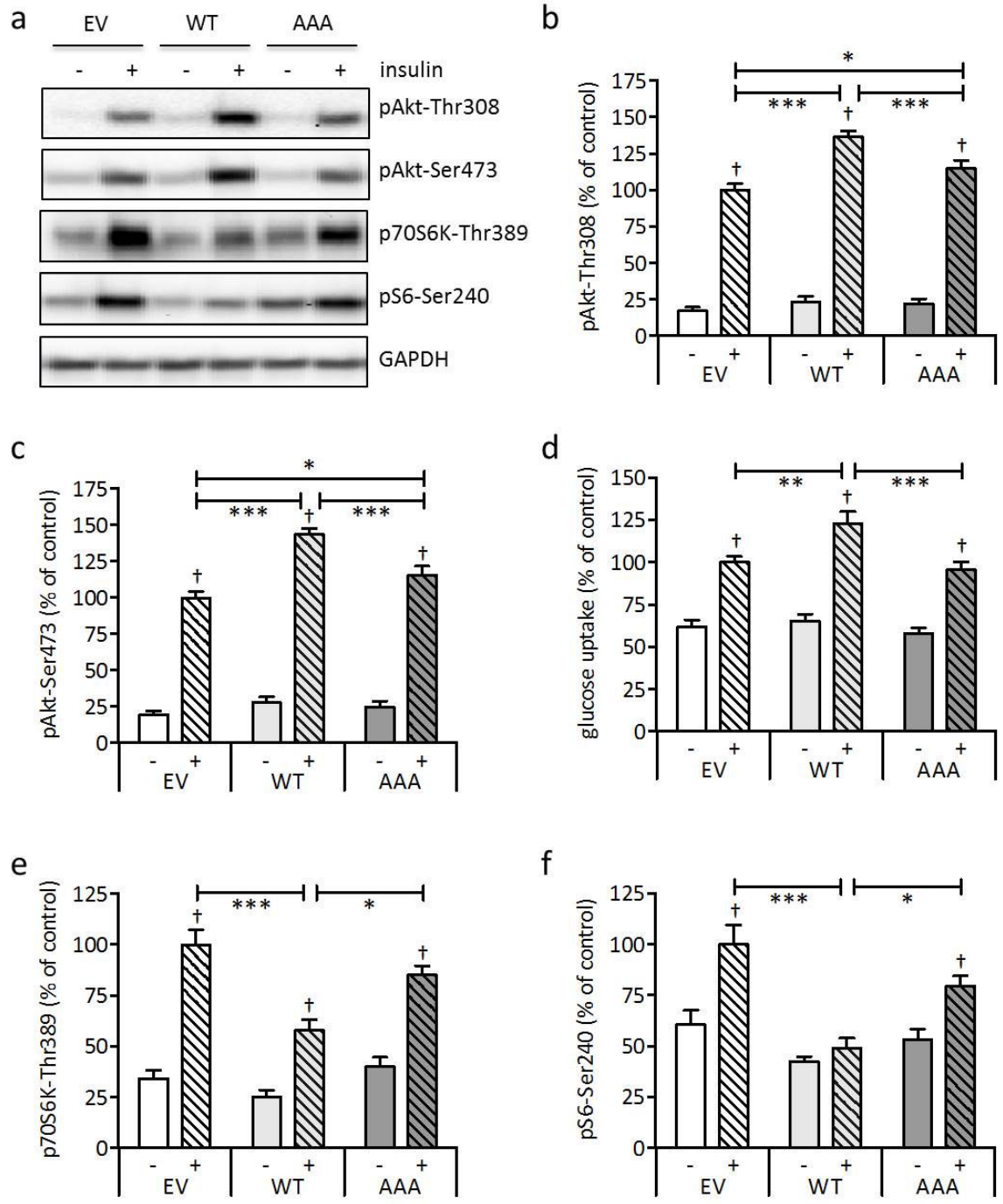

Figure 4.2 Effect of WT- and AAA-PRAS40 overexpression on insulin-mediated activation of the Akt and mTORC1 signaling pathway and the regulation of glucose uptake. Representative blots (A) and quantifications for the effect of wild type (WT) or mutant AAA-PRAS40 versus cell transduced with empty vector (EV) on insulin-mediated phosphorylation of Akt-Thr308 (B), Akt-Ser473 (C), glucose uptake (D), and insulin-induced phosphorylation of p70S6K-Thr389 (E) and S6-Ser240 (F). Phosphorylation signals were normalized for GAPDH abundance, which was not affected by the experimental conditions used. Data are presented as representative Western blots and bar graphs showing the mean ± standard error of the mean of at least 8 independent experiments using cells from different donors. Open bars depict basal (-) conditions, and hatched bars represent insulin-treated cells (+). The values obtained for EV-transduced insulin-treated cells were considered

as control and set at 100%. The effects of PRAS40 overexpression on insulin-mediated glucose uptake (D) are the mean ± standard error of the mean of 5 independent experiments using cells from different donors. The effects of WT- and AAA-PRAS40 expression on insulin action were analyzed using a two-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, $p<0.001$; **, $p<0.01$, *, $p<0.05$ versus EV-control cells; †, indicates $p<0.05$ for the effect of insulin- versus untreated-cells.

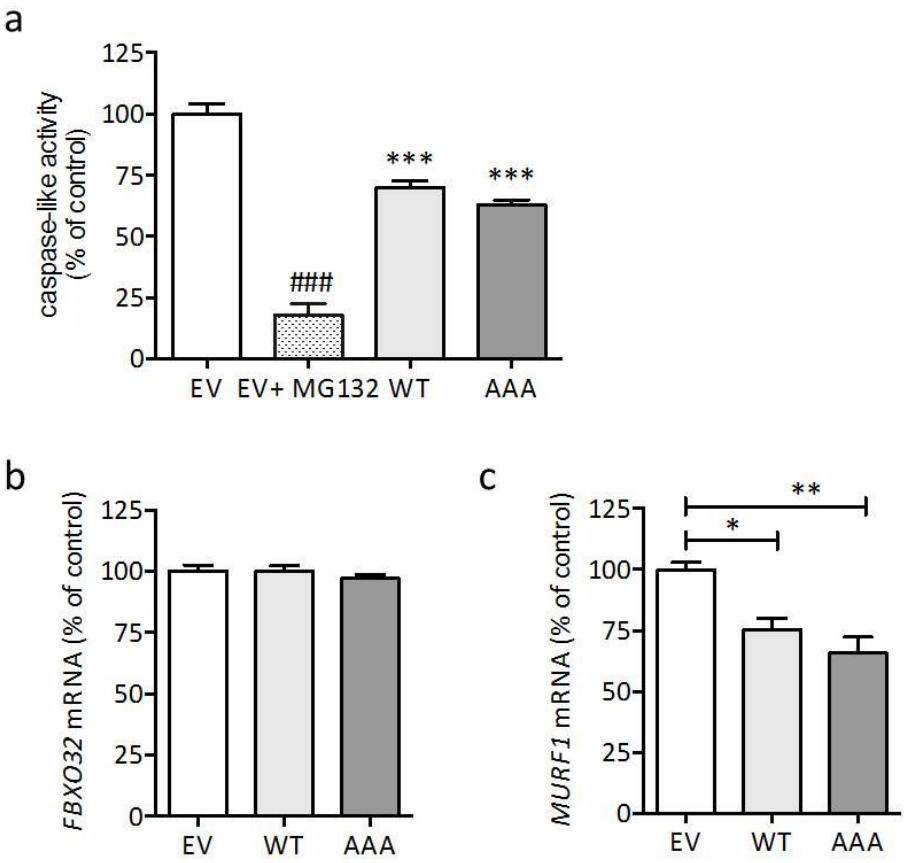

Figure 4.3 Effect of WT- and AAA-PRAS40 overexpression on proteasome activity. Human primary skeletal muscle cells were transduced with lentivirus particles either expressing an empty vector (EV), wild type PRAS40 (WT) or a mutant of PRAS40 (AAA) and analyzed for caspase-like proteasome activity (A), as well as mRNA expression of *FBXO32* (B) and *MuRF1* (C). As a control for the proteasome assay EV-transduced cells were treated with the proteasome inhibitor MG132 for 15 min prior assay performance. Data are expressed as mean ± standard error of the mean of at least 4 independent experiments using cells from different donors The values obtained for EV-transduced cells were considered as control and set at 100%. Effects of WT- and AAA-PRAS40 overexpression on gene expression and proteasomal activity were analyzed by one-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, $p<0.001$; **, $p<0.01$, *, $p<0.05$ versus EV-control cells; ###, $p<0.001$ for the effect of MG132.

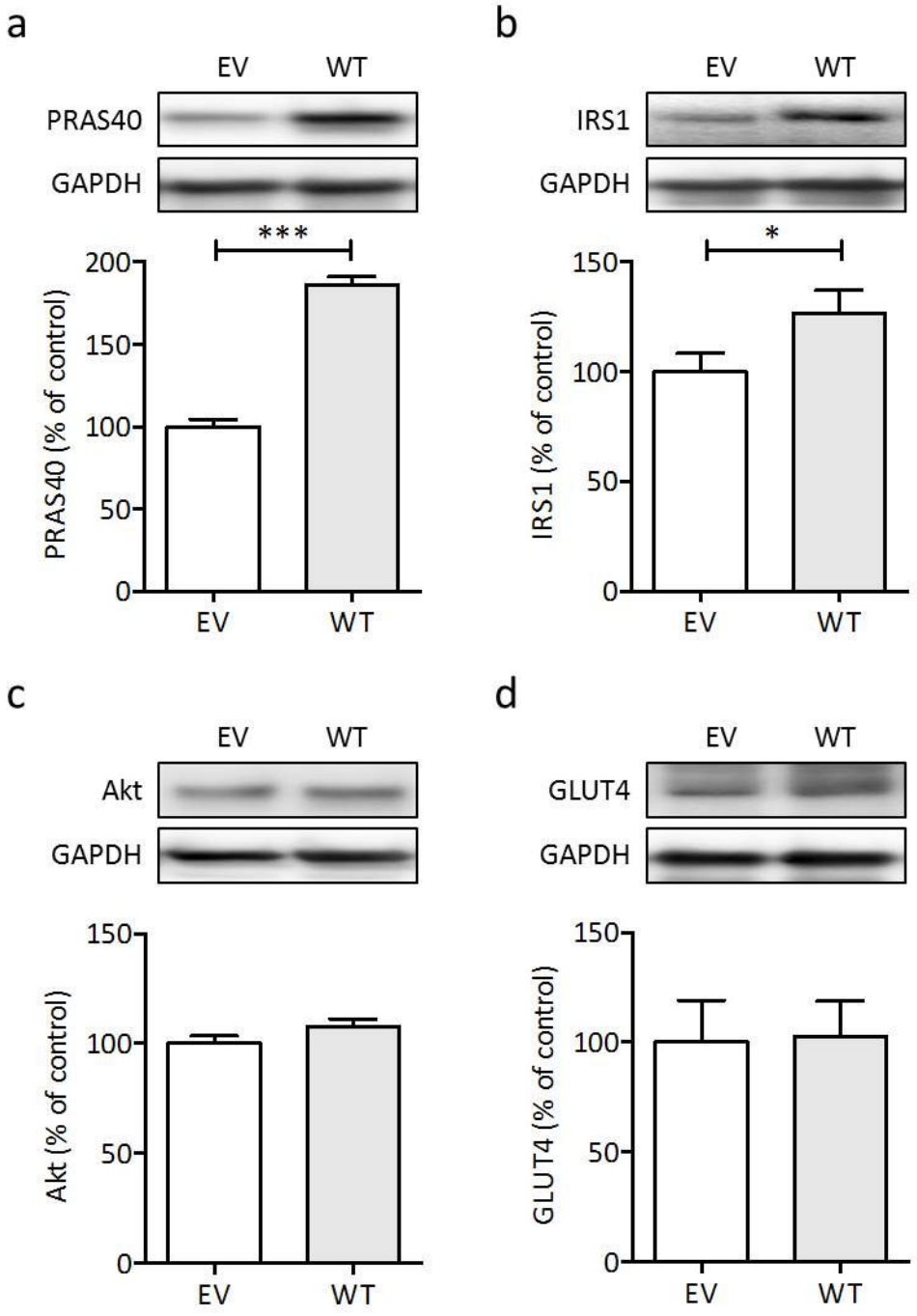

Figure 4.4 Effect of PRAS40 overexpression *in vivo* on protein expression of insulin signaling proteins. TA muscles were electroporated *in vivo* with empty vector (EV) or plasmid encoding wild type PRAS40 (WT). Lysates were analyzed for protein levels of PRAS40 (A), IRS1 (B), Akt (C), GLUT4 (D) (n=12). Signals were normalized for GAPDH protein levels, which were not affected by the experimental conditions used. Data are presented as representative Western blots and bar graphs showing the mean ± standard error of the mean. The values obtained for EV-transfected muscles were considered as control and set at 100%. Effects of WT-PRAS40 overexpression were analyzed by student's t-test. ***, $p<0.001$; *, $p<0.05$ versus EV-control muscle.

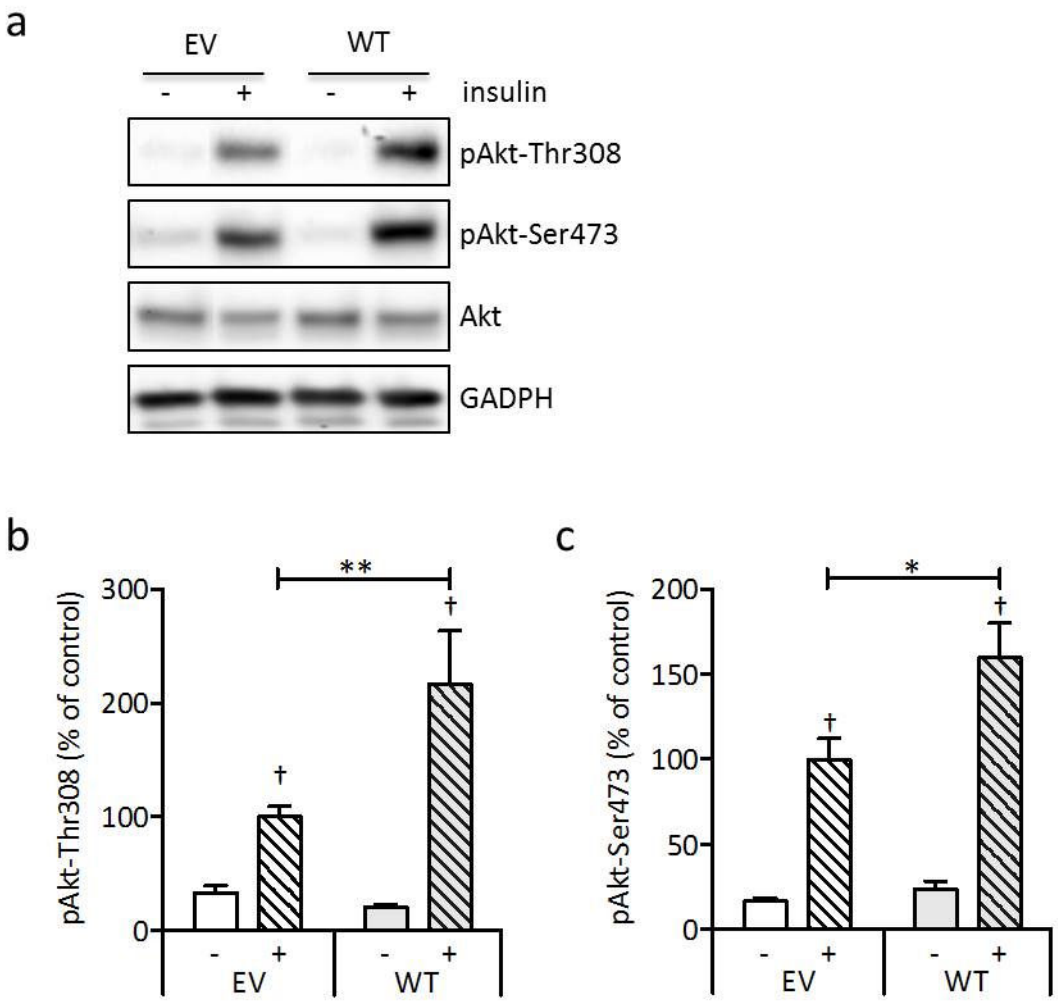

Figure 4.5 Effect of PRAS40 overexpression *in vivo* on insulin action. TA muscles were electroporated *in vivo* with empty vector (EV) or plasmid encoding wild type PRAS40 (WT). Lysates were analyzed for phosphorylation of Akt-Thr308 (B) and Akt-Ser473 (C). Signals were normalized for GAPDH protein levels, which were not affected by the experimental conditions used. Data are presented as representative Western blots for p-Akt-Thr308, p-Akt-Ser473, total Akt, and GAPDH (A), and bar graphs showing the mean ± standard error of the mean of 7 independent experiments with (+) and without (-) *ex vivo* insulin stimulation. The values obtained for EV-transfected muscles were considered as control and set at 100%. The effects of WT-PRAS40 expression on insulin action were analyzed using a two-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. **, $p<0.01$, *, $p<0.05$ versus EV-control muscles; †, indicates $p<0.05$ for the effect of insulin- versus untreated-muscle.

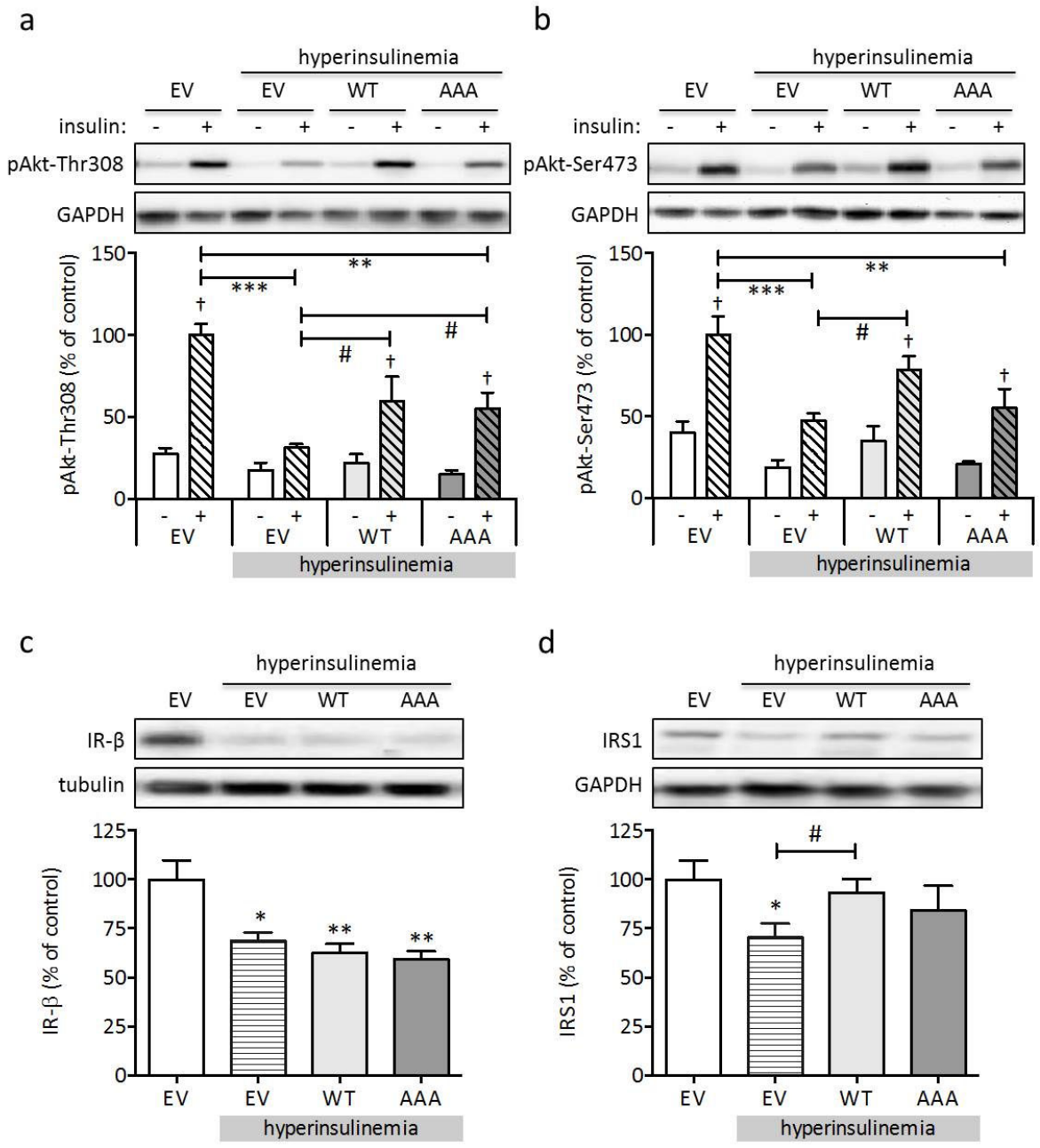

Figure 4.6 Effect of WT- and AAA-PRAS40 overexpression on hyperinsulinemia-induced inhibition of insulin signaling. Lysates from human primary skeletal muscle cells were transduced with lentivirus particles either expressing an empty vector (EV), wild type PRAS40 (WT) or a mutant of PRAS40 (AAA). When indicated cells were exposed to chronic hyperinsulinemia (50 nM, 24 h). Then cells were kept untreated (-) or stimulated with insulin (+) for analysis of phosphorylation of Akt-Thr308 (A), Akt-Ser473 (B), and protein expression of IRβ (C) and IRS1 (D). Signals were normalized for GAPDH or tubulin abundance, which was not affected by the experimental conditions used. Data are presented as representative Western blots and bar graphs showing the mean ± standard error of the mean of at least 4 independent experiments using cells from different donors. Open bars depict basal conditions, and hatched bars represent insulin-treated cells. The values obtained for EV-infected insulin-treated cells were considered as control and set at 100%. The effects of WT- and AAA-PRAS40 expression on insulin action were analyzed using two-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, $p<0.001$; **, $p<0.01$, *, $p<0.05$ versus EV-control cells; †, indicates $p<0.05$ for the effect of insulin- (+) versus untreated-cells (-); #, indicates $p<0.05$ for the effect of PRAS40 expression versus EV under conditions of hyperinsulinemia.

