Tour d'AMPK

Citation for published version (APA):

Oligschläger, Y. (2015). Tour d'AMPK: Glycogen-cytoplasmic cycling of AMP-activated protein kinase. [Doctoral Thesis, Maastricht University]. Uitgeverij BOXPress. <https://doi.org/10.26481/dis.20151127yo>

Document status and date:

Published: 01/01/2015

DOI:

[10.26481/dis.20151127yo](https://doi.org/10.26481/dis.20151127yo)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Tour d'AMPK

Glycogen–cytoplasmic cycling of
AMP-activated protein kinase

© Yvonne Oligschläger, Maastricht 2015

Cover Design/Layout: Yvonne Oligschläger/Paul Oligschläger

Thesis Layout: Yvonne Oligschläger

Printed by: Proefschriftmaken.nl || Uitgeverij BOXPress

Published by: Uitgeverij BOXPress, 's Hertogenbosch

ISBN: 978-90-9029337-0

The research described in this thesis was carried out at the Department of Molecular Genetics as part of the research School CARIM and the Faculty of Health, Medicine and Life Sciences of Maastricht University. The experimental work executed for this thesis is part of a wider project funded by the VIDI-Innovational Research Grant scheme from the Netherlands Organization of Scientific Research (NWO-ALW 864.10.007; to D. Neumann). In particular, the studies presented here were performed in close collaboration with a second PhD candidate, Marie Miglianico, thus producing inevitable overlap in the experimental chapters of the two theses.

Tour d'AMPK

Glycogen–cytoplasmic cycling of
AMP-activated protein kinase

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. dr. L.L.G. Soete,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 27 november 2015 om 10.00 uur

door

Yvonne Oligschläger

Geboren te Velsen op 6 december 1987

Promotor

Prof. dr. J.F.C. Glatz

Co-promotores

Dr. D. Neumann

Dr. J.J.F.P. Luiken

Beoordelingscommissie

Prof. dr. E. Blaak (voorzitter)

Dr. R.C. Langen

Prof. dr. T. Larsen (University of Tromsø, Norway)

Prof. dr. C. Schalkwijk

Dr. B. Schroen

ABN-AMRO

AUTO HARINGS

BROUQUA
Tweewielers

Rijksweg 5a • 4701 MG Margraten • Tel. 043-4589089

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

Contents

	Preface	7
Chapter 1	General Introduction – Tour d’AMPK: <i>Grand Départ</i>	9
Chapter 2	The recruitment of AMP-activated protein kinase to glycogen is regulated by autophosphorylation	35
Chapter 3	The interaction between AMPK β 2 and the PP1-targeting subunit R6 is dynamically regulated by intracellular glycogen content	63
Chapter 4	FRET-assisted reporting of intracellular AMPK activity based on Fluorescence Lifetime Imaging Microscopy	85
Chapter 5	Displacing AMPK from glycogen by pharmacological targeting of its carbohydrate-binding module	107
Chapter 6	General Discussion – Tour d’AMPK: <i>Arrivée Finale</i>	139
	Summary	163
	Samenvatting	167
	Valorization	173
	List of abbreviations	181
	Dankwoord	185
	Curriculum Vitae	193

PREFACE

AMP-activated protein kinase (AMPK) is a heterotrimeric protein consisting of α -, β - and γ -subunits that coordinates and maintains cellular and whole-body energy homeostasis. Metabolic stresses, such as nutrient deprivation, muscle contraction, exercise and hypoxia, increase the intracellular AMP to ATP ratio promoting the activation of AMPK. In turn, activated AMPK will phosphorylate a series of downstream proteins involved in switching off ATP-requiring anabolic pathways (*e.g.*, glycogen synthesis, fatty acid synthesis, cholesterol synthesis, and hepatic gluconeogenesis), while simultaneously switching on ATP-producing catabolic pathways (*e.g.*, muscle glucose and fatty acid transport, glycolysis, fatty acid oxidation, mitochondrial biogenesis). Therefore, AMPK is considered as key therapeutic drug target for treating metabolic diseases such as obesity, insulin resistance and type 2 diabetes.

Scientific scope and outline of the thesis

The research described in this thesis provides insight into the molecular mechanisms determining the subcellular localization of AMPK with a focus on the cytoplasmic *versus* glycogen-bound AMPK pool, and elucidates the significance of localized AMPK in regulating glycogen metabolism.

Chapter 1 introduces the macromolecule glycogen and gives a compact overview of AMPK, a key metabolic enzyme, regarding its tissue-specific function and distribution, subcellular localization and role in glycogen metabolism. Studies on the carbohydrate-binding module (CBM) of the β -subunit of AMPK identified the β -threonine-148 (Thr-148) as a phosphorylation site. Autophosphorylation of this specific residue prevented AMPK from binding to glycogen (**Chapter 2**). Therefore, Thr-