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Contribution to chapter 4

Journal: Molecular Endocrinology (submitted)

Impact factor (2012): 4.746

Contribution: Total: 80%

Conceived / designed experiments: 80%

Performed experiments: 70%

(cultivation of human skeletal muscle cells, production of lentivirus, lentiviral infection, preparation of plasmids, western blot analysis, RNA isolation and quantitative real-time PCR, glucose uptake assay, proteasome activity assay,
together with A.C.: *In vivo* muscle electroporation (IVE), *ex vivo* insulin stimulation in isolated muscles)

Analyzed data: 90%

Contributed to discussion: 70%

Wrote the manuscript: 90%

Reviewed / edited manuscript: 80%

Authorship: 1st author

CHAPTER 5

Study 4:

Proline-rich Akt substrate of 40-kDa contains a nuclear export signal

Proline-rich Akt substrate of 40-kDa contains a nuclear export signal†

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Abstract

The proline-rich Akt substrate of 40-kDa (PRAS40) has been linked to the regulation of the activity of the mammalian target of rapamycin complex 1 as well as insulin action. Despite these cytosolic functions, PRAS40 was originally identified as nuclear phosphoprotein in Hela cells. This study aimed to detail mechanisms and consequences of the nucleocytosolic trafficking of PRAS40. Sequence analysis identified a potential leucine-rich nuclear export signal (NES) within PRAS40. Incubation of A14 fibroblasts overexpressing human PRAS40 (hPRAS40) resulted in nuclear accumulation of the protein. Furthermore, mutation of the NES mimicked the effects of leptomycin B, a specific inhibitor of nuclear export, on the subcellular localization of hPRAS40. Finally, A14 cells expressing the NES-mutant showed impaired activation of components of the Akt-pathway as well as of the mTORC1 substrate p70 S6 kinase after insulin stimulation. This impaired insulin signaling could be ascribed to reduced protein levels of insulin receptor substrate 1 in cells expressing mutant NES. In conclusion, PRAS40 contains a functional nuclear export signal. Furthermore, enforced nuclear accumulation of PRAS40 impairs insulin action, thereby substantiating the function of this protein in the regulation of insulin sensitivity.

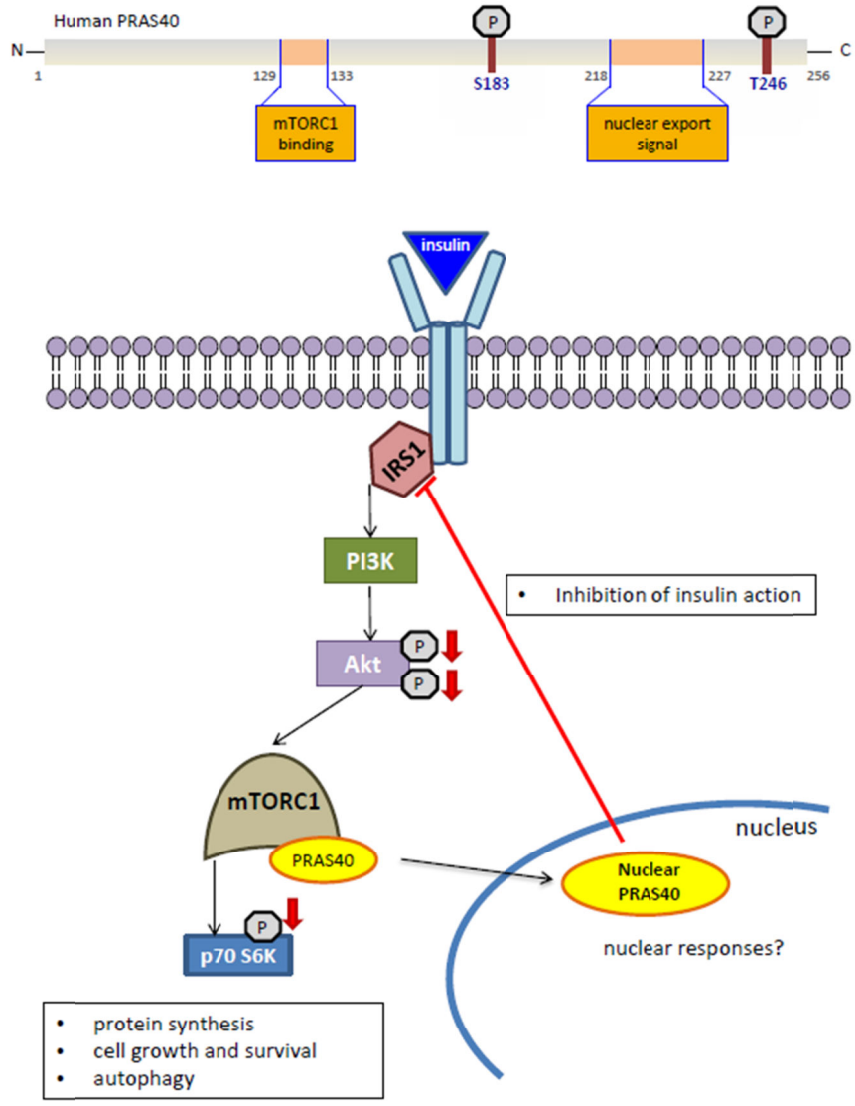
Highlights

- PRAS40 contains a nuclear export signal
- Enforced nuclear accumulation of PRAS40 impairs insulin action
- PRAS40 regulates insulin action by affecting IRS1 protein levels.

Keywords

PRAS40, Akt, insulin action, subcellular localization, nuclear export signal

Graphical Abstract



Introduction

The proline-rich Akt substrate of 40-kDa is a component of the mammalian target of rapamycin complex 1, which on its turn regulates a plethora of anabolic pathways, like control of cell growth, cell survival, proliferation, metabolism, and autophagy (1,2). In response to extracellular stimuli, such as insulin and amino acids, PRAS40 becomes phosphorylated on multiple residues, which leads to dissociation of PRAS40 from the mTORC1 complex (3,4). The function of PRAS40 within the mTORC1 is incompletely understood. Silencing and overexpression studies aimed detailing the function of PRAS40 within the mTORC1 complex have led to conflicting results, with both inhibitory and stimulatory roles ascribed to PRAS40 in the regulation of mTORC1 activity (5-10). Yet, a study in *Drosophila melanogaster* shed light on this controversy by suggesting that the regulation of mTORC1 activity by PRAS40 is tissue-specific and dependent on post-translational modification of the protein (11).

In previous reports, we could demonstrate that PRAS40 is a major substrate for Akt in tissues involved in the regulation of insulin sensitivity, and that the insulin-mediated phosphorylation of PRAS40 is impaired under conditions of insulin resistance, including skeletal muscle from patients with type 2 diabetes and high-fat diet fed animals (12,13). Furthermore, the knockdown of PRAS40 expression resulted in the proteasome-mediated degradation of the insulin receptor substrate 1 in human skeletal muscle, which was accompanied by inhibition of the insulin-mediated activation of the Akt signaling pathway regulating GLUT4-mediated glucose uptake (14).

Despite its participation in these cytosolic cellular processes, it is noteworthy that PRAS40 was originally identified as a nuclear phosphoprotein in HeLa cells (15). This nuclear localization of PRAS40 was also observed by immunological staining of both cultured cell lines and target tissues for insulin action with antibodies recognizing PRAS40 phosphorylated on Thr246 (12). These observations indicate that PRAS40 can shuttle between the cytosol and the nucleus. Accordingly sequence analysis identified a potential leucine-rich nuclear export signal (NES) in the carboxyterminal part of the protein. In the present study we aimed to assess the functionality of the NES using the nuclear export inhibitor leptomycin B and by overexpression of PRAS40 with a mutated NES. Because of the regulatory function of PRAS40 in insulin action, we further examined whether the enforced nuclear localization of PRAS40 impacts on insulin action under normal conditions and following exposure to palmitate, an inhibitor of insulin action.

Methods

Plasmids

The pRRL plasmid expressing human PRAS40 under control of a cytomegalovirus promoter and green fluorescent protein behind and IRES has been described elsewhere (13). Via mutagenesis PCR, the leucine residues at the positions 225 and 227 in human PRAS40 were mutated into alanine residues to generate mutant L225A/L227A-hPRAS40. The correct sequence of the mutant was verified by sequence analysis.

Cell culture and incubations

This study was conducted in A14 fibroblasts, which are NIH3T3 cells stably expressing $1.0x10^6$ insulin receptors per cell (16). Cells were grown in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum, penicillin and streptomycin (all from Invitrogen, Carlsbad, CA, USA). When indicated A14 cells, which were seeded at a density of 300000 cells per well in a 6-well plate, were transfected the following day at 40% confluence with 1.2µg of empty pRRL vector or pRRL containing wild type or mutant PRAS40 using Attractene (Qiagen, Hilden, Germany) following the manufacturer's instructions. Following the transfection, the cells were incubated overnight in DMEM containing 0.30 mM fatty acid free bovine serum albumin, or exposed to 0.75 mM palmitate bound to fatty acid free serum albumin a in ratio 2.5:1 as described (13). Then, cells were exposed to bovine insulin (Sigma Aldrich, St. Louis, MA, USA) (10 nM, 10 min) for analysis of insulin action. For immunofluorescence experiments, cells were exposed for 6 hours to the nuclear export inhibitor leptomycin B (LMB, 10 nmol/l) (Biomol, Plymouth meeting, PA, USA) when indicated.

Animals

Rat skeletal muscle was obtained from a previously published study (17). This investigation conformed to the guide for the care and use of laboratory animals, as published by the National Institute of Health (NIH publ. no 85-23, revised 1996) and the regulations of the institutional animal care and use committee. Following a 6 h fast, rats received an intraperitoneal injection with saline or insulin (10 U/kg body weight; Actrapid 100 U/ml; Novo Nordisk, Alphen aan den Rijn, The Netherlands) 30 min before being sacrificed by decapitation. Skeletal muscles were rapidly removed, snap frozen in liquid nitrogen-cooled isopentane and stored at −80°C until further analysis.

Immunofluorescence

For immunofluorescence, cells were grown on coverslips and fixed in 3.7% formaldehyde. Residual cross-linking was quenched using NH_4Cl and cells were permeabilized using 0.1% Triton X-100. Coverslips were blocked (0.2% BSA) and incubated overnight with hPRAS40 antibodies (AHO131, Invitrogen, Carlsbad, California, USA) followed by 2 h with Cy3-conjugated anti-mouse IgG (Jackson Immunochemicals, West Grove, PA, USA) prior to mounting with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI). Pictures were taken with a CCD CoolSnap K4 camera (Photometrics, Tucson, AZ, USA) on a Leica DM 5500 B microscope. Confocal pictures were taken on a Leica TCS SP2 system using a Dm IRBE confocal microscope with maximal magnification. In each independent experiment, images from 2 to 4 cells were analyzed with Leica Confocal Software (version 2.5).

Subcellular fractionation

Nuclear proteins were extracted from A14 fibroblasts and rat skeletal muscle as described previously (18). Protein content was determined using the BCA protein assay kit (Pierce Rockford, IL, USA). Purity of the cell fractions was checked by Western blotting with α/β-tubulin and lamin A/C as cytosolic and nuclear markers, respectively.

Western blot analysis

For western blot analysis, cells were lysed in 100 mM Tris-Cl (pH 6.8), 3% (w./vol.) SDS, and 10% (vol./vol.) glycerol. Protein content was determined using the bicinchoninic acid (BCA) protein assay kit. Expression and phosphorylation of proteins was then analyzed by SDS-PAGE and western blotting as described (14) using the following antibodies: Akt, Akt-phospho-Ser473, Akt-phospho-Thr308, FOXO3a-phospho-Thr32, p70 S6 kinase-phospho-Thr389, GAPDH, PRAS40, lamin A/C and α/β-tubulin (all from Cell Signalling Technology, Danvers, MA, USA), insulin receptor β-subunit (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and IRS1 as previously described (19). Signals were detected by enhanced chemiluminescence using Immobilon western blot detection reagents (Millipore Corporation, Billerica, MA, USA) on a Versadoc 4000 MP work station (BioRad, Munich, Germany), and quantitated using Quantity One analysis software (BioRad, version 4.6.7).

RNA-isolation and quantitative real-time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and transcribed into cDNA using the GoScript® reverse transcription system (Promega, Mannheim, Germany). Gene expression levels were determined by real-time PCR using GoTaq® qPCR Master Mix (Promega) on a Step One Plus Cycler (Applied Biosystems, Carlsbad, CA, USA). IRS1 gene expression was analyzed using QuantiTect primer assays from Qiagen (Hilden, Germany). Hypoxanthine phosphoribosyltransferase 1 (HPRT1) primers with the following sequences: forward-TGACACTGGCAAAACAATGCA, reverse-GGTCCTTTTCACCAGCAAGCT were designed using the NCBI Primerblast tool and ordered from Eurogentec (Seraing, Belgium). Expression levels were calculated using the ΔΔCt method using HPRT1 as housekeeping gene. The expression levels of HPRT1 showed no variations in response to the various conditions applied in this study.

Statistical analysis

Data are presented as means ± SEM. Significant differences were determined by two-way ANOVA (post-hoc test, Bonferroni multiple comparison test) or two-tailed Students *t* test using Prism5 (GraphPad, La Jolla, CA, USA) software. Values of $P<0.05$ were considered statistically significant.

Results

PRAS40 contains a functional nuclear export sequence

Analysis of the protein sequence of PRAS40 for potential motifs regulating the trafficking between the cytoplasm and the nucleus led to the identification of a potential 10 amino acid sequence in the carboxyterminal part of the protein. The IAASMRALVL-sequence located between amino acid residue 218 and 227 in human PRAS40 matches the sequence for a leucine-rich nuclear export signal (Figure 5.1a) (20). This potential NES-sequence is identical among the PRAS40 homologs identified in *Homo sapiens*, *Bos taurus*, *Rattus norvegicus*, and *Mus musculus*, slightly altered in *Xenopus tropicalis*, and *Danio rerio*, but conserved to a lesser extent in *D. melanogaster* (Figure 5.1b).

To assess the functionality of the NES, we first compared the localization of a PRAS40-mutant in which the critical leucine residues at positions 225 and 227 (L225A/L227A) were replaced by alanine with the localization of wild type PRAS40. Immunofluorescence staining of A14 cells showed

that mutant L225A/L227A-PRAS40 is mainly found in the nucleus, whereas wild type PRAS40 has a predominant cytosolic localization (Figure 5.2a). Quantification of images obtained using confocal microscopy showed that 65% of PRAS40 immunoreactivity was observed in the nucleus of L225A/L227A-PRAS40 expressing cells versus 2.6% in cells expressing wild type PRAS40 (Figure 5.2b). We further confirmed this distribution of wild type and mutant PRAS40 by fractionation and Western blot analysis (Figure 5.2c). In a subsequent experiment, we assessed whether the nuclear export inhibitor leptomycin B affects the localization of wild type PRAS40. As shown in Figure 5.2a, exposure to leptomycin B increased the amount of PRAS40 immunoreactivity in the nucleus in cells expressing wild type PRAS40 versus untreated cells. Quantification of images obtained using confocal microscopy showed that 30% of wild type PRAS40 was found in the nucleus following leptomycin B incubation (Figure 5.2b).

Finally, we examined the subcellular localization of endogenous PRAS40 both in A14 fibroblasts and rat skeletal muscle. Western blot analysis of cytosolic and nuclear fractions prepared from A14 fibroblasts showed that PRAS40 is present in both compartments. Insulin incubation had no impact on the distribution of PRAS40 between the cytosol and the nucleus, but did increase the level of PRAS40 phosphorylated on Thr246 (Figure 5.3a). Comparable data were obtained in a more physiological context, i.e. rat skeletal muscle. Figure 5.3b shows the presence of PRAS40 in both cytosolic and nuclear fractions prepared from rat skeletal muscle following saline injection. Insulin injection had no impact on the subcellular distribution of total PRAS40, but increased the amount of phosphorylated PRAS40 both in the cytosol and the nucleus (Figure 5.3b).

Nuclear accumulation of PRAS40 and insulin action

Because of the recently identified regulatory role of PRAS40 in insulin action (14), we next studied whether enforced nuclear localization of PRAS40 affects the expression and phosphorylation of components of the insulin signaling system. The expression of either wild type or L225A/L227A-PRAS40 had no impact on the protein levels of the insulin receptor, and Akt as compared to A14 cells transfected with the empty vector (Figure 5.4). However, the protein level of IRS1 was significantly lower in L225A/L227A-PRAS40 expressing cells versus cells transfected with wild type PRAS40 or the empty vector (Figure 5.4). Importantly, no effect of enforced nuclear localization of PRAS40 was observed on mRNA expression of IRS1 (Supplementary figure 5.6). The reduction in IRS1 protein levels in L225A/L227A-PRAS40 expressing cells associated with an impaired insulin-mediated phosphorylation of Akt-Thr308, Akt-Ser473, and of the Akt-regulated substrate FOXO3a-Thr32 in L225A/L227A-PRAS40 expressing cells versus cells transfected with wild type PRAS40 or the empty

vector (Figure 5.5). Furthermore, the enforced nuclear expression of PRAS40 inhibited the insulin-mediated phosphorylation of the mTORC1-substrate p70 S6 kinase-Thr389 (Figure 5.5). Exposing the cells to palmitate prior to insulin treatment had no additional effect on the inhibition of insulin action observed in L225A/L227A-PRAS40 expressing cells (Figure 5.5). In contrast, palmitate was found to lower IRS1 protein levels and to blunt the insulin-mediated phosphorylation of Akt, FOXO3a, and p70 S6 kinase in cells expressing the empty vector or wild type PRAS40 (Figure 5.4 and 5.5).

Discussion

This report describes the identification of a nuclear export sequence within PRAS40. The increased nuclear abundance of PRAS40 in cells exposed to leptomycin B or expressing mutant L225A/L227A-PRAS40 demonstrates that this nuclear export sequence is functional. Furthermore, we showed that enforced nuclear expression of PRAS40 impairs insulin action in a way similar to that previously observed in primary human skeletal muscle cells in which PRAS40 was silenced, i.e. involving a reduction in the protein abundance of IRS1.

The presence of a functional nuclear export sequence adds PRAS40 to the list of components of the mTORC1-signaling pathway displaying a nucleocytoplasmic distribution and/or possessing a nuclear export sequence (21-27). Among these are the other subunits of the mTORC1 complex, such as mTOR itself, raptor, and mLST8, as well as upstream regulators of mTORC1 activity, such as Akt and TSC2, and the downstream effectors p70 S6 kinase and ribosomal protein S6 (21-27). Raptor, which acts as substrate binding scaffold within mTORC1, is highly abundant in the nucleus (25). However, mTOR and mLST8, like PRAS40 as demonstrated here, are more abundant in the cytosol (25). Furthermore, the nuclear localization of mTOR, and its regulated proteins p70 S6 kinase and the ribosomal protein S6 is cell cycle dependent with low levels during G_0, and increased nuclear abundance during G_0 to G_1-progession (28,29). In the present study, we did not analyze whether the nuclear appearance of PRAS40 is cell cycle dependent. Nevertheless, given the potential role of PRAS40 in the regulation of mTORC1 activity, one may propose that nuclear PRAS40 impacts on the regulation of processes ascribed to nuclear mTOR, such as the RNA-polymerase I- and III-mediated transcription of rDNA and tRNA (30).

In the present study we further examined whether alterations in the subcellular distribution of PRAS40 affect insulin sensitivity. In some cell types, including 3T3L1 adipocytes, HepG2 cells and primary human skeletal muscle cells, the knockdown of PRAS40 lowers the protein levels of IRS1 and consequently impairs the insulin-mediated activation of the Akt signaling pathway (8,14,31). Expression of mutant L225A/L227A-PRAS40 in A14 fibroblasts impaired insulin action similar to that

observed after PRAS40 silencing (14). One report ascribed the inhibition of insulin action to an increased basal phosphorylation p70 S6 kinase, which on its turn promotes the serine phosphorylation of IRS1 that may result in the degradation of this protein (8). However, expression of mutant L225A/L227A-PRAS40 had no effect on the basal phosphorylation of p70 S6 kinase, whereas in insulin-stimulated cells even a decreased phosphorylation was observed. Similar findings have been reported following PRAS40 silencing in multiple studies (9,14,32,33), and it seems plausible that the blunted phosphorylation of p70 S6 kinase is a direct consequence of the inhibition of Akt, which also regulates insulin-mediated mTORC1 activation (2). Instead the effects of L225A/L227A-PRAS40 on insulin action may be more in line with the proposed novel function for PRAS40 in the regulation of IRS1 protein levels through the proteasome (14). This hypothesis is supporting by the finding that enforced nuclear PRAS40 localization has no impact on IRS1 mRNA expression, indicating a posttranscriptional regulation of IRS1. In primary human skeletal muscle cells we observed that silencing PRAS40 led to degradation of IRS1 through activation of the proteasome (14). One may speculate that the as yet unidentified factor responsible for the inhibitory action of PRAS40 on the activity of the proteasome is translocated along with PRAS40 in cells with enforced nuclear expression of PRAS40. This mechanism would also explain why expression of wild type PRAS40 has no effect on insulin action in A14 fibroblasts, which has also been reported previously in other cell types (9). Irrespective of the mechanism via which L225A/L227A-PRAS40 impairs insulin action, one should note that these data have been obtained in overexpressing experiments, and that it remains to be clarified whether the amount of endogenous PRAS40 translocating to the nucleus is sufficient to impact on cytosolic insulin action.

Another issue that remains to be addressed is how PRAS40 is transported to the nucleus. We could not identify a nuclear localization signal with the PRAS40 sequence. Notably, proteins smaller than 60-kDa can freely move between the cytoplasm and the nucleus, and since PRAS40 is a 40-kDa protein it may be able to shuttle independent of active transport between both compartments. Nevertheless, expression of wild type PRAS40 in A14 cells resulted in a predominant cytosolic localization of the protein while the co-expressed green fluorescent protein was found both in the nucleus and the cytosol. This indicates that an alternative mechanism may participate in the translocation of PRAS40 to the nucleus. Possible mechanisms regulating the nuclear import of PRAS40 could be the interaction with other proteins, such as 14-3-3 proteins or raptor (3,4,34). Multiple studies have implicated 14-3-3 proteins in the control of nucleocytoplasmic shuttling (35,36), and various reports have demonstrated the binding of PRAS40 to 14-3-3 proteins, albeit upon phosphorylation of PRAS40 (9,37,38). Neither in A14 cells nor in rat skeletal muscle, the distribution of PRAS40 between the cytosol and the nucleus was affected by insulin, thereby make a

contribution of 14-3-3 proteins in this process less likely. Alternatively, the transport of PRAS40 to the nucleus may involve in complex with raptor. Raptor is essential for the binding of unphosphorylated PRAS40 to the mTORC1 complex and has a predominant nuclear localization (25). With the use of additional mutant forms of PRAS40 we have attempted to investigate the importance of the key phosphorylation sites within PRAS40 as well as the so-called TOS-motif that is implicated in raptor binding. However, all mutants investigated showed nuclear accumulation in cells exposed to leptomycin B (supplementary figure 5.7), suggesting that the nuclear import of PRAS40 is independent of phosphorylation and binding to raptor.

Conclusion

This study demonstrates that PRAS40 contains a functional nuclear export sequence, and that enforced nuclear accumulation of PRAS40 impairs insulin action. Our findings corroborate a function of cytosolic PRAS40 as regulator of insulin action. Yet, further studies are required to elucidate the cellular processes affected by nuclear PRAS40.

Authors contributions

CW and EBMN, designed the study, conducted the experiments, analyzed the data and wrote the paper. MMLL, FC, DHdW, and GCMvdZ performed experiments, analyzed the data and contributed to the discussion. JAM, MD, and BG supervised the experiments, analyzed the data and reviewed the manuscript. DMO designed the study, acquired funding, supervised the study, analyzed the data and wrote the paper. DMO is the guarantor of this work and has full access to the entire dataset and takes full responsibility for the integrity of the data and the accuracy of the data analysis. All authors have seen and approved the final version of the manuscript.

Duality of interest

The authors declare that they have no conflict of interest.

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Figures

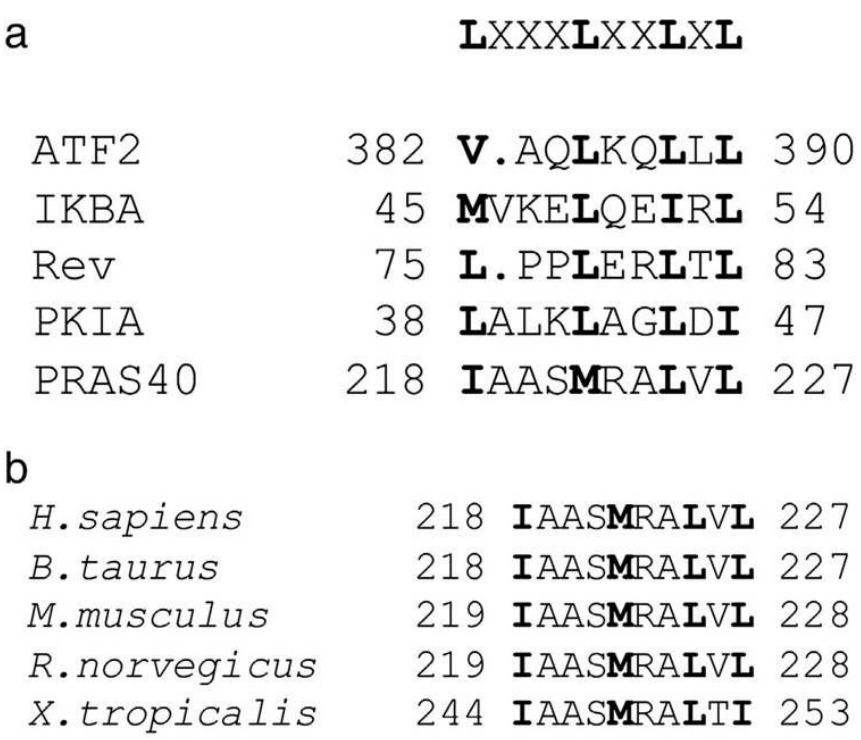

Figure 5.1 PRAS40 contains a nuclear export signal. (a). Alignment of the nuclear export signal of PRAS40 with those found in human activating transcription factor 2 (ATF2), inhibitor of nuclear factor κBα (IκBα), the rev protein of simian human immunodeficiency virus (Rev), and cAMP-dependent protein kinase inhibitor α (PKIA). In the consensus sequence for a leucine-rich nuclear export sequence above the aligned sequences, the X represents any amino acid, while the leucine (L) can be replaced by any other large hydrophobic amino acid (20). (b) Alignment of the nuclear export signal of PRAS40 with PRAS40 homologues found in other species.

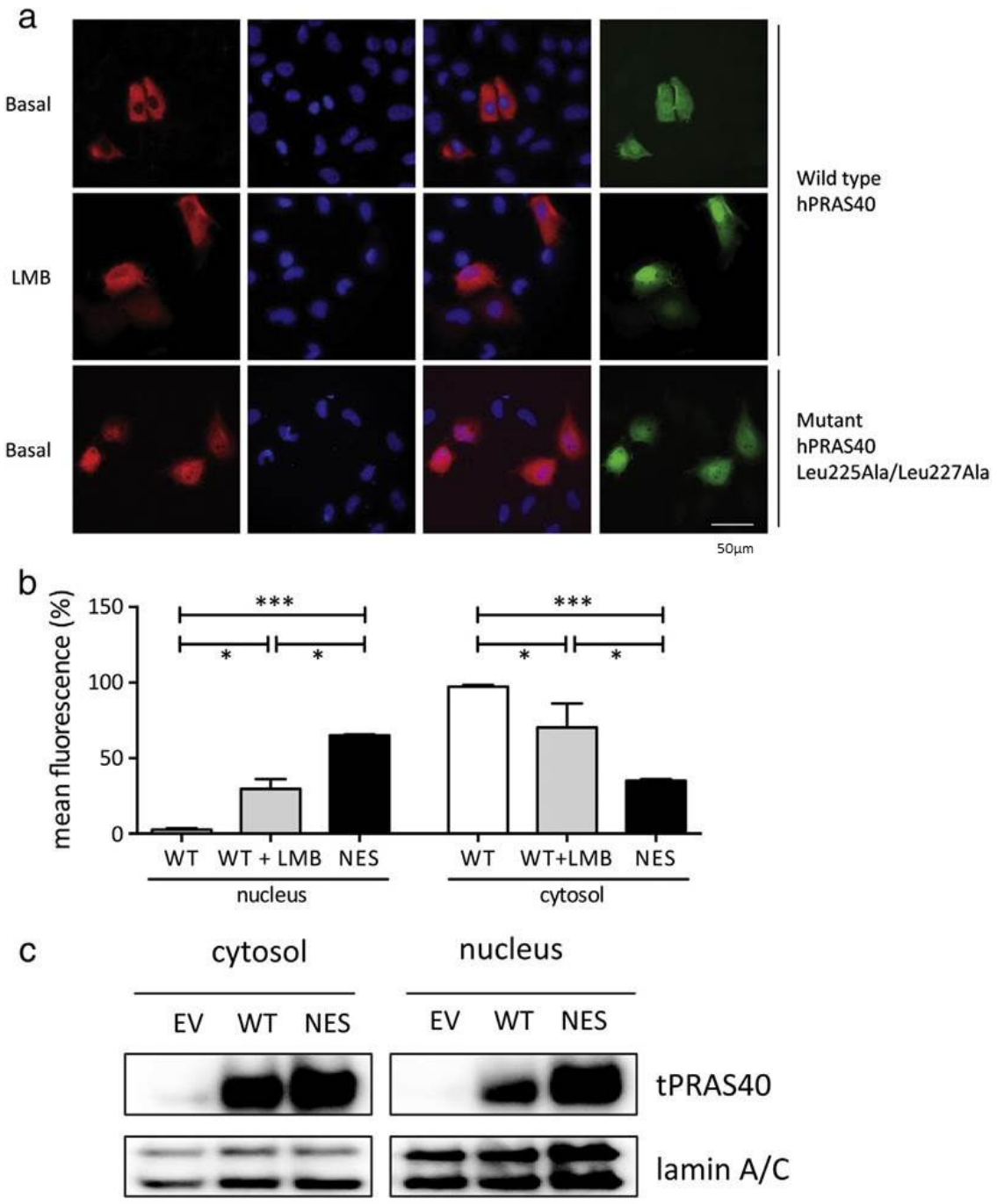

Figure 5.2 Functionality of the nuclear export sequence in PRAS40. (a) A14 fibroblasts were transfected with wild-type hPRAS40 or mutant L225A/L227A-hPRAS40. Transfected cells are positive for green fluorescent protein (GFP; green). Cells were untreated (basal) or incubated with LMB (6 h, 10 nM), fixed, permeabilized and incubated with anti-hPRAS40. Bound PRAS40 antibody was visualized with anti-mouse conjugated Cy3 secondary antibodies (red). DNA was stained with DAPI (blue). Photographs are representative of three independent experiments. The scale bar equals 50 µm. (b) Quantification of fluorescence detected with confocal microscopy in the nucleus and cytoplasm for the hPRAS40 antibody in A14 cells transfected with wild-type hPRAS40 that were kept untreated (open bars), treated with LMB (gray bars), or transfected with mutant L225A/L227A-hPRAS40 (NES; black bars). Data are expressed as mean ± SEM (n=3); *: $p<0.05$; **, $p<0.001$ for the indicated comparisons. (c) Cytosolic and nuclear extracts from A14 fibroblasts which were transfected with empty vector (EV) wild-type hPRAS40 (WT) or mutant L225A/L227A-hPRAS40 (NES) were prepared as described in the experimental procedures. Lysates were used for Western Blot analysis to detect total PRAS40, whereas purity of the fractions was determined using antibodies against tubulin and lamin A/C, respectively.

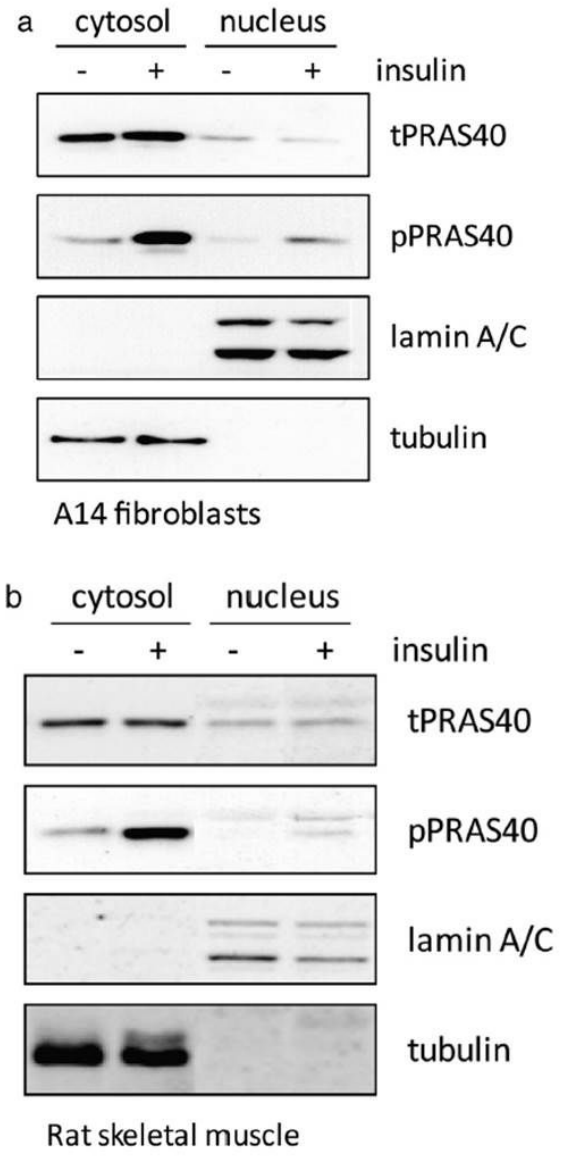

Figure 5.3 Localization of PRAS40 in A14 fibroblasts (a) and rat skeletal muscle (b). A14 cells were serum starved during 6 h, and were left untreated (-) or stimulated with 100 nM insulin for 5 min (+). Following a 6 hr fast, rats received an intraperitoneal injection with saline or insulin (10 U/kg body weight), and were sacrificed 30 min later. Cytosolic and nuclear extracts from A14 fibroblasts and rat skeletal muscles were prepared as described in the experimental procedures. Ten micrograms of protein lysate was loaded on a SDS-PAGE gel. Western blot analysis was performed using phospho-specific antibodies against PRAS40-Thr246. Purity of the fractions was determined by reprobing the western blots with antibodies against tubulin and lamin A/C, respectively. Representative blots are shown of three independent experiments.

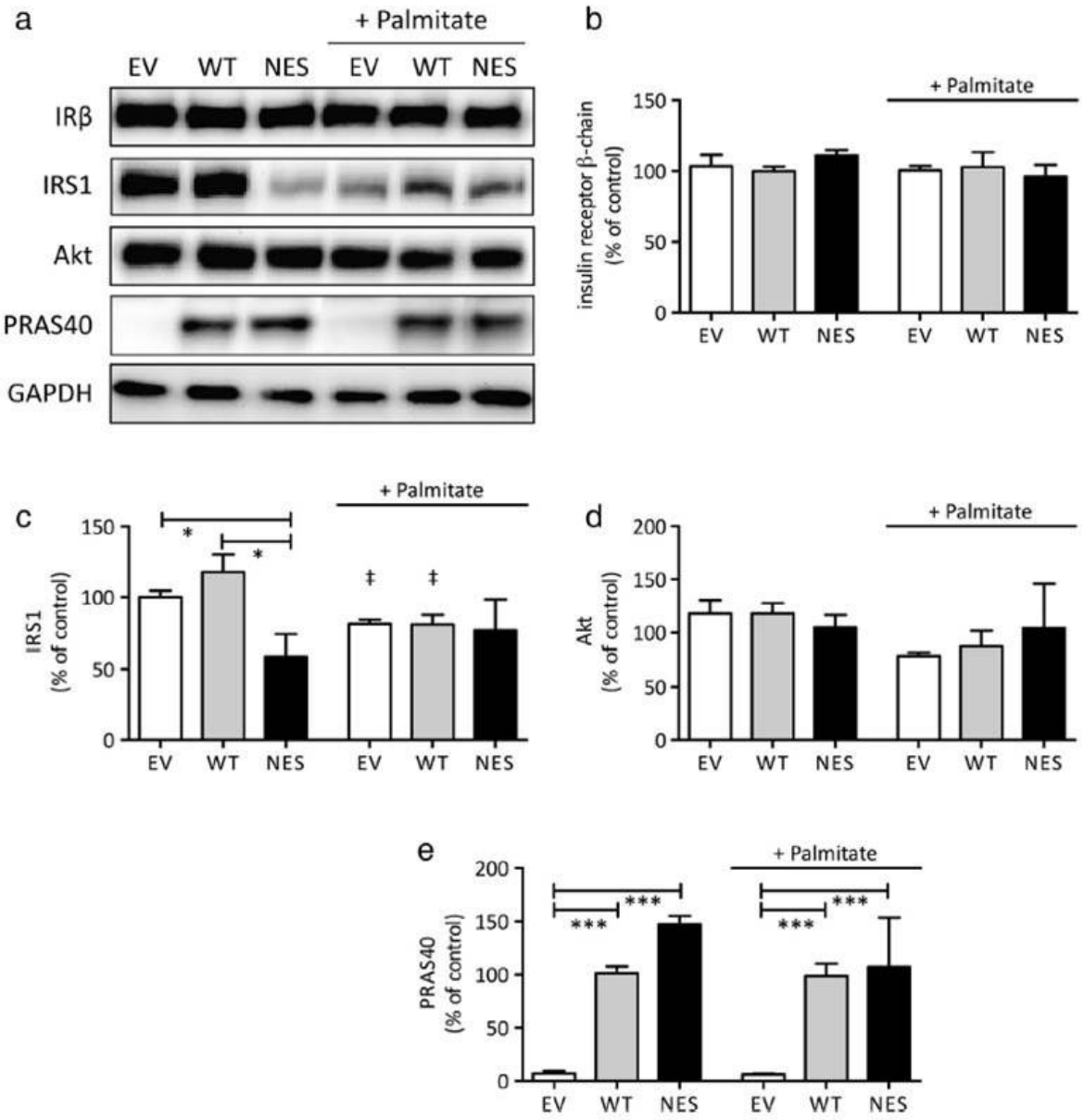

Figure 5.4 Protein levels of insulin signaling components in cells expressing wild type or mutant L225A/L227A-PRAS40. A14 fibroblasts were transfected with an empty vector (EV), or a plasmid encoding wild type (WT) or L225A/L227A-PRAS40 (NES), followed by overnight exposure to 0.3 mM BSA or 0.75 mM palmitate. Shown are representative western blots and bar graphs for the protein levels of the insulin receptor β-subunit (IRβ), IRS1, Akt, and PRAS40. Signals were normalized for GAPDH levels, which were not altered by the experimental conditions. Data are expressed as mean ± SEM (n=4). *: $p<0.05$, ***, $p<0.001$ for the indicated comparisons; ‡: $p<0.05$ for the effect of palmitate.

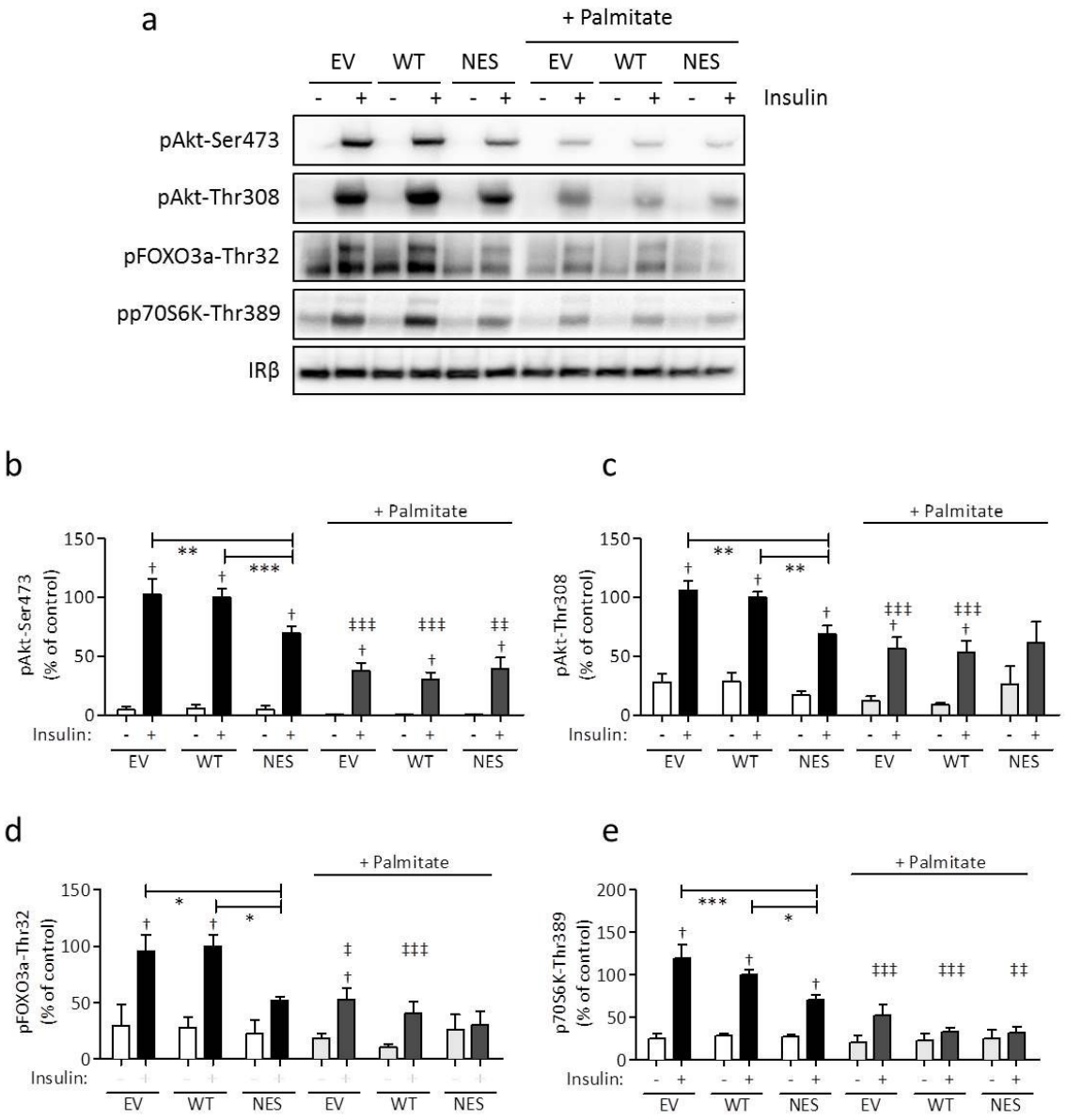

Figure 5.5 Phosphorylation of insulin signaling components in cells expressing wild type or mutant L225A/L227A-PRAS40. A14 fibroblasts were transfected with an empty vector (EV), or a plasmid encoding wild type (WT) or L225A/L227A-PRAS40 (NES), followed by overnight exposure to 0.3 mM BSA or 0.75 mM palmitate. Shown are representative western blots and bar graphs for the phosphorylation of Akt-Ser473, Akt-Thr308, FOXO3a-Thr32 and p70 S6 kinase-Thr389. Signals were normalized for GAPDH levels, which were not altered by the experimental conditions. Data are expressed as mean ± SEM (n=4). *: p<0.05, **, p<0.01, ***, p<0.001 for the indicated comparisons; ‡‡‡, p<0.001, ‡‡, p<0.01, ‡, p<0.05 for the effect of palmitate; †, p<0.05 for the effect of insulin versus basal.

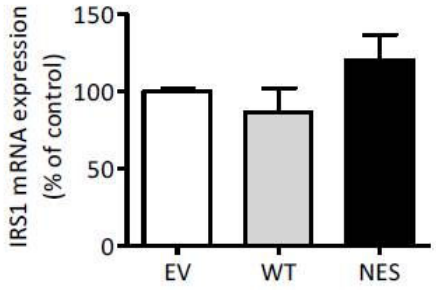

Figure 5.6 (supplementary) Effect of overexpression of WT- and NES-PRAS40 on IRS1 mRNA expression. A14 fibroblasts were transfected with an empty vector (EV), or a plasmid encoding wild type (WT) or L225A/L227A-PRAS40 (NES). Two days after transfection, total RNA was extracted and analyzed for IRS1 mRNA expression. Data are presented as mean ± standard error of the mean (n=3), and were analyzed by ANOVA followed by Bonferroni analysis for multiple comparisons.

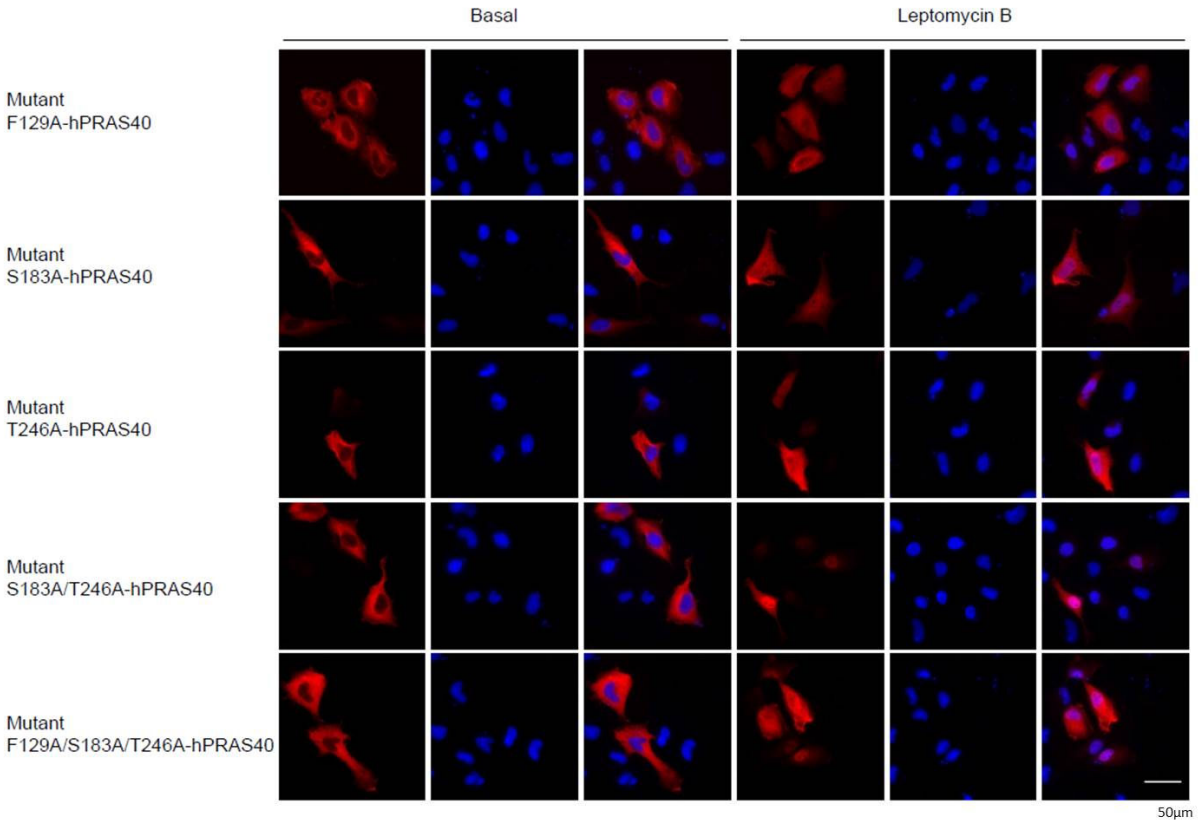

Figure 5.7 (supplementary) Effect of leptomycin B on the nuclear localization of mutant forms of PRAS40. A14 fibroblasts were transfected with wild-type hPRAS40 or mutant F129A-hPRAS40, S183A-hPRAS40, T246A-hPRAS40, S183A/T246A-hPRAS40, or F129A/S183A/T246A-hPRAS40. Following transfection, cells were kept untreated (basal) or incubated for 6 h with 10 nM leptomycin B. PRAS40 was visualized by immunofluorescence using PRAS40 antibodies, which on its term were visualized with anti-mouse conjugated Cy3 secondary antibodies (red). DNA was stained with DAPI (blue). Photographs are representative of three independent experiments. The scale bar equals 50 µm.

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Contribution to chapter 5

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Conceived / designed experiments: 50%

Performed experiments: 50%

(cultivation and transfection of A14 fibroblasts, preparation of plasmids, western blot analysis, RNA isolation and quantitative real-time PCR, immunofluorescence, subcellular fractionation)

Analyzed data: 70%

Contributed to discussion: 80%

Wrote the manuscript: 60%

Reviewed / edited manuscript: 80%

Authorship: shared 1st author

CHAPTER 6

Study 5:

Effects of Sfrp5 on cytokine release and insulin action in primary human adipocytes and skeletal muscle cells

Effects of Sfrp5 on cytokine release and insulin action in primary human adipocytes and skeletal muscle cells †

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Abstract

Secreted frizzled-related protein (Sfrp5) was described as adipokine with anti-inflammatory and insulin-sensitizing properties in mouse models. However, the mechanism of Sfrp5 action, especially in humans, is largely unknown. Therefore, cytokine release and insulin signaling was analyzed to investigated the impact of Sfrp5 on inflammation and insulin signaling in primary human adipocytes and skeletal muscle cells (hSkMC) *in vitro*. Sfrp5 had no impact on the release of interleukin (IL)-6, monocyte chemoattractant protein 1 (MCP-1), and adiponectin from human adipocytes and of IL-6 and IL-8 from hSkMC. In tumor necrosis factor (TNF)α-treated adipocytes, Sfrp5 reduced IL-6 release by 49% ($p<0.05$), but did not affect MCP-1 and adiponectin release. In MCP-1-treated hSkMC, the secretion of IL-6 and IL-8 was unaltered. In untreated adipocytes, Sfrp5 decreased the insulin-mediated phosphorylation of Akt-Ser473 by 28% and Akt-Thr308 by 38% (both $p<0.01$) as well as of its substrates GSK3α-Ser21 by 37% ($p<0.001$) and PRAS40-Thr246 by 34% ($p<0.05$), respectively. TNFα impaired insulin action in adipocytes to a similar extent as Sfrp5, but there was no additional effect when Sfrp5 and TNFα were combined. In contrast, neither in untreated, nor in MCP-1 treated hSkMC, Sfrp5 affected insulin signaling. In conclusion, Sfrp5 lowered IL-6 release from human insulin-resistant adipocytes, but not under normal conditions, and decreased insulin sensitivity in human adipocytes. Sfrp5 did not act on hSkMC. Thus, the cellular actions of Sfrp5 seem to depend on the type of tissue as well as its inflammatory and metabolic state.

Key words

Adipose tissue, muscle, insulin action, inflammation, Sfrp

Funding

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Introduction

Chronic, low-grade inflammation in adipose tissue induced by obesity is characterized by an aberrant release of hormones, cytokines and chemokines. These factors affect insulin sensitivity not only in an auto-/paracrine fashion in adipose tissue but also in an endocrine manner in liver and skeletal muscle. Several pro-inflammatory cytokines and chemokines such as tumor necrosis factor (TNF)α, monocyte chemotactic protein (MCP)-1 and chemerin, which participate in the detrimental crosstalk between adipose tissue and skeletal muscle and progression of insulin resistance during obesity, were identified (1-3). In contrast, the knowledge about anti-inflammatory cytokines remains limited. Currently, only adiponectin and omentin have been linked to improved insulin sensitivity and are downregulated in obesity and type 2 diabetes (T2D) (4).

Recent studies in mice also suggest an anti-inflammatory and anti-diabetic function for secreted frizzled-related protein 5 (Sfrp5). Sfrp5 antagonizes wingless-type MMTV integration site family member (Wnt)5a in the non-canonical Wnt-signaling pathway (5). Importantly, Sfrp5-deficiency in mice results in deterioration of high-calorie diet-induced glucose intolerance, hepatic steatosis and macrophage infiltration in adipose tissue. Conversely, acute administration of Sfrp5 to obese and diabetic mice improved glucose tolerance and adipose tissue inflammation (6). However, one report demonstrated decreased mRNA levels of Sfrp5 (6), whereas others reported increased Sfrp5 expression in obese mice (7-10). Also studies in humans on Sfrp5 yielded conflicting results. In Chinese subjects, both reductions and increases in circulating Sfrp5 levels between obese and T2D patients versus control participants were reported (11-13), while no differences were observed between lean and obese Caucasian subjects (14, 15). Furthermore, Sfrp5 gene expression in adipose tissue was unaffected by obesity (16). We recently reported a positive association of Sfrp5 with insulin resistance and markers of oxidative stress in mostly overweight and obese Caucasians, indicating that the function of Sfrp5 in humans may be dependent on the subjects' metabolic and inflammatory state (14). Therefore, the aim of this study was to elucidate the mechanism of Sfrp5 action in primary human adipocytes and skeletal muscle cells (hSkMC) by assessing the impact of Sfrp5 on insulin signaling and release of inflammatory proteins under basal culture conditions and following inflammation-induced insulin resistance.

Material and Methods

Cell culture

Primary human adipocytes were differentiated from cryopreserved human white preadipocytes (PromoCell, Heidelberg, Germany) isolated from subcutaneous adipose tissue from five healthy Caucasian donors (5 females aged 31-58 years). For induction of differentiation, the medium was replaced by PromoCell preadipocyte differentiation medium for 72h. Then, the medium was changed to PromoCell adipocyte nutrition medium to complete differentiation and was exchanged every 2-3 days. On day 15-17 of differentiation adipocytes were used for experiments.

Primary hSkMC isolated from the rectus abdominis muscle of four healthy Caucasian donors (2 females, 2 males aged 16-37 years) were obtained as proliferating myoblasts from PromoCell or Lonza (Basel, Switzerland) Differentiation into myotubes was initiated by replacing the growth medium by αMEM containing 2% horse serum (Gibco, Berlin, Germany). Cells were routinely starved on serum-free αMEM on day 6 of differentiation and then used for experiments. (17).

Differentiated cells were incubated (i) with or without 10 or 100 ng/ml Sfrp5 (R&D Systems, Wiesbaden, Germany) for 24h (adipocytes) or 18h (hSkMC), (ii) with or without 100 ng/ml Sfrp5 for 4h and then adipocytes were exposed to 5 nM TNFα (24h) (Sigma-Aldrich, St Louis, MO) (adipocytes) while hSkMC were exposed to 2 ng/ml MCP-1 (PeproTech, Hamburg, Germany). The Sfrp5 concentrations used match those reported in the circulation in human clinical studies (11-15). For analysis of insulin signaling, cells were stimulated with 100 nM insulin (porcine insulin, Sigma Aldrich) (10 min) following cytokine treatment.

Analysis of inflammation in primary human adipocytes and skeletal muscle cells

Interleukin (IL)-6, IL-8, IL-15, monocyte chemotactic protein-1 (MCP)-1 and adiponectin were measured in cell culture supernatants using Quantikine ELISA kits (R&D Systems). Detection limits for the Quantikine ELISAs for human IL-6, IL-8, IL-15, MCP-1 and total adiponectin were 1.6 pg/ml, 15.6 pg/ml, 2.0 pg/ml, 7.5 pg/ml and 1.6 ng/ml, respectively. Concentrations for all cytokines in cell culture supernatants were above the respective detection limit with the exception of IL-15 for which concentrations did not exceed the detection limit under the described cell culture conditions.

Analysis of insulin signaling in primary human adipocytes and skeletal muscle cells

For insulin signaling, cell lysates were analyzed by Western blotting as described (17). Membranes were incubated with antibodies recognizing Akt-phospho-Ser473, Akt-phospho-Thr308, glycogen synthase kinase 3 (GSK3) α/β-phospho-Ser21/9 and proline-rich Akt-substrate of 40-kDa (PRAS40) phospho-Thr246 (all from Cell Signaling Technology, Danvers, MA). The phosphorylation signals were normalized for equal loading by reprobing the membranes with antibodies for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology).

Statistical analysis

Differences in cytokine concentrations between various treatments were analyzed by Friedman´s test followed by Dunn´s multiple comparison test. For analysis of effects on insulin action, the insulin-stimulated condition in the absence of cytokines was set at 100%. Thereafter, differences were analyzed using two-way ANOVA followed by Bonferroni multiple comparison analysis. All data are presented as mean ± SEM. Statistical analyses were performed using Prism 6 (GraphPad, LA Jolla, CA) software. P-values of $p<0.05$ were considered as statistically significant.

Results

Sfrp5 reduces IL-6 release from human adipocytes but has no impact on myokine release from skeletal muscle cells

Under basal culture conditions, Sfrp5 did not affect the release of IL-6, MCP-1, and adiponectin from primary human adipocytes, and of IL-6 and IL-8 from hSkMC, respectively (Figure 6.1). Exposing adipocytes to Sfrp5 prior to TNFα incubation lowered the secretion of IL-6 by 49% ($p<0.05$), but had no impact on the release of MCP-1 and adiponectin as compared to cells exposed to TNFα only (Figure 6.2a-c). Preincubation of hSkMC with Sfrp5 prior to incubation with MCP-1 had no effect on IL-6 or IL-8 release (Figure 6.2d-e). Levels of IL-15 were below the detection limit under all experimental conditions for hSkMC.

Sfrp5 inhibits insulin signaling in primary human adipocytes but not in skeletal muscle cells

Treating primary human adipocytes with Sfrp5 had no effect on the basal phosphorylation of Akt-Thr308, Akt-Ser473 and its substrates GSK3α-Ser21 and PRAS40-Thr246 (Figure 6.3a-d). However, the insulin-mediated increases in Akt-Thr308, Akt-Ser473, GSK3α-Ser21, and PRAS40-Thr246 were reduced in cells pre-incubated with Sfrp5 by 38%, 28%, 37%, and 34%, respectively (Figure 6.3a-d). Because some studies ascribed an anti-diabetic and anti-inflammatory action to Sfrp5 (6, 15), we subsequently analyzed the effect of Sfrp5 on insulin signaling in TNFα-treated adipocytes. TNFα alone decreased insulin-mediated phosphorylation of Akt-Ser473, Akt-Thr308, GSK3α-Ser21 and PRAS40-Thr246 (by 47%, 42%, 28%, and 41%, respectively). Yet, there was no restoration of insulin action in adipocytes exposed to Sfrp5 together with TNFα (Figure 6.3). Sfrp5 did not affect insulin signaling in hSkMC, neither under basal culture conditions nor following MCP-1-induced insulin resistance (Figure 6.4).

Discussion

The present study shows that Sfrp5 impairs insulin signaling in adipocytes under basal culture conditions. Furthermore, Sfrp5 reduced IL-6 release from TNFα-treated adipocytes. In contrast to adipocytes, Sfrp5 did not act on hSkMC. This suggests that the cellular function of Sfrp5 is tissue-specific, and dependent on the metabolic and inflammatory state of the target tissue.

Studies toward the (molecular) mechanism of Sfrp5 action in tissues critical for metabolic control are limited and have yielded conflicting results. Several studies reported the induction of Sfrp5 expression during differentiation of 3T3L1 adipocytes and in rodent models of genetic and/or diet-induced obesity (7-10), and propose a role for Sfrp5 in the adipocyte growth via suppression of the Wnt-pathway and inhibition of adipocyte mitochondrial metabolism (9). However, the observed inhibition of IL-6 release from TNFα-treated human adipocytes by recombinant Sfrp5 in the present study suggests a protective function for Sfrp5, and fits to the reduction of transcript levels of inflammatory cytokines, including IL-6, observed in adipose tissue from insulin-resistant *ob/ob*, but not wild-type mice, following adenovirus-mediated Sfrp5 expression (6). Furthermore, a study on Asian subjects on 89 normal glucose tolerant and 87 subjects with type 2 diabetes found a negative association between plasma levels of Sfrp5 and IL-6 ($r=-0.438$, $p<0.01$) (12). Unfortunately, this study did not mention whether this relation was different between controls and subjects with type 2 diabetes (T2D). A study on a smaller, mostly overweight or obese Caucasian population reported no association between circulating Sfrp5 and IL-6 levels (14). Thus, more detailed studies involving

additional rodent models as well as validation of interaction of Sfrp5 levels with pro-inflammatory cytokines in human clinical samples are clearly required to substantiate the notion that metabolic and/or inflammatory disturbances impact on the mode of Sfrp5 action.

The present study further showed that Sfrp5 inhibits insulin action in primary human adipocytes under basal culture conditions. In support of a role for Sfrp5 in impairing insulin action are data from a clinical study on obese subjects without diabetes in which circulating Sfrp5 levels were found to be associated with HOMA-IR ($r=0.32$, $p<0.05$) (14), and a study on Chinese subjects which reported increased circulating Sfrp5 levels in patients with T2D as compared to subjects without diabetes (13). In contrast, two other studies on Asians showed decreased circulating Sfrp5 levels in patients with T2D versus subjects with normal glucose tolerance, and reported a negative association between plasma levels of Sfrp5 and HOMA-IR ($r=-0.446$, $p<0.01$) (12) and ($r=-0.444$, $p<0.001$) (11). Also in mice conflicting data have been reported. One study showed that loss of functional Sfrp5 mitigated increases in serum leptin levels, as well as the induction of glucose intolerance, and insulin resistance after high-fat feeding (9). In contrast, Sfrp5 deficiency led to severe glucose intolerance and further impaired insulin-stimulated phosphorylation of Akt in adipose tissue following high-fat feeding as compared to wild-type mice (6). This was associated with increased activation of the c-Jun N-terminal kinase (JNK) signaling pathway, which inhibits insulin action via phosphorylation of Ser307 of insulin receptor substrate 1 (18, 19). In line with observations in 3T3L1 adipocytes (9), we observed that Sfrp5 had no effect on JNK phosphorylation in primary human adipocytes, neither under basal conditions nor following exposure to TNFα (data not shown). A limitation of the present study is that we did not examine all pathways potentially involved in the induction of insulin resistance, such as the NFκB-cascade or activation of the proteasome. The value of such investigations would however benefit from recognition of the confounding factors that could explain the conflicting data observed in the in vivo studies.

Another aspect that should be considered in this context is that we failed to observe any effect of Sfrp5 on hSkMC. Consequently, the action of Sfrp5 may be tissue-specific. Sfrp5 is known to act as an antagonist for Wnt5a, which in turn activates Wnt-signaling through binding to the frizzled receptors (5). One may hypothesize that hSkMC do not produce Wnt5a, and are therefore unresponsive to Sfrp5. However, we found that mRNA levels of Wnt5a are higher in hSkMC compared to adipose tissue (data not shown). Furthermore, Wnt5a expression is increased in regenerating mouse muscles (20). However, there is nothing known from the literature about effects of Sfrp5 and/or Wnt5a on inflammation and insulin action in hSkMC. This suggests that Wnt5a might not be the only target molecule for Sfrp5 and that there might be other molecules and/or receptors for Sfrp5 associated with its mechanisms of action.

Conclusion

Sfrp5 attenuated insulin action in adipocytes under normal conditions and reduced IL-6 release in TNFα-treated adipocytes, but did not act on hSkMC. The mode of action of Sfrp5 in inflammation and insulin resistance might depend on the kind of tissue and defined inflammatory and metabolic circumstances of the site of action.

Figures

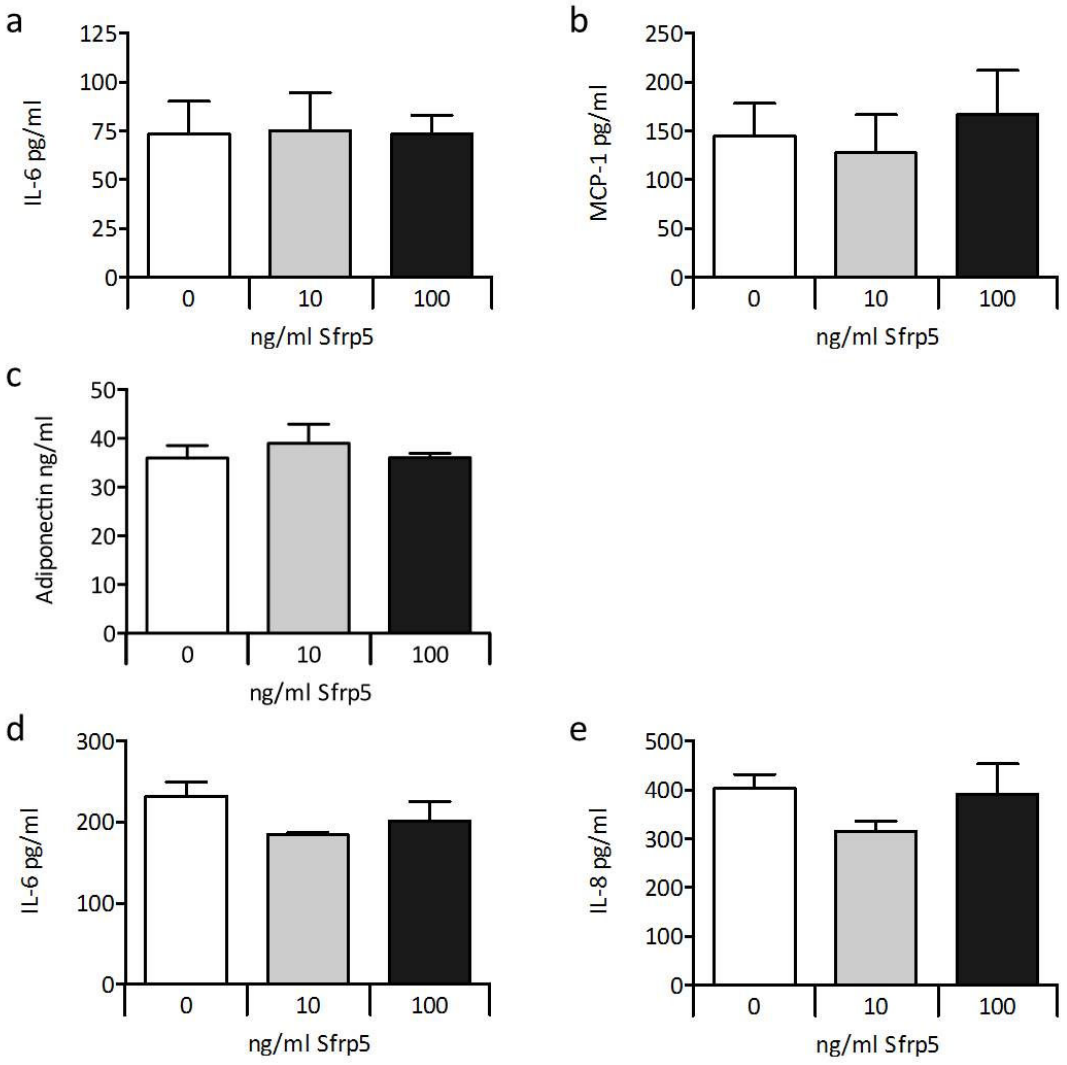

Figure 6.1 Effect of Sfrp5 on adipokine and myokine release (unstimulated conditions). Primary human adipocytes (a-c) and primary human skeletal muscle cells (d-e) were exposed to increasing amounts of Sfrp5 for 24 h. Cytokine release by the adipocytes and myotubes was quantified by ELISA, and expressed as mean ± standard error of the mean (a-c: n=5; d-e: n=4).

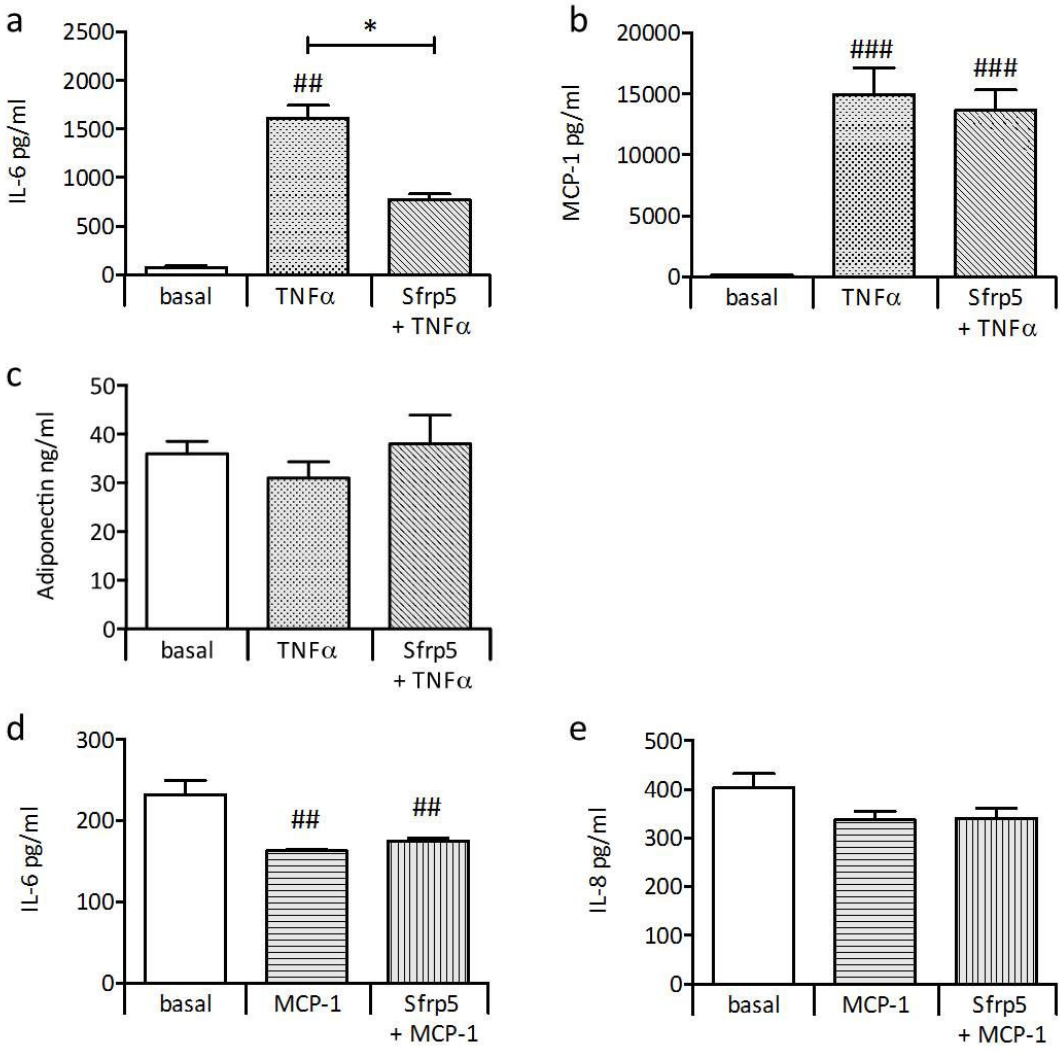

Figure 6.2 Effect of Sfrp5 on adipokine and myokine release from cytokine-stimulated cells. Primary human adipocytes (a-c) and primary human skeletal muscle cells (d-e) were exposed to Sfrp5 (4h; 100 ng/ml) prior to incubation with TNFα (24h; 5 nmol/l) or MCP-1 (18h; 2 ng/ml). Cytokine release by the adipocytes and myotubes was quantified by ELISA, and expressed as mean ± standard error of the mean (a-c: n=5; d-e: n=4). Differences among the various conditions were analyzed by Friedman´s test followed by Dunn´s multiple comparison test; ### indicates $P<0.001$; ##, $P<0.01$ versus cells kept untreated (basal); *, $P<0.05$ for the effect of the Sfrp5 incubation versus TNFα alone.

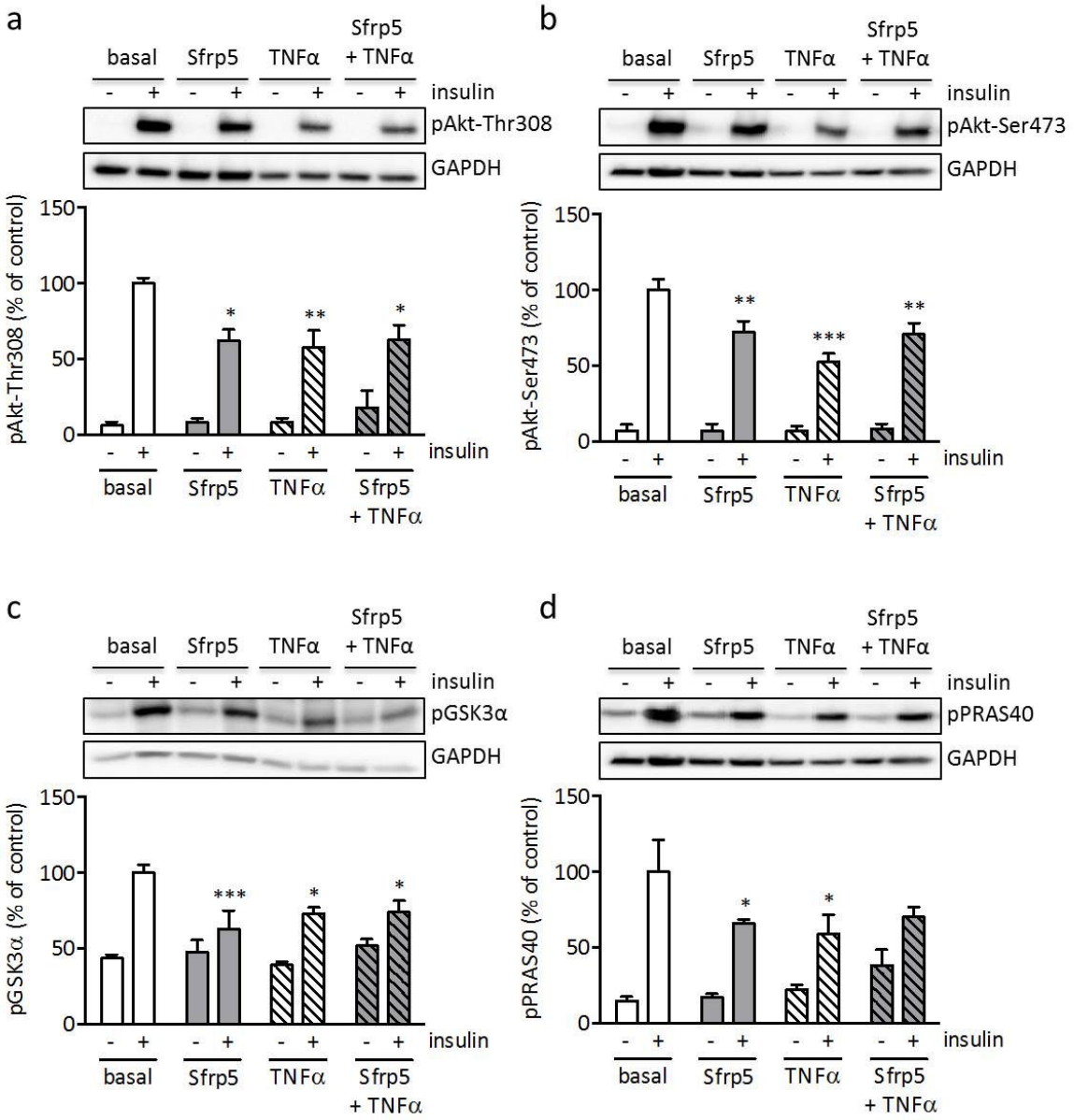

Figure 6.3 Effect of Sfrp5 on insulin action in primary human adipocytes. Primary human adipocytes were kept untreated (basal), exposed to 100 ng/ml Sfrp5 for 24h, or to 5 ng/ml TNFα for 24h with or without a 4h preincubation with 100 ng/ml Sfrp5. Then, when indicated (+) cells were stimulated with insulin (10 min; 100 nM). Cell lysates were analyzed for phosphorylation of Akt-Thr308 (a), Akt-Ser473 (b), GSK3α-Ser21 (c), and PRAS40-Thr246 (d) by Western blotting. Phosphorylation signals were normalized for GAPDH protein abundance and expressed as mean ± standard error of the mean of five independent experiments using cells from different donors. The values obtained for untreated insulin-treated cells were considered as control and set at 100%. Differences among groups were calculated by two-way ANOVA followed by Bonferroni multiple comparison analysis. ***, p<0.001; **, p<0.01; *, p<0.05 versus basal.

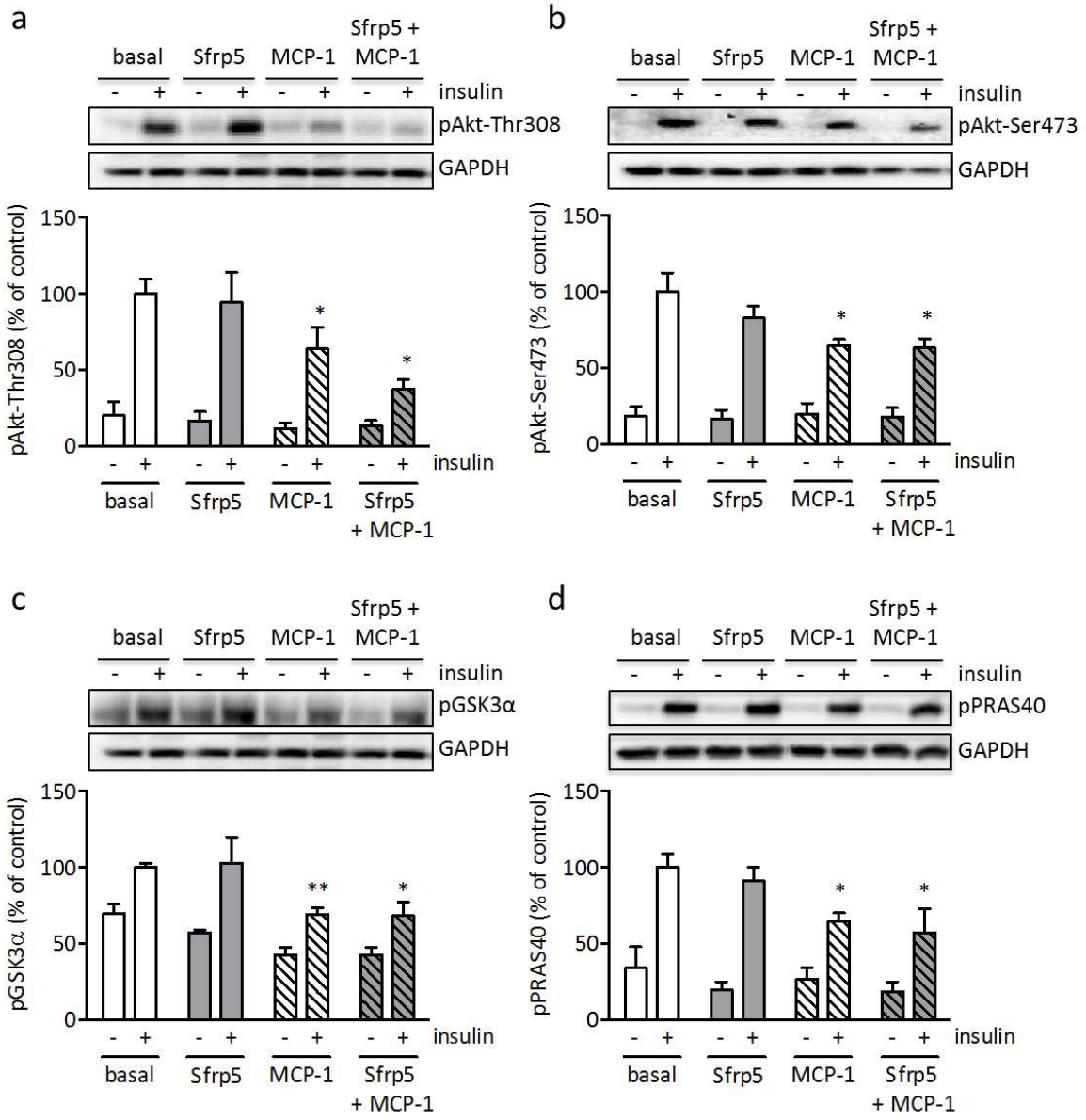

Figure 6.4 Effect of Sfrp5 on insulin action in primary human skeletal muscle cells. Primary human skeletal muscle cells were kept untreated (basal), exposed to 100 ng/ml Sfrp5 for 24h, or to 2 ng/ml MCP-1 for 18 h with or without a 4h preincubation with 100 ng/ml Sfrp5. Then, when indicated (+) cells were stimulated with insulin (10 min; 100 nM). Cell lysates were analyzed for phosphorylation of Akt-Thr308 (a), Akt-Ser473 (b), GSK3α-Ser21 (c), and PRAS40-Thr246 (d) by Western blotting. Phosphorylation signals were normalized for GAPDH protein abundance and expressed as mean ± standard error of the mean of five independent experiments using cells from different donors. The values obtained for untreated insulin-treated cells were considered as control and set at 100%. Differences among groups were calculated by two-way ANOVA followed by Bonferroni multiple comparison analysis. **, $p<0.01$; *, $p<0.05$ versus basal

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Contribution to chapter 6

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Contribution: Total: 50%

Conceived / designed experiments: 50%

Performed experiments: 50%

(cultivation and treatment of human skeletal muscle cells, western blot analysis)

Analyzed data: 50%

Contributed to discussion: 50%

Wrote the manuscript: 40%

Reviewed / edited manuscript: 60%

Authorship: shared 1st author

CHAPTER 7

General Discussion

Insulin is an essential factor in maintaining adequate glucose levels. Therefore, insulin resistance is the most important pathophysiological feature of T2D. The development of insulin resistance in peripheral tissues such as liver, fat and skeletal muscle is an early event during the progression of T2D and occurs long before clinical symptoms appear. The intermediates involved in insulin signaling are pleiotropic and their role in physiological and pathophysiological insulin action is often undefined. For that reason one important issue of diabetes research is to understand the normal insulin signaling cascade as well as to identify and characterize the multiple mechanisms involved in the disturbance of insulin action. Since skeletal muscle is one of the major insulin-sensitive organs, identification of new modulators of insulin action in skeletal muscle is of crucial importance. This knowledge will be essential for the development of new strategies to improve insulin sensitivity or to prevent insulin resistance. PRAS40 has been identified as one of the most prominent substrates for Akt in skeletal muscle. Initial studies investigating the function of PRAS40 yielded conflicting results and left many aspects regarding the precise function of PRAS40 unaddressed. Therefore, the aim of the present research in this thesis was to further characterize the potential function of PRAS40 in insulin signaling in skeletal muscle. Special attention was paid on modification of PRAS40 phosphorylation under conditions of insulin resistance (see 7.1) as well as investigation of the impact of different PRAS40 protein levels on mTORC1 signaling and insulin action (see 7.2). Finally, the impact of posttranslational modifications and cellular localization of PRAS40 for its function was investigated and discussed (see 7.3).

7.1. Phosphorylation of PRAS40 as a biomarker for insulin resistance

PRAS40 is highly conserved in higher species and ubiquitously expressed in all tissues, with highest expression in human liver and heart (182). The phosphorylation site Thr246 at the C-terminus of PRAS40 shows a high conservation in higher species (chapter 2, Fig. 2.1). Akt has been identified as the major kinase promoting Thr246 phosphorylation of PRAS40. Kovacina et al. could demonstrate for the first time that cells lacking Akt1 and Akt2 exhibit a diminished ability to phosphorylate PRAS40 at Thr246 after platelet-derived growth factor (PDGF) stimulation (183). In line with these findings, tumor cells, which display a constitutively active Akt through loss of PTEN, showed elevated levels of Thr246-phosphorylated PRAS40 (187). Finally, several studies investigating new cancer drugs, which target the PI3K/Akt pathway, used phosphorylation of PRAS40 at Thr246 as a marker for efficiency of the inhibitors (187-191). Therefore, PRAS40 phosphorylation at Thr246 was postulated to be an early event during carcinogenesis and was suggested to be used as a detection marker for cancer development (190). Given the pivotal role of Akt in insulin signaling, PRAS40 phosphorylation could

also function as a marker for insulin sensitivity. PRAS40-Thr246 is among the most prominent substrates for Akt in target tissues for insulin action and Nascimento et al. have already demonstrated a strong link between *in vivo* phosphorylation of PRAS40 at Thr246 and insulin resistance (182). Phosphorylation of PRAS40 was enhanced after insulin stimulation in rat, mouse and human insulin target tissues and this effect was blunted under conditions of insulin resistance (117,182,192). Additionally, *ex vivo* incubation of rat soleus muscle with palmitate was shown to decrease insulin-stimulated phosphorylation of PRAS40-Thr246 (193). Finally, phosphorylation of PRAS40 was improved by excess weight loss in skeletal muscle following hyperinsulinemia in obese patients with T2D (194).

7.1.1 Impact of MCP-1 and chemerin on Akt-dependent PRAS40 phosphorylation

To extend the findings about PRAS40-Thr246 as a marker for insulin sensitivity, we investigated the impact of factors associated with insulin resistance, such as MCP-1 and chemerin (151-153,155,156,158), on PRAS40 phosphorylation at Thr246 in human skeletal muscle cells. We found that both MCP-1 and chemerin reduced insulin-mediated phosphorylation of PRAS40 at Thr246 (Fig. 7.1 a, c). MCP-1 and chemerin were previously shown to promote insulin resistance in skeletal muscle via induction of different signaling mechanisms, including inhibitory serine/threonine phosphorylation of IRS1, and activation of inflammatory pathways such as ERK1/2, JNK, and NFκB (153,156,195). Furthermore, both MCP-1 and chemerin impaired insulin-mediated Akt phosphorylation at both Ser473 and Thr308 (chapter 3, Fig. 3.2 a, b) resulting in diminished propagation of the insulin signal from Akt to PRAS40. Based on our results, one may speculate that the improvement of insulin-mediated phosphorylation of PRAS40-Thr246 in patients with T2D after substantial weight loss (194) could be mediated by the loss of adipose tissue, which is linked to decreased secretion of cytokines and chemokines, such as chemerin and MCP-1 (174,196). In line with this, weight loss was already found to decrease serum levels of MCP-1 (197) and chemerin (161) in obese subjects. However, changes in secretion of these adipokines were not measured in this study (194).

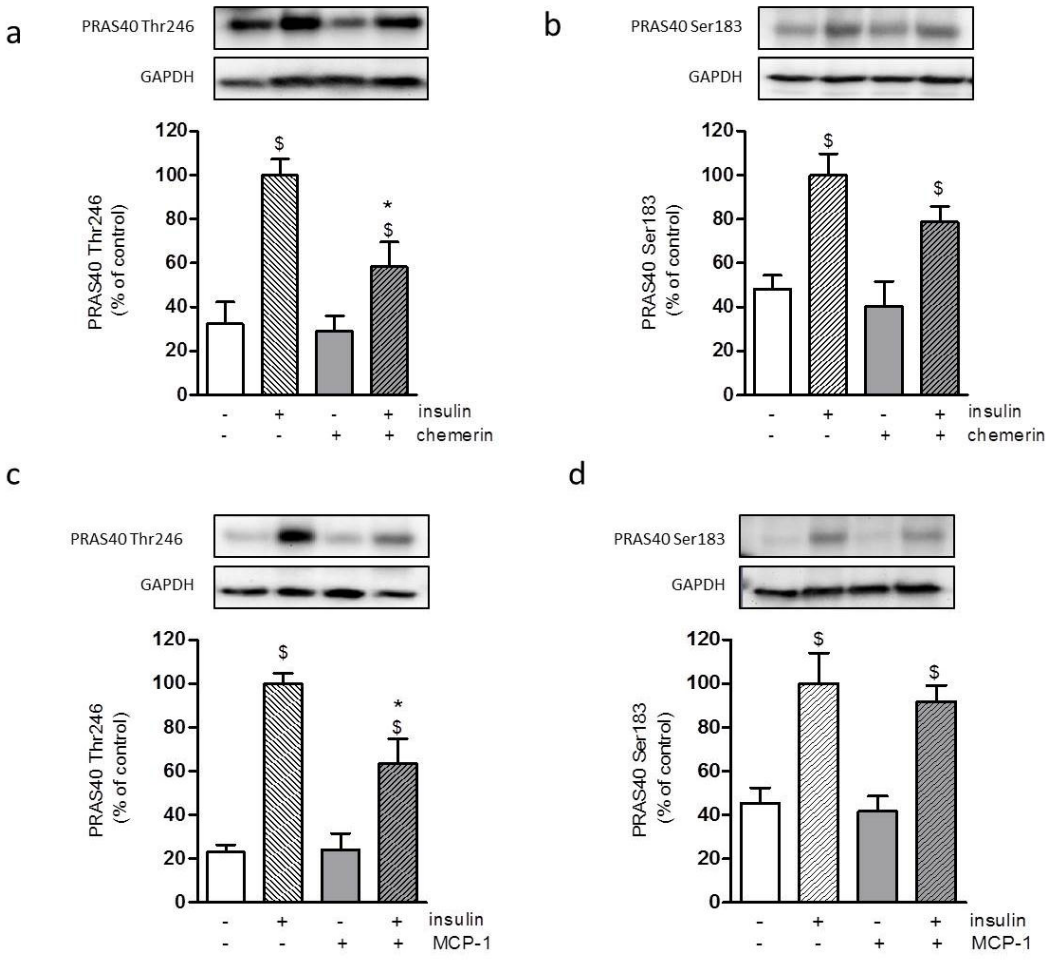

Figure 7.1 Effect of chemerin and MCP-1 on insulin-stimulated phosphorylation of PRAS40. Primary human skeletal muscle cells from different donors were differentiated for seven days and serum starved for 24h. Incubation of cells with 2µg/ml chemerin (a,b) or 2ng/ml MCP-1 (c,d) was performed for 24h. After that, cells were stimulated with 100nM insulin for 10 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-PRAS40 Thr246 (a,c) or anti-PRAS40 Ser183 (b,d) antibody. All data are normalized to the level of GAPDH protein expression and are expressed relative to the insulin-stimulated control. Data are means ± SEM with n>3 experiments. *p<0.05 versus insulin-stimulated control. $, p<0.05 insulin-stimulated versus basal.

7.1.2 Akt-independent phosphorylation of PRAS40-Thr246

Besides MCP-1 and chemerin, we also investigated the impact of adipocyte-conditioned medium (CM) on PRAS40 phosphorylation at Thr246 (Fig. 7.2). CM is generated by collecting culture media from differentiated adipocytes after 48 h of cultivation, thus containing all factors secreted by adipocytes during that period of time. As described previously (198-200), incubation of human skeletal muscle cells with CM induces insulin resistance on the level of insulin-mediated Akt and GSK3 phosphorylation. Although we did observe impaired insulin-mediated phosphorylation of Akt, CM

surprisingly increased both basal and insulin-stimulated phosphorylation of PRAS40 at Thr246 (Fig. 7.2). Due to the fact that CM contains a variety of factors modulating e.g. extracellular matrix, immune response, inflammation, metabolism, oxidative stress or angiogenesis, one might speculate that CM contains factors which activate kinases contributing to PRAS40 phosphorylation at Thr246 in an Akt-independent way.

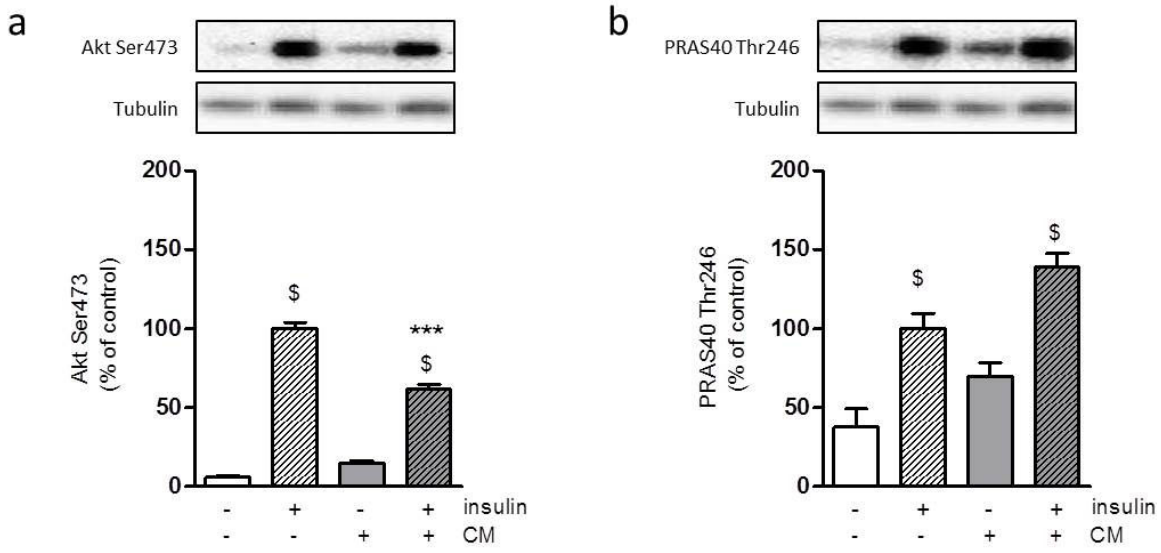

Figure 7.2 CM decreases insulin-mediated phosphorylation of Akt at Ser473 but increases phosphorylation of PRAS40-Thr246. Primary human skeletal muscle cells from different donors were differentiated for seven days and serum starved for 24h. Incubation of cells with CM was performed for 24h. Thereafter, cells were stimulated with 100nM insulin for 10 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific anti-Akt Ser473 (a) or anti-PRAS40 Thr246 (b) antibody. All data are normalized to the level of Tubulin protein level and are expressed relative to the insulin-stimulated control. Data are means ± SEM with n=7 experiments. The impact of CM was analyzed using two-way ANOVA followed by post-hoc Bonferroni testing for multiple comparisons. ***, $P<0.001$ versus insulin-stimulated control. $, $P<0.05$ insulin-stimulated versus basal.

Indeed, the induction of PRAS40-Thr246 phosphorylation is neither confined to insulin nor to activation of the Akt pathway. For example follicle-stimulating hormone in Sertoli cells (201), IGF-1 in PC12 cells (202), growth hormone in preadipocytes (203), ethanol in myocytes (204), ceramide-1 in macrophages (205), exercise in human skeletal muscle cells (206) or several growth factors as well as nutrients, such as glucose and amino acids (207) increase PRAS40-Thr246 phosphorylation. In all cases the phosphorylation of PRAS40 at Thr246 was paralleled by the increase in the phosphorylation of Akt (201-206). Furthermore, phosphorylation of PRAS40-Thr246 was blunted after inhibition of Akt phosphorylation by PI3K inhibitors, such as wortmannin and LY294002 (201-203). However, PRAS40 phosphorylation at Thr246 can also be induced when Akt activation is impaired ((190) and Fig. 7.2).

Kovacina et al. have demonstrated that phosphorylation of endogenous PRAS40 could be induced by PDGF in mouse embryonic fibroblasts (MEFs) from wild type mice and those lacking Akt2 (183). MEFs lacking Akt1 displayed a slight decrease in PDGF-stimulated Thr246 phosphorylation, whereas those cells lacking both Akt1 and Akt2 showed the largest decrease, however phosphorylation was not fully abolished. This study did not assess the impact of Akt3, which can also promote PRAS40-Thr246 phosphorylation (208). However, Akt3 is predominantly found in testis, brain and kidney (53) but not in classical insulin-sensitive tissues like liver, muscle and fat (209). These findings raise the possibility that other kinases can also promote PRAS40-Thr246 phosphorylation. Indeed, leucine induces phosphorylation of PRAS40 at Thr246 in hearts of wild type mice but neither affects Ser473 nor Thr308 phosphorylation of Akt. Interestingly, this leucine-mediated PRAS40 phosphorylation is entirely dependent on the presence of PDK1. The authors hypothesized that PDK1 may stimulate PRAS40-Thr246 phosphorylation through recruitment and/or activation of a protein kinase from the AGC-kinase family different from Akt (210). In line with this, the AGC-kinase protein kinase A (PKA) has been linked to the induction of PRAS40 phosphorylation at Thr246 after stimulation of rat thyroid PC Cl3 cells with thyroid hormone (211). In addition to PKA, PIM1 has been demonstrated to increase PRAS40 phosphorylation at Thr246. PIM1 is a serine/threonine kinase that is described to act as a potent mediator of cell survival, proliferation and differentiation (212). Importantly, the substrates recognition sequence of PIM1 is very similar to that of Akt, indicating that these kinases may share many cellular substrates (213). Enforced overexpression of PIM1 increases phosphorylation of PRAS40 at Thr246 in FDCP1 cells (a murine non leukemic cell line). This phosphorylation was independent of Akt, which could be confirmed by an *in vitro* kinase assay demonstrating a direct phosphorylation of PRAS40-Thr246 by PIM1 (212).

Alternatively, a recent study reported that AMPK is involved in dephosphorylation of PRAS40 at Thr246 (214). Together with our data, this demonstrates that phosphorylation of PRAS40-Thr246 is regulated by a network of kinases, which have to be characterized in further studies.

7.1.3 Phosphorylation of PRAS40 on other residues

In addition to Thr246, multiple stimuli, including insulin and amino acids can increase the phosphorylation of PRAS40 at Ser183 (215). The phosphorylation of PRAS40-Ser183 is mediated by the mTORC1 (216,217), and accordingly wortmannin, rapamycin, glucose withdrawal as well as amino acid starvation were found to decrease Ser183 phosphorylation. A study by Nascimento et al. further reported a complete inhibition of insulin-mediated PRAS40 Ser183 phosphorylation in skeletal muscle and hearts of HFD-fed rats as well as in A14 fibroblasts treated with low

concentrations of palmitate (216). However, in our study on hSkMC, MCP-1 and chemerin failed to impair insulin-mediated phosphorylation of PRAS40-Ser183 (Fig 7.1 b, d). This may be explained by the fact that MCP-1 has no impact on mTORC1 signaling, as illustrated by the observation that MCP-1 neither affects the phosphorylation of p70S6K and S6 nor the protein abundance of Grb10 (chapter 3, Fig. 3.3). We also measured the effect of chemerin on insulin-mediated activation of mTORC1 and could not detect a significant reduction of mTOR and p70S6K phosphorylation either (data not shown). This indicates that phosphorylation of PRAS40 at Ser183 is only an indicator for impaired insulin action, if mTORC1 signaling is also affected. It has further been postulated that PRAS40-Thr246 phosphorylation facilitates efficient phosphorylation of PRAS40 Ser183 by mTORC1 (216). This cannot be confirmed by our data. Here, impairment of PRAS40 Thr246 by chemerin and MCP-1 does not affect full phosphorylation of Ser183 (Fig 7.1). A possible explanation for this discrepancy could be that our study was conducted in cells expressing endogenous levels of PRAS40, whereas the study by Nascimento et al. (216) was performed on cells overexpressing mutant forms of PRAS40. One may speculate in this context, that the expression of ectopic mutant T246A-PRAS40 is still capable of inhibiting the mTORC1 complex.

Importantly, PRAS40 also possesses further phosphorylation sites, which are induced by insulin and are probably regulated by mTORC1 as well (chapter 2, Fig. 2.1 and (207,218)). However, until today no antibodies against these phosphorylation sites are commercially available. Hence investigations toward the impact of these phosphorylation sites on PRAS40 function or if they are affected in terms of insulin resistance are still missing. Therefore, we cannot exclude some effect of MCP-1 or chemerin on PRAS40 phosphorylation at other phosphorylation sites.

7.1.4 Effect of the potential novel insulin-sensitizing protein Sfrp5 on PRAS40 phosphorylation

Sfrp5 belongs to the family of secreted frizzled-related proteins, which has been described to function mainly in antagonizing wingless-type MMTV integration site family member 5a (Wnt5a). In contrast to other members of the Wnt family, such as Wnt1, Wnt3 or Wnt7, which activate the transcription factor β-catenin, Wnt5a induces the β-catenin-independent pathway, in which small G-proteins like Rac and Rho as well as protein kinases, such as protein kinase C, are activated (219). Wnt5a is well described to be involved in developmental processes of different organs and regulation of postnatal cell function, however full understanding of Wnt5a signaling, regulation and participation in related diseases remains unclear. With regard to metabolic disorders, it is known that Wnt5a participates in adipogenesis (220) and functions in stimulating inflammatory processes (219).

Ouchi et al. could demonstrate for the first time that Wnt5a protein levels are increased in adipose tissue of mice fed with a HFD and *ob/ob* mice (172). Interestingly, in the same study, Sfrp5 has been reported to act in an anti-inflammatory and insulin sensitizing manner by antagonizing Wnt5a. Furthermore, Sfrp5 has been shown to be secreted by adipocytes and was decreased in conditions of obesity. Therefore, Sfrp5 has been postulated to be a new anti-inflammatory adipokine with similar properties to adiponectin, a feature which evokes our as well as other groups´ interest. However, our results investigating the impact of Sfrp5 on inflammatory and insulin signaling in human adipocytes and skeletal muscle cells (chapter 6) failed to confirm these beneficial properties of Sfrp5. Sfrp5 alone neither altered the secretion of proinflammatory immune mediators, like IL-6 and MCP-1, nor of adiponectin (chapter 6, Fig. 6.1). However, pre-incubation of adipocytes with Sfrp5 down-regulated TNFα-induced IL-6 release, while MCP-1 and adiponectin release were not altered by Sfrp5 pre-incubation (chapter 6, Fig. 6.2). With regard to this point our results are in line with a study performed in mice, where Sfrp5 overexpression decreased TNFα, IL6 and MCP-1 mRNA levels in adipose tissue of obese but not lean wild type mice (172). This indicates that Sfrp5 may only act in an anti-inflammatory manner under conditions of metabolic and inflammatory disturbances. With our study we could show for the first time that Sfrp5 inhibits insulin signaling in primary human adipocytes. This was demonstrated by decreased insulin-stimulated Akt and GSK3 phosphorylation as well as by inhibition of insulin-mediated phosphorylation of PRAS40 at Thr246 (chapter 6, Fig. 6.3). As indicated previously, phosphorylation of PRAS40 at Thr246 is an eligible marker for disturbances of insulin signaling downstream of PI3K/Akt. This decrease in insulin sensitivity in adipocytes by Sfrp5 could be confirmed by a study conducted in 47 healthy subjects, where circulating levels of Sfrp5 positively associated with decreased insulin sensitivity (measured by HOMA-IR) and oxidative stress (170). Furthermore, other members of the Sfrp family such as Sfrp4 have been linked to T2D. In serum, Sfrp4 was associated with elevated fasting glucose and impaired insulin sensitivity (221), indicating that there could be a general mechanism of the way Sfrp proteins modulate insulin sensitivity.

Interestingly, no alteration of insulin action was observed in human skeletal muscle cells after Sfrp5 incubation at baseline or in combination with MCP-1 (chapter 6, Fig. 6.4). While PRAS40-Thr246 phosphorylation was impaired by MCP-1, Sfrp5 was ineffective in this cell type. Until now, no studies investigating the impact of Sfrp5 on skeletal muscle are available. However, it is well known that Wnt5a participates in muscle regeneration following injury by regulating satellite cell differentiation as well as determining fiber types of newly formed myotubes (222). Therefore, Wnt5a signaling is functional in skeletal muscle cells and might be regulated by Sfrp5 as well. Further studies investigating this difference in tissue-specific effects of Sfrp5 by e.g. analyzing the expression of

frizzled receptors or by investigating the impact of Wnt5a in combination with Sfrp5 should be performed in the future.

In mice, Sfrp5 has been reported to be exclusively secreted by adipocytes and has therefore been described to be a new adipokine (172). Nevertheless, Sfrp5 protein levels measured in the supernatant of differentiated human adipocytes were below the detection limit of the only commercially available ELISA. Furthermore, we have measured high mRNA expression of Sfrp5 in human pancreas and liver (data not shown), indicating that Sfrp5 is not exclusively expressed by adipocytes. In line with these results, Sfrp5 serum levels were similar in obese and non-obese men and not affected by weight loss after gastric bypass surgery (unpublished data). In addition, Ehrlund et al. reported that mRNA levels of Sfrp5 in white adipose tissue are low and unaltered by obesity. Importantly, this study demonstrated that Sfrp5 is not actively secreted from human white adipose tissue (169). Therefore, in contrast to mice, Sfrp5 cannot be considered as an adipokine in humans. However, further studies are crucial to investigate the reason for these controversial data as well as to analyze the underlying mechanism how and under which conditions Sfrp5 impairs insulin action.

In summary, phosphorylation of PRAS40 at Thr246, but not at Ser183, is a promising biomarker for insulin sensitivity in skeletal muscle and probably in human adipocytes. However, it should be kept in mind that kinases other than Akt can participate in PRAS40-Thr246 phosphorylation.

7.2. Potential Function of PRAS40

7.2.1 Impact of PRAS40 on mTORC1 signaling

7.2.1.1 Absence of PRAS40 indirectly affects mTORC1 activity

PRAS40 was first described to be an Akt substrate and 14-3-3 binding protein in 2003 (183). Nevertheless, the function of this novel Akt substrate remained unknown for several years. New aspects of the potential function of PRAS40 were generated after identification of PRAS40 as a component and substrate of the mTORC1 (217,223-225). Several groups have postulated that PRAS40 functions in the regulation of mTORC1 activity. This hypothesis was strengthened by observations that overexpression of wild type PRAS40 resulted in decreased mTORC1 activity, indicated by inhibition of phosphorylation of the mTORC1 substrates S6K and 4E-BP1, while knockdown of PRAS40 had the opposite effect (223-226). However, other attempts to detail the

function of PRAS40 in mTORC1 activation have led to conflicting results while demonstrating that PRAS40 also seemed to be important for mTORC1 activity (186,204,227,228). As already discussed in study 1 (chapter 2), the reason for these discrepancies between the reports is unknown. Our results analyzing the effect of PRAS40 knockdown (chapter 3) and enforced nuclear localization of PRAS40 (chapter 5) on mTORC1 signaling are in line with a requirement of PRAS40 for full mTORC1 activity. We could demonstrate that insulin-mediated phosphorylation of p70S6K-Thr389, pS6-Ser240 and p4E-BP1-Thr37/46 was significantly decreased in PRAS40 knockdown cells (chapter 3, Fig. 3.3 and Fig. 3.4). Additionally, protein levels of Grb10, a newly identified mTORC1 substrate (90,97-99) whose stabilization is increased when it becomes phosphorylated by mTORC1, was significantly down-regulated in PRAS40-KD cells (chapter 3, Fig. 3.3). Although we have not directly confirmed a decrease in mTORC1 activity by performing an *in vitro* mTORC1 activity assay, these results demonstrate that mTORC1 signaling is inhibited after PRAS40 knockdown. Furthermore, mutation of the nuclear export sequence within PRAS40, resulting in decreased PRAS40 protein levels in the cytosol, diminished insulin-stimulated p70S6K-Thr389 phosphorylation (chapter5, Fig.5.5e). It is important to realize, however, that these effects likely result from a secondary response induced by impaired activation of the PI3K/Akt/TSC2 pathway (chapter 3, Fig. 3.2 and chapter 5, Fig. 5.5) rather than from direct inhibition of mTORC1 activity by reduced PRAS40 protein levels. The fact that we have not observed any effect of PRAS40 knockdown or enforced nuclear localization on basal phosphorylation of the mTORC1 substrates further supports this conclusion. However, it still remains elusive, if a possible increase in mTOR enzymatic activity early on during the course of PRAS40 absence results in stimulation of feedback mechanisms known to decrease IRS1/PI3K/Akt/TSC2 signaling pathway and therefore induces subsequent inhibition of insulin to stimulate the mTORC1 signaling pathway.

Interestingly, in a study performed in C2C12 mouse myocytes, knockdown of PRAS40 diminished mTORC1 activity and protein synthesis via decreasing the protein level of mLST8 (also known as GβL), a component of mTORC1 and an important factor for full activation of mTORC1 (204). In addition, PRAS40 knockdown increased the activity of AMPK. On the one hand, AMPK can directly inhibit mTORC1 activity by phosphorylation of raptor at Ser722 and Ser792, which results in binding of phosphorylated raptor to 14-3-3 proteins and inhibition of mTORC1. On the other hand, AMPK indirectly suppresses mTORC1 activity via its action on TSC2 (86). Due to the decreased phosphorylation of TSC2 in our study mediated by decreased Akt activity, we can exclude that the decrease in mTORC1 activity is induced via the AMPK/TSC2 axis. Furthermore, in preliminary experiments we could not observe any significant effects of PRAS40 knockdown on AMPK-Thr172 or raptor-Ser792 phosphorylation (data not shown). It has been postulated that PRAS40 may be

essential for the assembly and integrity of the mTORC1 complex (228,229). For that reason, we also analyzed raptor protein abundance in PRAS40 knockdown and control cells without detecting any differences between the groups (chapter 3, Fig 3.8). However, we have not investigated the protein levels of the other components of the mTORC1, such as mLST8, as well as if binding of these factors to each other is disturbed in the absence of PRAS40.

7.2.1.2 Overexpression of PRAS40 directly inhibits mTORC1 activity

Enhanced expression of wild type PRAS40 in differentiated human skeletal muscle cells (chapter 4) demonstrated that mTORC1 activity is inhibited in PRAS40 overexpressing cells, which was indicated by reduced insulin-stimulated p70S6K phosphorylation at Thr389 and S6-Ser240 phosphorylation (chapter 4, Fig. 4.2). Similar results were obtained in studies performed in 3T3-L1 adipocytes (226) and HEK cells (217,223,228). Importantly, due to increased activity of the IRS1/Akt axis induced by PRAS40 overexpression (see 7.2.2), these inhibitory effects on mTORC1 activity seem to be directly mediated by PRAS40. In contrast to the effects of PRAS40 knockdown and enforced nuclear localization of PRAS40 on mTORC1 activity, which could result from inhibition of the IRS1/AKT/TSC2 pathway, PRAS40 overexpression modulates the mTORC1 activity independent of the IRS1/AKT/TSC2 axis. PRAS40 has been reported to directly interact with raptor, the regulatory component of the mTORC1, through its TOR signaling (TOS) motif (217,223,225,228). It is proposed that binding of PRAS40 to raptor sequesters mTORC1 from other substrates, such as p70S6K and 4E-BP1, and therefore inhibits their activation. However, PRAS40 association with mTORC1 appears to require the presence of both, mTOR and raptor, since disruption of the mTOR-raptor interaction releases PRAS40 from the mTORC1 (229). Phosphorylation of PRAS40 weakens the binding of PRAS40 to raptor and results in dissociation of PRAS40 from the mTORC1, enabling the binding and phosphorylation of other mTORC1 substrates. Until today, only one animal model for PRAS40 loss of function has been described to address the question if PRAS40 indeed modulates mTORC1 activity directly (230). By generating PRAS40 knockout *Drosophila,* Pallares-Carstes et al. could demonstrate that PRAS40 seems to modulate mTORC1 activity in a tissue-specific manner. In particular, PRAS40 affects TORC1 in ovary and testis of *Drosophila,* whereas PRAS40 is present but unable to modulate TORC1 activity in other tissues (230). However, when PRAS40 was overexpressed inhibition of TORC1 was also observed in most of the other tissues. In line with these results, a study conducted in mouse muscle cells (C2C12) demonstrated that reduction of PRAS40 protein levels in myoblasts impairs mTORC1-mediated protein synthesis, whereas PRAS40 knockdown in differentiated myotubes did not affect protein synthesis, indicating a developmental-specific effect of PRAS40 in muscle cells

(186) (see also 7.3.2). Importantly, all our studies were conducted in differentiated human skeletal muscle cells. It will be interesting to investigated possible differences of PRAS40 knockout or overexpression between undifferentiated and differentiated muscle cells in future studies.

In summary, the role of PRAS40 in the regulation of mTORC1 activity seems to be more complicated than anticipated. PRAS40 is present in all tissues but seems to be switched on and off in a tissue-and developmental-specific manner. Posttranslational modifications of PRAS40 could be responsible for this switch (for more details see 7.3.2). However, due to the fact that PRAS40 also modulates the IRS1/Akt/TSC2 pathway, alterations of the PRAS40 protein abundance also affects mTORC1 activity via this indirect way.

7.2.2 Impact of PRAS40 on insulin sensitivity

7.2.2.1 PRAS40 modulates insulin sensitivity by regulating IRS1 protein abundance

The mTORC1 pathway is well known to participate in physiological feedback regulation of insulin signaling. Various studies indicated that chronic activation of the mTORC1/S6K signaling in obesity and diabetes may promote insulin resistance by affecting IRS1 (110-114). Due to the proposed modulating effects of PRAS40 on mTORC1, this thesis aimed to analyze if alterations of PRAS40 protein abundance affects insulin signaling. Like studies regarding the function of PRAS40 in mTORC1 regulation, investigations about the impact of PRAS40 on insulin action have also revealed controversial results (chapter 2). While in HEK-293 cells neither PRAS40 overexpression nor knockdown affected insulin action (228), knockdown of PRAS40 in 3T3L1, HepG2 and C2C12 myotubes resulted in decreased insulin-mediated Akt phosphorylation and diminished IRS1 protein levels (204,224). These results are in line with our observations that PRAS40 knockdown, the absence of PRAS40 from the cytoplasm as well as overexpression of PRAS40 affect the activity of the insulin signaling pathway (chapter 3, chapter 4 and chapter 5). While the absence of PRAS40 (either due to silencing or due to enforced nuclear localization) inhibited insulin action in primary human skeletal muscle cells and A14 fibroblasts, the overexpression of PRAS40 increased insulin sensitivity in myotubes *in vitro*. Furthermore, to the best of our knowledge, results presented in this thesis have demonstrated for the first time that PRAS40 overexpression also improved insulin sensitivity *in vivo* in skeletal muscle. Importantly, the changes of insulin sensitivity by PRAS40 were mainly induced by affecting IRS1 protein abundance. mRNA levels of IRS1 were not altered by PRAS40 knockdown or

overexpression, indicating that PRAS40 modulates IRS1 levels at the posttranslational level (see section 7.2.3).

The IRS proteins have been implicated as crucial intermediates in insulin signaling and insulin-regulated glucose metabolism. They are essential for the promotion of glucose uptake and the regulation of genes important for the utilization of glucose for energy production as well as for the biosynthesis of macromolecules such as proteins, lipids and nucleic acids, which are required for cell growth and proliferation (231). Although defects at any step of the insulin signaling cascade between the IR and GLUT4 translocation can impair insulin sensitivity, it is widely accepted that defects in IRS1 represents a pivotal feature in insulin resistance (106,108-110). Multiple studies, also ones conducted in humans, have provided evidence that insulin signaling defects at the level of IRS1 are directly associated with insulin resistance and T2D (232). For instance, IRS1 phosphorylation was significantly altered in skeletal muscle from T2D patients (233). Moreover, not only tyrosine phosphorylation of IRS1 was reduced in conditions of insulin resistance, but also IRS1 protein levels are lower in skeletal muscle from morbidly obese insulin-resistant subjects or from women with gestational diabetes (232,234). Importantly, we could demonstrate that the insulin-resistant state in PRAS40 knockdown cells was entirely restored when IRS1 protein levels were normalized to control levels (chapter 3, Fig. 3.6). In addition, WT-PRAS40 overexpression protected against chronic insulin-induced insulin resistance via increasing IRS1 protein levels and thereby normalizing IRS1/PI3K/Akt signal transduction induced by insulin (chapter 4, Fig. 4.6). In summary, this finding verified that a decrease in IRS1 protein abundance is likely to underlie the altered insulin action by PRAS40 and not a consequence thereof.

Nevertheless, some studies also doubt this central role of IRS1. While obese IRS1 heterozygous knockout mice were troubled with profound insulin resistance compared to their obese wild type littermates, no differences regarding insulin sensitivity was observed between healthy (non-obese) *IRS*$^{+/-}$ and wild type mice (235). In line with this, shRNA-mediated silencing of IRS1 in mouse muscle did not impair glucose disposal (61). Similarly, decreased IRS1 activity did not impair full Akt phosphorylation by insulin in muscle tissue of insulin-resistant mice (236). It should be noted in this context that IRS1-deficient rodents show up-regulation of related IRS-proteins, like IRS2 and IRS3, which could take over the function of IRS1 (232,237,238). In line with the observations in IRS1-deficient mice, studies conducted in women with gestational diabetes (234) or polycystic ovary syndrome (PCOS) (239), who are also characterized by insulin resistance, a decrease in IRS1 protein levels in skeletal muscle biopsies was accompanied by an increase in IRS2 protein expression, suggesting a compensatory role of IRS2 trying to overcome the loss of IRS1. Because IRS1 is the most

prominent IRS isoform in skeletal muscle and adipose tissue (48), the activity or protein level of IRS2 has not been investigated in the studies conducted in this thesis.

Insulin-stimulated activation of the IR also mediates mitogenic effects via activation of the MAPK pathway. Importantly, insulin defects in T2D appear to be selective for the metabolic pathway (232). Skeletal muscles from obese insulin-resistant and moderately obese T2D subjects are characterized by a profound impairment of the IRS/PI3K pathway, but generally display a normal signal transduction along the MAPK pathway (233,240). However, as the impact of PRAS40 on insulin-mediated activation of the MAPK axis has not been analyzed here, a role of PRAS40 in the modulation of this insulin-regulated pathway cannot be ruled out. Therefore, the impact of PRAS40 on IRS2 as well as on the MAPK pathway should be investigated in future studies.

7.2.2.2 PRAS40 affects IRS1 in an mTORC1-independent way

A key finding of this thesis is that the modulation of insulin action by PRAS40 is not mediated by alterations in mTORC1 activity. Contrary to our initial hypothesis, alterations in PRAS40 protein abundance did not affect IRS1 via activation of the mTORC1-mediated feedback mechanism, such as suggested from a study conducted in 3T3L1 and HepG2 cells (224), which reported an increased phosphorylation of p70S6K in the absence of PRAS40. These investigators proposed that the reduction of IRS1 protein levels in these cells is mediated by induction of an inhibitory feedback loop due to the hyperactivity of the mTORC1/p70S6K pathway. Indeed, constitutive activation of mTORC1/p70S6K is believed to be one of the crucial mechanisms of insulin resistance (241). In skeletal muscle of obese rats and mice, hyperactivity of the mTORC1 pathway has been observed (111,117), which was linked to an increased inhibitory phosphorylation of IRS1 and impaired PI3K activation. In addition, inhibition of mTORC1 *in vitro* and in mice on a HFD displayed a reduction in inhibitory serine phosphorylation of IRS1, thus allowing optimal stimulation of IRS1 tyrosine phosphorylation by the IR and subsequent activation of the PI3K (113,241). In addition, depletion of S6K1 protected mice against obesity and insulin resistance due to up-regulation of the oxidative phosphorylation pathway (e.g. via increasing the number of mitochondria) as well as increased insulin sensitivity (113). Finally, a newly identified substrate of mTORC1, Grb10, has also been postulated to mediate feedback mechanisms on the IR and IRS1 (97-99). It was postulated that phosphorylation of Grb10 by mTORC1 affects the stability of this protein and triggers the induction of Grb10-mediated feedback loops (90). Depletion of Grb10 increases insulin sensitivity, whereas overexpression suppresses insulin signaling via binding of Grb10 to the IR and blocking tyrosine phosphorylation of IRS1 (90,99,242). The work presented in this thesis showed that PRAS40

knockdown in hSkMCs was associated with decreased insulin-stimulated phosphorylation of p70S6K and 4E-BP1 as well as reduced Grb10 protein abundance compared to control cells (chapter 3, Fig. 3.3). Crucially, inhibition of p70S6K did not restore IRS1 protein levels and insulin signaling in cells lacking PRAS40 (chapter 3, Fig. 3.4). In addition, enforced localization of PRAS40 in the nucleus also decreased IRS1 protein abundance as well as insulin-mediated phosphorylation of p70S6K at Thr389 (chapter 5, Fig. 5.5). Together these data clearly demonstrate that PRAS40 alters insulin sensitivity in an mTORC1-independent way. In support of these data, Hong-Brown et al. have reported that the absence of PRAS40 was accompanied with a decreased IRS1 protein abundance and a simultaneous down regulation of mTOR as well as p70S6K activity in C2C12 myotubes (204). Overexpression of WT-PRAS40 increased IRS1 protein levels but simultaneously inhibited mTORC1 activity (chapter 4, Fig. 4.1-2). Thus, one may speculate that PRAS40 overexpression increased IRS1 abundance via inhibition of an mTORC1-mediated feedback-loop. However, considering all data presented in this thesis, it is more convincing that PRAS40 modulates IRS1 and mTORC1 activity via two independent mechanisms (see also 7.3.2).

It had long been postulated that the p70S6K-IRS1 feedback loop was insufficient to explain the powerful negative control of insulin action by mTORC1, leading to the identification of the mTORC1/Grb10 mechanism to fill the first gap in understanding this process (99). Although PRAS40 regulates insulin signaling independently of mTOR, Grb10 and p70S6K, we have identified PRAS40 as a new component of the mTORC1 signaling network, which also participates in feedback mechanisms regulating insulin sensitivity.

Our results further demonstrate that alterations in PRAS40 protein abundance have a significant impact on insulin sensitivity *in vivo* on the one hand and in different cell types *in vitro* on the other hand via affecting IRS1 protein abundance. This indicates that not only phosphorylation of PRAS40 but also total protein expression of PRAS40 may act as a biomarker for insulin sensitivity. In this respect, we have conducted experiments to examine whether PRAS40 protein abundance is altered between insulin-sensitive and insulin-resistant subjects. However, preliminary analysis of PRAS40 protein levels in skeletal muscle biopsies from healthy subjects and patients with the metabolic syndrome or type 2 diabetes provided no evidence for significant deregulations of PRAS40 protein levels among these groups (data not shown). In addition, investigations concerning possible gene variants within the PRAS40 gene (*Akt1S1*) and their association with insulin resistance and T2D are still lacking, indicating the necessity to further characterize the impact of alterations in PRAS40 protein expression for the progression of insulin resistance in humans.

7.2.3 Impact of PRAS40 on the 26S proteasome

7.2.3.1 PRAS40 alters mRNA expression of the E3 ligases *MuRF1* and *FBXO32*

Up to date, a variety of mechanisms have been reported which modulate the activity of IRS1, like protein/protein interactions, inhibitory phosphorylation, dephosphorylation or other posttranslational modifications as well as targeted degradation by the proteasome (43,105). Early studies in the 1990s have already linked an increased proteasome activity with enhanced degradation of IRS1 after prolonged insulin exposure (136,243). Although some kinases involved in inhibitory IRS1 phosphorylation as well as E3 ligases have already been identified to be associated with increased proteasomal degradation of IRS1, regulation of this process still remains to be under investigation. Based on results presented in this thesis, we have obtained first hints indicating that MuRF1 (chapter 3, Fig. 3.5; chapter 4, Fig. 4.3) and in part also FBXO32 (also known as atrogin 1) (chapter 3, Fig. 3.5), two muscle specific E3 ligases, participate in the regulation of IRS1 stability. While the decrease in IRS1 protein levels in PRAS40 knockdown cells was associated with an increased mRNA expression of *MuRF1* and *FBXO32*, mRNA levels of *MuRF1* were reduced after PRAS40 overexpression, resulting in elevated IRS1 protein abundance. Nevertheless, evidences that MuRF1 and FBXO32 act as genuine IRS1-E3 ligases have not been obtained so far. In contrast, E3 ligases CUL7, F-box only protein 40, Cbl-b and F-box/WD repeat-containing protein 8 have been identified to participate in IRS1 degradation (244-246). Cbl-b knockout mice are protected from diet-induced obesity and insulin resistance likely due to inhibition of IRS1 degradation (247,248). Mitsugumin 53 (MG53), a muscle- and heart-specific E3 ligase has recently been identified to induce IR and IRS1 degradation via activation of the 26S proteasome. Knockout of MG53 in mice resulted in protection against HFD-induced obesity, insulin resistance and other metabolic disorders, whereas overexpression of MG53 caused insulin resistance, obesity, hypertension and dyslipidemia (249). Thus, future investigation must show if one of these E3 ligases are also involved in PRAS40-mediated IRS1 degradation.

7.2.3.2 PRAS40 affects *MuRF1* and *FBOX32* expression in an mTORC1-independent way

One may speculate that the increased expression of the E3 ligases in PRAS40 knockdown cells results from an inhibition of mTORC1 activity. This hypothesis is supported by a study conducted in mTOR-heterozygous mice (250). Skeletal muscles of these animals displayed an enhanced expression of both E3 ligases, *MuRF1* and *FBXO32*. Furthermore, inhibition of mTORC1 by rapamycin in primary

rat hepatocytes was associated with reduced inhibition of the ubiquitin-proteasome system by amino acids and insulin (251). In line with this, Tong et al. have reported that rapamycin-treatment increased basal protein expression of MuRF1 and FBXO32 in C2C12 myotubes (252). These reports suggested that mTORC1 activity is negatively correlated with the expression of E3 ligases and ubiquitin-dependent degradation of proteins. In addition, Grb10, which was also decreased in PRAS40 knockdown myotubes, has been identified to protect the vascular endothelial growth factor receptor 2 (VEGF-R2) against proteasomal degradation by inhibiting the ubiquitin ligase neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) (253). These data would support the idea that PRAS40 knockdown induced increased mRNA expression of *MuRF1* and *FBXO32* via inhibition of mTORC1. However, several facts argue against this hypothesis: i) As mentioned above, decreased mTORC1 activity in PRAS40 knockdown cells seemed to be a secondary event due to inhibition of the PI3K/Akt/TSC2 axis. Therefore, decreased mTORC1 activity as well as reduced Grb10 protein levels have to be considered as a result and not as the primary cause for IRS1 degradation. ii) According to the hypothesis, decreased *MuRF1* expression in PRAS40 overexpressing myotubes should be accompanied by elevated mTORC1 activity, which in turn protects against IRS1 degradation. However, activity of mTORC1 is significantly reduced in WT-PRAS40 expressing cells. iii) Overexpression of both, WT and mutant PRAS40, reduced *MuRF1* mRNA levels in skeletal muscle cells but only overexpression of WT-PRAS40 and not of AAA-PRAS40 affected mTORC1 activity (see also section 7.3.2). iv) Although expression of *MuRF1* and *FBXO32* was elevated in mTOR-heterozygous muscles, no impact of this genotype on IRS1 protein levels has been observed (250). v) Finally, genetic deletion or knockdown of raptor in skeletal muscle, which results in activation of mTORC1, even caused a decrease in *MuRF1* and *FBXO32* expression (254). Therefore, it is unlikely that PRAS40 modulates expression of E3 ligases via mTORC1.

Expression of *MuRF1* and *FBXO32* are both regulated amongst others by the FOXO transcription factor signaling pathway (255). As mentioned above (see 1.2.1 and Fig. 1.3), Akt activation results in phosphorylation of FOXO proteins, leading to translocation of FOXO from the nucleus and inhibition of their transcriptional functions. Therefore, decreased Akt activity might reduce FOXO phosphorylation and increase *MuRF1* and *FBXO32* mRNA expression. However, other studies pointed out that translocation of FOXO proteins may also be regulated independently of Akt (250,256,257). The absence of PRAS40 from the cytoplasm in A14 fibroblast inhibited insulin-mediated phosphorylation of Akt and FOXO3a-Thr32 (chapter 5, Fig. 5.5), which may affect expression of the E3 ligases. However, it is difficult to discriminate if decreased FOXO phosphorylation in NES-PRAS40 expressing cells is a consequence of inhibition of the IRS1/PI3K/Akt axis or a potential cause for increased IRS1 degradation, if it is activated in an Akt-independent way.

Unfortunately, mRNA levels of *MuRF1* and *FBXO32* have not been analyzed in this study. In order to obtain further insight into the participation of FOXO proteins in PRAS40-mediated regulation of *MuRF1* and *FBXO32*, insulin-mediated phosphorylation of FOXO should be analyzed in skeletal muscle cells lacking or overexpressing PRAS40.

7.2.3.3 PRAS40 alters the activity of the 26S proteasome

Importantly, in this thesis we provide evidence that PRAS40 not only increases mRNA expression of E3 ligases but also affects proteasome activity (chapter 3, Fig 3.5; chapter 4, Fig. 4.3). In addition, the increase in proteasome activity in PRAS40 knockdown cells is responsible for the induction of insulin resistance. Inhibition of the proteasome using the specific inhibitor MG-132 entirely restored IRS1 protein levels as well as insulin-mediated Akt phosphorylation in cells lacking PRAS40 (chapter 3, Fig. 3.6). Conversely, proteasomal activity was decreased in myotubes overexpressing PRAS40 (chapter 4, Fig. 4.3). Although proteasomal degradation of a variety of insulin signaling targets, such as IRβ, Glut4 or Akt have been reported (146,249,258), PRAS40 only affected the degradation of IRS1. Neither protein abundances of Akt, IRβ nor Glut4 was altered in the absence of PRAS40 or after PRAS40 overexpression (chapter 3, Fig. 3.1; chapter 4, Fig. 4.1; chapter 5, Fig. 5.4) In addition, overexpression of PRAS40 restored hyperinsulinemia-induced reduction of IRS1 but did not alter IRβ protein levels, resulting in a persisting decrease of IRβ abundance in PRAS40 overexpressing cells after chronic insulin treatment. As mentioned above, this specificity of proteasomal degradation could be mediated via activation of IRS1-specific E3 ligases by PRAS40, which remain to be identified in the future.

Although PRAS40-mediated activation of the proteasome seemed to exclusively target IRS1, we cannot rule out that PRAS40 may also regulate the protein stability of other IRS isoforms. For instance, IRS2 has been reported to be also targeted by the proteasome (133,259-261). Chronic exposure of pancreatic β-cells to glucose induced mTOR-mediated inhibitory phosphorylation of IRS2, which resulted in its proteasomal degradation (259). In addition, different cell culture models of insulin resistance, such as osmotic stress or chronic exposure to insulin or IGF1, have been reported to induce IRS2 degradation in 3T3-L1 cells, Fao hepatoma cells and mouse embryo fibroblasts (260).

To conclude, in this thesis we could demonstrate for the first time that PRAS40 affects the activity of the proteasome, resulting in modulation of IRS1 protein stability. The underlying mechanisms, how PRAS40 interacts with E3 ligases and the 26S proteasome still need to be investigated in the future. Identification of new IRS1-specific E3 ligases, or investigations analyzing the impact of FOXO proteins, SOCS proteins, which have also been described to be involved in IRS1

degradation (262), as well as 14-3-3 proteins (see 7.3.1) could be possible first steps for a better understanding of this process.

7.3. Importance of PRAS40 localization and posttranslational modification for its function

7.3.1 Impact of PRAS40 localization on insulin action

Sequence analysis of PRAS40 has identified a potential leucine-rich nuclear export sequence (NES) in the carboxyterminal part of PRAS40 (207). Therefore, it was not surprising that PRAS40 was originally identified as a nuclear phosphoprotein in HeLa cells (185). In addition, immunohistochemical staining of hepatocytes and cardiomyocytes from insulin-treated rats with an antibody against PRAS40-Thr246 demonstrated a nuclear localization of this protein in both cell types (182). Exposure of A14 fibroblasts with the nuclear export inhibitor leptomycin B or mutation of the nuclear export sequence within PRAS40 resulted in increased nuclear abundance of PRAS40 (chapter 5, Fig. 5.2). However, the functional importance of PRAS40 translocation remained incompletely addressed. We found that mutation of the NES sequence affected insulin action in A14 fibroblasts (chapter 5). In this context, NES-PRAS40 expressing cells were characterized by decreased insulin-mediated phosphorylation of Akt and FOXO3a as well as p70S6K, indicating that mTORC1 signaling was additionally impaired. As mentioned above (see 7.2.1 and 7.2.2), the effect of NES-PRAS40 on insulin signaling was induced via down-regulation of IRS1 protein levels. Thus, NES-PRAS40 expression in A14 cells resembled the phenotype induced by PRAS40 knockdown in skeletal muscle cells.

Despite the presence of NES, no nuclear localization signal within PRAS40 could be identified so far. Therefore, mechanism and regulation of PRAS40 import into the nucleus remains to be addressed. Due to the fact that PRAS40 is a 40kDa protein and proteins smaller than 60kDa can freely shuttle between the cytoplasm and the nucleus, translocation of PRAS40 independently of an active transport would be conceivable. However, WT-PRAS40 was predominantly localized in the cytoplasma and did not shuttle into the nucleus indicating that PRAS40 translocation indeed displays a regulated process. Importantly, translocation of PRAS40 was independent of phosphorylation of PRAS40 as well as of its binding to raptor (chapter 5, supplementary data Fig. 5.7).

PRAS40 is not the only component of the mTORC1 displaying a different subcellular localization. Although mTOR functions mainly in the cytoplasm, mTOR and other mTORC1-associated

proteins as well as regulators of mTORC1 activity, such as Akt and TSC2, are also found in the nucleus (53,263-266). In this context, translocation mainly seemed to be dependent on specific phosphorylation events (264-266). In contrast, nuclear localization of total PRAS40 protein was not affected by insulin treatment (chapter 5, Fig. 5.3) but the amount of phosphorylated PRAS40 in the nucleus increased after insulin stimulation. So far, it is unknown which function PRAS40 may have in the nucleus. On the one hand, one may speculate that due to the regulating properties of PRAS40 on mTORC1 in the cytosol, PRAS40 may also function in the regulation of nuclear mTOR processes, such as alteration of rDNA and tRNA transcription (267). On the other hand, PRAS40 or phosphorylated PRAS40 may have a direct mTOR-independent function in the nucleus. The proline-rich regions in the amino-terminal of PRAS40 as well as sequences that have the potential to bind proteins containing Src homology 3 and/or WW domains (chapter 1 and (228)) may mediate different protein-protein interactions with so far unidentified nuclear binding partners. Therefore, it is difficult to clarify if the impact of NES-PRAS40 expression on insulin action is induced by a direct effect of enhanced PRAS40 abundance in the nucleus (for instance via affecting transcription of E3 ligases and thereby promoting IRS1 degradation) or by a secondary effect induced by the absence of PRAS40 from the cytoplasm (mimicking silencing of PRAS40). Further studies investigating the function of PRAS40 in the nucleus as well as the regulation of PRAS40 import into the nucleus are necessary to address these questions.

7.3.2 Impact of PRAS40 phosphorylation as well as raptor-binding on PRAS40 function

A study conducted in *Drosophila* has described for the first time that function of PRAS40 may differ among various tissues (230). The authors proposed that the phosphorylation state of PRAS40 might be differently regulated in the tissues and therefore define PRAS40 function. Comparable data were obtained in cultured cell lines, as illustrated by the observation that PRAS40 knockdown induced different phenotypes in undifferentiated C2C12 myoblasts versus differentiated myotubes (186). Thus, the function of PRAS40 might depend on posttranslational modifications such as phosphorylation events and might be differently regulated in distinct tissues or during cell differentiation. To assess the impact of phosphorylation on PRAS40 function, we generated a mutant form of PRAS40 (AAA-PRAS40), in which both key phosphorylation sites, Thr246 and Ser183, as well as the TOS motif at Phe129 were substituted to Ala resulting in loss of phosphorylation at these sites as well as prevention of PRAS40 binding to raptor (chapter 4). Mutation of the phosphorylation sites Thr246 and Ser183 has been reported to alter PRAS40 function via affecting its binding to 14-3-3

proteins (see 7.3.3). In contrast, preventing PRAS40-raptor binding has been linked to decreased PRAS40-mediated inhibition of mTORC1 activity (226). In addition, mutation of Ser183 also abolished binding of PRAS40 to raptor, resulting in increased mTORC1 activity (217). In line with these results, overexpression of AAA-PRAS40 in differentiated human skeletal muscle cells did not impair insulin-stimulated activation of mTORC1, demonstrated by unchanged phosphorylation of p70S6K and S6 compared to insulin-treated control cells (chapter 4, Fig. 4.2). Conversely, overexpression of WT-PRAS40 inhibited insulin-stimulated phosphorylation of these mTORC1 targets, demonstrating that PRAS40 function on mTORC1 depends on intact phosphorylation sites as well as on binding of PRAS40 to mTORC1.

Importantly, this AAA-PRAS40 helped us to further substantiate that the effect of PRAS40 on insulin action is independent of mTORC1 signaling. No differences between WT- and AAA-PRAS40 expressing cells were observed regarding the expression of *MuRF1* as well as the activity of the proteasome (chapter 4, Fig. 4.3). In both myotubes the proteasomal machinery was inhibited, resulting in increased IRS1 protein abundance. These results further support the indication, that PRAS40 has a dual function in that it can regulate mTORC1 activity on the one hand and proteasomal activity on the other hand, which both seemed to be independent of each other.

However, some issues remain to be addressed in this context. Although expression of AAA-PRAS40 has an insulin-sensitizing effect, the activity of this mutant is not similar to that of WT-PRAS40. Furthermore, in contrast to WT-PRAS40 expressing cells, AAA-PRAS40 expressing cells fail to overcome hyperinsulinemia-induced insulin resistance. These data suggest that for full beneficial effect of PRAS40 on insulin sensitivity, phosphorylation at Ser183 and Thr246 and/or binding to raptor is required. In addition, PRAS40 exhibits at least eight additional (potential) phosphorylation sites (chapter 2), which might participate in regulation of PRAS40 function as well in its localization. Furthermore, the potential existence of inhibitory phosphorylation sites within PRAS40 was hypothesized to be involved in regulation of PRAS40 function (230). Finally, posttranslational events other than phosphorylation might impact PRAS40. Application of tools for prediction of posttranslational protein modifications (http://prosite.expasy.org/prosite.html, (268)) revealed that PRAS40 might also undergo N-glycosylation as well as N-myristoylation. This indicates that further studies, for instance involving the generation of different PRAS40 mutants, in particular individual mutation of the phosphorylation sites, are required to analyze the impact of posttranslational modification on PRAS40 function.

7.3.3 Potential role of 14-3-3 proteins for PRAS40 function

14-3-3 binding proteins are highly conserved proteins which are expressed in all eukaryotic cell types (269). All seven isoforms (α/β, ε, η, γ, τ/θ, δ/ζ, σ) are reported to regulate a broad range of cellular processes such as cell death, cell migration, cell-cycle, cytoskeletal dynamics and especially cellular signaling pathways. Up to now, more than 500 potential 14-3-3 binding partners have been identified (269). Recognition and binding of target proteins by 14-3-3 proteins depends on a putative peptide sequence (RSXpSXP, p represents phosphorylated serine, X any amino acid) characterized by a phosphorylated serine residue (270). Due to the large number of 14-3-3 interacting partners, the particular function of 14-3-3 proteins is difficult to define. Binding of 14-3-3 proteins can induce conformational changes within target proteins, modulate interactions of the target protein with other proteins or facilitate protein-protein interaction by acting as scaffold proteins (271). Furthermore 14-3-3 proteins can directly alter enzyme activity of target proteins, shield against dephosphorylation, modulate subcellular localization of their cargos (269,272-274) or mediate proteasomal degradation of their binding proteins (261,275,276).

PRAS40 has been identified to be one of the 500 binding partners of 14-3-3 proteins (183,228). Phosphorylation of PRAS40 at Thr246 and additionally at Ser183 is essential for binding of PRAS40 to 14-3-3 proteins (see chapter 2, (183,228,277)), demonstrating that PRAS40 binding to 14-3-3 proteins is Akt- and mTORC1-dependent. In line with this, mutations within mTORC1 binding sites markedly impaired 14-3-3 binding to PRAS40 (228,277). Formation of PRAS40/14-3-3 protein complexes has been proposed to function in PRAS40-mediated mTORC1 regulation. Thus, phosphorylation of PRAS40 by Akt and mTORC1 induces the dissociation of PRAS40 from mTORC1 through its binding to 14-3-3 proteins. This dissociation is believed to be responsible for elevated mTORC1 activity via increasing the binding capacity of mTOR for its other substrates, such as 4E-BP1, Grb10 or p70S6K (chapter 2, (229)). However, other reports have demonstrated that binding of PRAS40 to 14-3-3 protein is not required in order to repress the inhibitory function of PRAS40 on mTORC1 activity (223,278). Therefore, it remains to be addressed if binding of PRAS40 to 14-3-3 proteins indeed affects its regulatory function on mTORC1.

As mentioned above, 14-3-3 proteins also participate in nuclear translocation of proteins (270,272-274). Thus, 14-3-3 proteins may participate in regulation of PRAS40 localization. However, we reported that mutation of PRAS40 phosphorylation sites did not impact PRAS40 localization (chapter 5, supplementary data, Fig. 5.7). In addition, insulin treatment of A14 fibroblasts did not alter PRAS40 abundance in the nucleus, demonstrating that participation of 14-3-3 proteins in PRAS40 translocation is rather unlikely.

Finally, the interaction of PRAS40 and 14-3-3 proteins may participate in modulation of IRS1 protein abundance by PRAS40. Neukamm et al. have recently published that cAMP-stimulated

binding of 14-3-3 to IRS2 protects IRS2 from proteasomal degradation in HEK293 cells (261). They hypothesize that interaction of 14-3-3 and IRS2 results in a conformational change that obscures the recognition motif for E3 ligases, thus preventing IRS2 degradation. The stretch implicated in 14-3-3 binding within IRS2 seems conserved in IRS1, indicating that 14-3-3 binding to IRS1 could protect the protein against degradation. However, other reports indicated that binding of 14-3-3 proteins to IRS1 inhibits insulin-stimulated PI3K activation (279) and plays an integral role in the process of insulin desensitization via sequestering IRS1 in subcellular compartments (280). It remains to be investigated whether changes in PRAS40 levels impact on IRS1/14-3-3 complex formation and thereby affects insulin sensitivity. Therefore, it could be of great importance to alter 14-3-3 protein abundance simultaneously to PRAS40 to verify the role of 14-3-3 proteins in PRAS40 function.

7.4. Potential role of PRAS40 in other tissues and diseases

Due to the central role of the IRS1/PI3K/Akt and mTORC1 pathway in insulin signal transduction, PRAS40 may also have an impact on insulin action in tissues other than skeletal muscle. Furthermore, these signaling pathways also participate in progression of disorders other than metabolic disturbances, such as cancer. Therefore, the results of our studies might provide new insights into the possible impact of PRAS40 on progression of other metabolic complications as well as other diseases.

As mentioned in the introduction (see 1.1.3), progression of T2D is associated with insulin resistance in skeletal muscle, adipose tissue and liver as well as pancreatic β-cell dysfunction. Although insulin signaling in skeletal muscle cells, adipocytes and hepatocytes results in different outcomes for the respective tissue (e.g. glucose uptake in skeletal muscle or inhibition glucose production in the liver), metabolic function of insulin in all insulin-sensitive tissues is mainly transmitted through IRS activation. Importantly, PRAS40 is ubiquitously expressed in all tissues examined so far, for instance including brain, cardiac muscle, liver and adipose tissue (chapter 2, (229)). Thus, PRAS40 may also affect insulin action in these tissues via similar mechanisms as observed in skeletal muscle cells (chapter 3 and 4). In addition, the IR-induced signaling cascade including activation of the PI3K-, MAPK- as well as mTORC1 pathway is required for insulin-stimulated mitogenesis of pancreatic β-cells (281,282). Especially IRS2 has been demonstrated to play a crucial role in maintaining β-cell homeostasis and function (283). Therefore, it might be of great importance to investigate the potential modulating effects of PRAS40 on β-cell function. Endothelial dysfunction is a key event in the pathogenesis of diabetic micro- and macrovascular disorders and is induced by hyperglycemia, excess exposure to FFAs as well as insulin resistance

(284). Preliminary results obtained from insulin- or VEGF-treated umbilical vein endothelial cells (HUVECs) indicate that PRAS40 also regulates Akt-and mTORC1 signaling in these cells (data not shown). However, datasets have to be expanded in the future. Finally, PRAS40 has been demonstrated to be a crucial mediator of glomerular hypertrophy, a process linked to progression of diabetic nephropathy (chapter 2 and (285-288)). In summary, these results suggest that PRAS40 might also influence the progression of various disorders linked to T2D and MetS.

Nevertheless, PRAS40 has already been linked to other diseases which are not directly associated with metabolic disorders. Investigations of the role of PRAS40 in carcinogenesis have already aroused great interest. Akt activation is one of the most frequent alterations observed in human cancer, resulting in promotion of tumorigenesis by inhibiting apoptosis (229,289). In addition, deregulation of the mTORC1 signaling pathway is an essential mediator of cancer progression and tumor formation and is induced through the loss of PTEN, mutations in TSC/TSC2 complex, hyperactivity of IRS/PI3K and/or overexpression of Akt (290). On the one hand, Akt-dependent phosphorylation of PRAS40 at Thr246 has been reported to be elevated in several cancer cell lines as well as in meningiomas and malignant melanomas. Therefore, PRAS40-Thr246 has been identified to act as an eligible biomarker for the investigation of the efficiency of novel inhibitors of the PI3K-pathway as new therapeutic options (for details see chapter 2). On the other hand, PRAS40 has also been reported to directly affect tumor development. Knockdown of PRAS40 in UACC 903 cells (human melanoma cells), which were subsequently injected subcutaneously into mice, resulted in decreased tumorigenic potential of these melanoma cells. In particular, targeting PRAS40 inhibited melanoma tumor development by elevating apoptosis rates in these tumor cells (208). In line with this, cell proliferation and metastatic growth of Ewing sarcoma family tumors (ESFT) cells were suppressed by siRNA-mediated PRAS40 knockdown (291). Additionally, the anti-apoptotic properties of PRAS40 have also been identified to have an impact on the development of embryoid bodies during human amniotic fluid stem cell (AFS) differentiation (292). In contrast to these reports, one study has postulated that PRAS40 promotes apoptosis, because knockdown of PRAS40 prevented TNFα-induced apoptosis in HeLa cells (225). This indicates again that PRAS40 function in the regulation of apoptosis and proliferation differs between different types of tumors and cells and might be regulated via posttranslational modifications or alterations in PRAS40 localization.

Finally, PRAS40 has been demonstrated to be associated with the progression of neuronal diseases, in particular Alzheimer´s diseases. Accumulation and toxicity of β-amyloid (Aβ) in the brain is considered to be a significant component of the onset and progression of Alzheimer´s diseases. Wnt 1 inducible signaling pathway protein 1 (WISP1) expression has been reported to be upregulated after Aβ exposure and to play a role in protection of microglial cells against Aβ (293). Shang et al.

have reported that the neuroprotective function of WISP1 is mediated by the inhibition of PRAS40 resulting in increased mTORC1 activity. Similar to WISP1 treatment, gene reduction of PRAS40 decreased microglial cell injury, genomic DNA degradation as well as externalization of membrane phosphatidylserine residues during Aβ exposure. In line with this identification of a neuroprotective function of PRAS40, overexpression of PRAS40 reduced neuronal apoptosis after cerebral ischemia in mice as well as increased the survival of motor neurons after spinal cord injury in rats (184,294,295).

7.5. Conclusion and perspectives

The balance and regulation of insulin action is essential for the maintenance of glucose homeostasis and is disturbed in patients with T2D. The term insulin resistance describes conditions in which insulin becomes increasingly ineffective in decreasing blood glucose levels due to disturbances of insulin action in the peripheral tissues. Especially, induction of insulin resistance in skeletal muscle is of great importance, since this organ is mainly responsible for postprandial clearance of glucose from the circulation. We and others have demonstrated that phosphorylation of PRAS40 is a suitable biomarker for insulin resistance. Although kinases which phosphorylate PRAS40 independently of Akt have been identified, PRAS40-Thr246 phosphorylation sufficiently reflects Akt activity in skeletal muscle cells. In line with this, decreased insulin-stimulated phosphorylation of PRAS40 displayed the insulin resistance state in Sfrp5-treated adipocytes.

In this thesis we have demonstrated for the first time that PRAS40 is a novel modulator of insulin action in skeletal muscle *in vivo* and *in vitro* and that alterations in protein abundance as well as localization of this protein affect insulin sensitivity. In addition, PRAS40 overexpression protects against hyperinsulinemia-induced insulin resistance in skeletal muscle, which depends on the capability of PRAS40 to bind mTORC1 as well as to be phosphorylated after insulin stimulation. The effects of PRAS40 on insulin action are induced by alterations in IRS1 protein abundance. Importantly, PRAS40-mediated regulation of insulin action is independent of its function on the mTORC1 activity and is not transmitted by mTORC1-induced feedback mechanisms on IRS1. In contrast, protein abundance of IRS1 is regulated by PRAS40-mediated alterations of the 26S proteasome activity. In this context, IRS1 seems to be exclusively targeted for proteasomal degradation, since protein levels of other insulin signaling targets, such as Akt, IRβ or Glut4 are unaltered. The results presented here indicate that PRAS40 possesses a dual function: One the one hand, depending on its phosphorylation, PRAS40 inhibits mTORC1 activity. In this context, PRAS40-mediated regulation of mTORC1 is able to uncouple mTORC1 activity from the PI3K/Akt/TSC2 pathway, since inhibition of mTORC1 by PRAS40 overexpression overcomes increased IRS1/PI3K/Akt

activity in skeletal muscle cells. On the other hand, PRAS40 modulates the IRS1/PI3K/Akt pathway and insulin sensitivity. Although the effects on the proteasomal machinery are mediated independently of posttranslational phosphorylation of PRAS40 as well as of its binding to mTORC1, beneficial effects of PRAS40 overexpression are only fully mediated if phosphorylation and binding to mTORC1 is intact (Fig. 7.3).

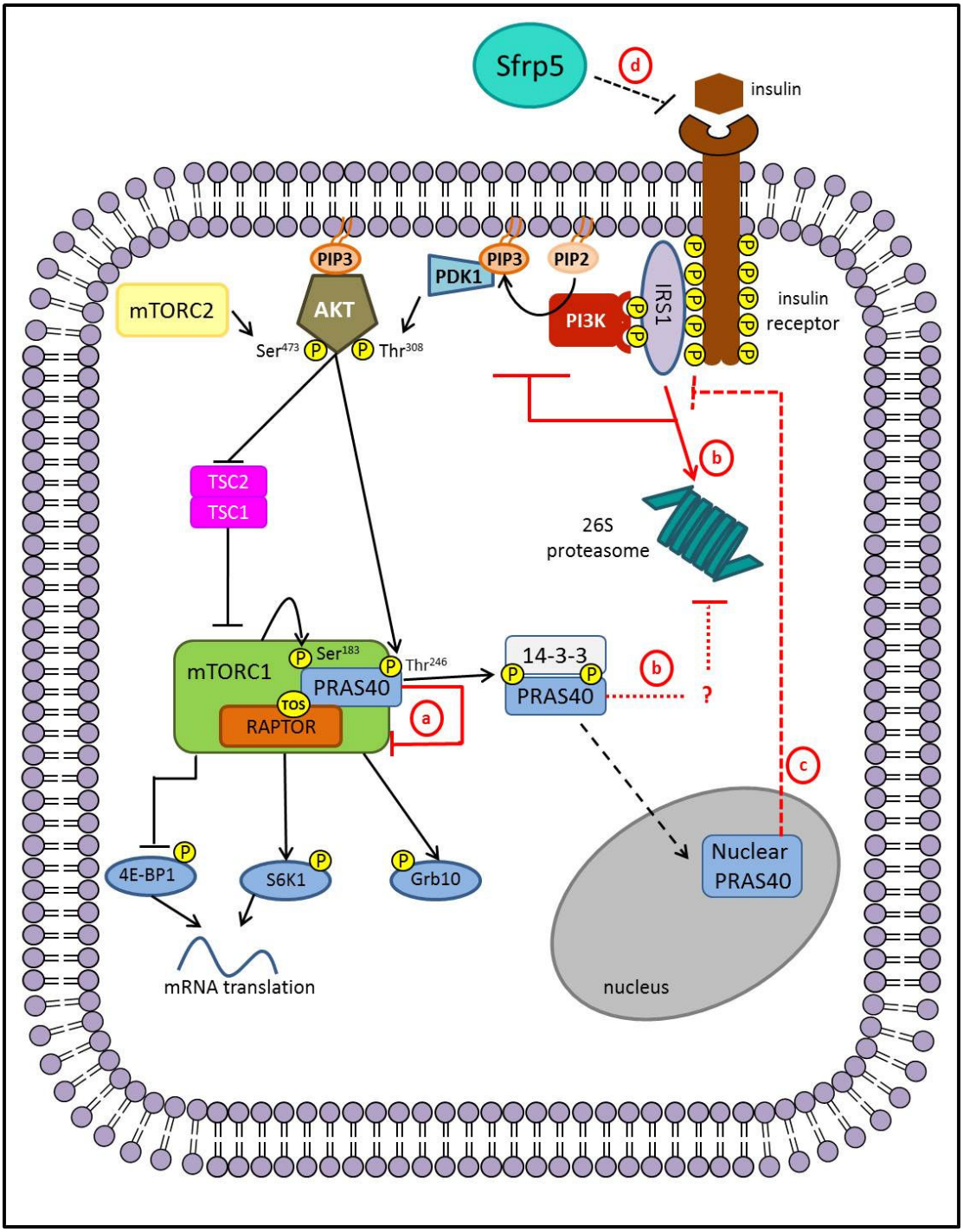

Figure 7.3 Summary of the presented work. The protein PRAS40 binds via its TOS-motif to raptor. Phosphorylation of Ser183 within this protein is mediated by mTORC1, whereas phosphorylation at Thr246 is induced by Akt. PRAS40 has a dual cellular function: (a) PRAS40 inhibits mTORC1 function. The suppressing

effect depends on the phosphorylation of PRAS40 as well on its binding to mTORC1. Phosphorylation of PRAS40, for instance induced by insulin, results in dissociation of PRAS40 from mTORC1 and thereby in the release of the suppressive constrain. Phosphorylated PRAS40 is believed to bind 14-3-3 proteins. However, the function of 14-3-3 proteins in modulating PRAS40 function is still unclear. In addition, PRAS40 also modulates the activity of the proteasomal machinery and thereby alters IRS1 protein abundance (b). IRS1 degradation is highly linked to disturbances in insulin-mediated activation of the PI3K/Akt pathway. The mechanism by which alterations in PRAS40 protein abundance affect proteasomal-mediated degradation of IRS1 still needs to be identified. Furthermore, PRAS40 possesses a nuclear export sequence (c). Enforced accumulation of PRAS40 in the nucleus is associated with decreased IRS1 protein levels exactly mimicking the results obtained in PRAS40 knockdown cells. Finally, phosphorylation of PRAS40 at Thr246 acts as a suitable marker for insulin sensitivity. In line with this, Sfrp5 (d), a newly identified inhibitor of insulin action in human adipocytes, reduces insulin-mediated phosphorylation PRAS40 at Thr246. However, the precise mechanism in which Sfrp5 affects insulin sensitivity needs to be address in the future.

Identifying the underlying mechanism by which PRAS40 affects proteasomal activity and how IRS1-specifity is achieved will be the challenge for the future. One attempt might be to find differences in the protein expression pattern between cells expressing different amounts of PRAS40 using proteomics approaches. Pilot experiments using the 2D-difference gel electrophoresis (DIGE) analysis, where the protein pattern of PRAS40 knockdown cells were compared to skeletal muscle cells treated with non-target siRNA, revealed that the abundance of six protein spots is altered in the absence of PRAS40 (Fig.7.4). In this context, PRAS40 knockdown increased the abundance of four protein spots, whereas the expression of two protein spots was down-regulated. The identity of these six proteins could not be established yet. However, these experiment have to be expanded (for instance via sub-fractionation of the cell lysates or restriction of the pH-gradient). Identification of these proteins and others could yield new insight into the mechanism of PRAS40 function in the cytosol as well as in the nucleus.

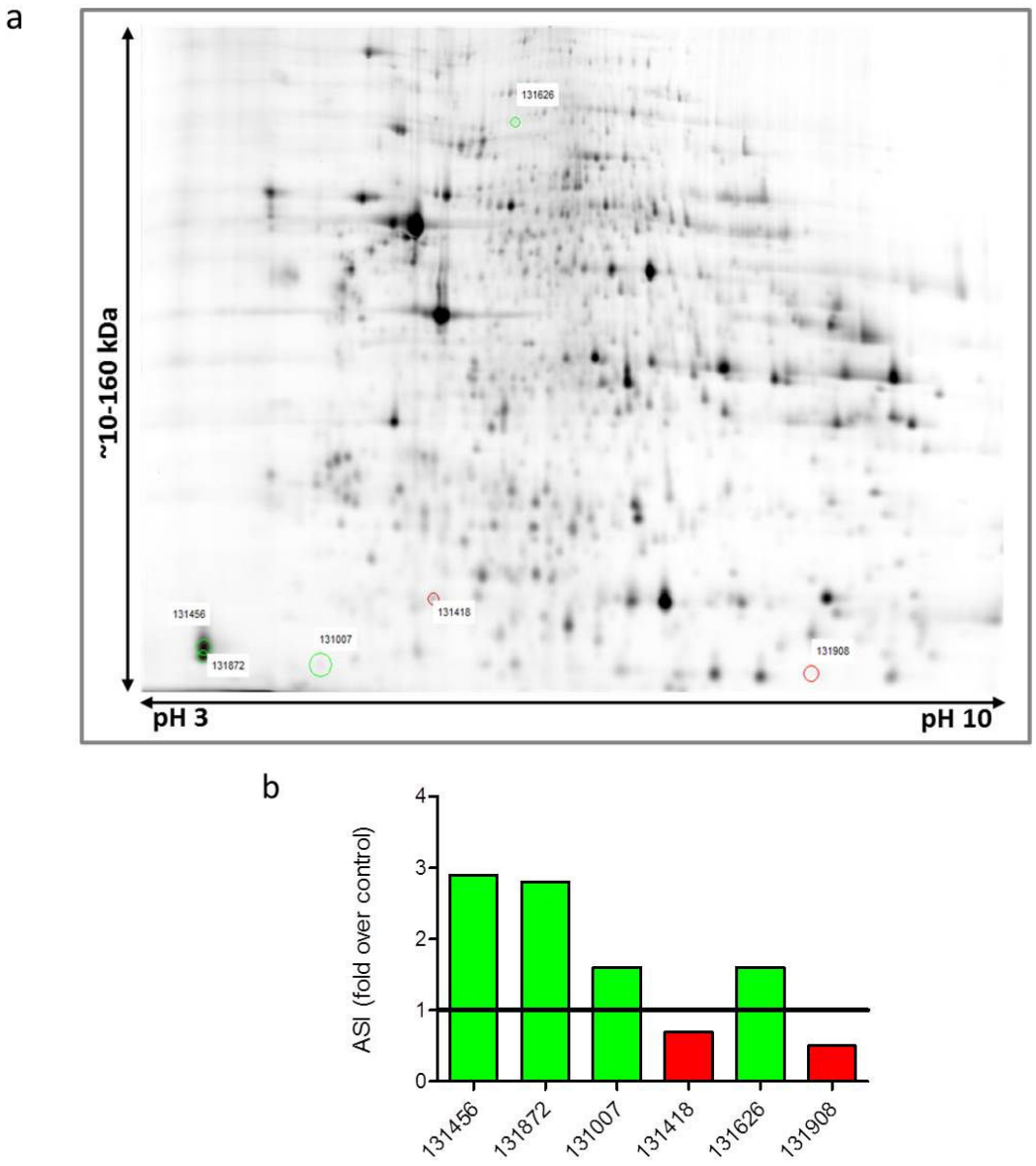

Figure 7.4 2D-DIGE of lysates from PRAS40 knockdown- and control cells. Human skeletal muscle cells were differentiated and transfected on day 3 of differentiation with either a non-target or PRAS40 siRNA. On day 7 of differentiation cells were lysed in 2D lysis buffer. Aliquots were subjected to 2D-DIGE analysis. Labeled samples (50 µg each) were separated in the first dimension by isoelectric focusing (IEF) using IPG strips (24 cm, pH 3-10 linear), followed by SDS-PAGE on 12.5% polyacrylamide gels (24 cm × 18 cm). Subsequently, images of protein pattern were acquired using a Typhoon 9400 laser scanner (resolution of 100 µm, photomultiplier tube of 550 V). Detection of protein spots and calculation of relative spot abundances were carried out automatically using Proteomweaver 4.0 image analysis software. a) Differential analysis revealed in total 923 spots of which 6 spots differ in the abundance marked with a circle and the unique superspotID. Red circles indicate less abundant, green circles more abundant after PRAS40 knockdown. b) Average spot intensity (ASI) of the six spots, which were regulated by PRAS40 knockdown, is expressed as fold over ASI of control cells. Proteins with the spot-IDs 131456, 131872, 131007, 131626 are up-regulated in the absence of PRAS40 (green), whereas the proteins with the spot-IDs 131418 and 131908 were down-regulated. Data were produced in collaboration with Dr. Sonja Hartwig.

Since the IRS1/PI3K/Akt as well as mTORC1 pathway are ubiquitously involved in insulin signal transduction, investigation of the impact of PRAS40 in other insulin target tissues might be of great interest. Generation of whole body as well as tissue-specific PRAS40 knockout mice may help to further characterize the role of PRAS40 in progression of insulin resistance and to clarify if the function of PRAS40 differs between the various tissues. Furthermore, overexpression of different mutant forms of PRAS40 *in vitro* and *in vivo* will help to further analyze the impact of posttranslational modifications on PRAS40 function. In particular, mutations of different phosphorylation sites as well as mutations within protein structures, which were uninvestigated so far (e.g. the proline-rich region of PRAS40) could create new insights for understanding PRAS40 function and might explain the controversy regarding PRAS40 in the literature. Finally, the function of nuclear PRAS40 has to be further investigated in the future. Therefore, challenging and inspiring work still lies ahead.

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Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis“ an der Heinrich-Heine-Universität Düsseldorf erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, 15. August 2013

Claudia Wiza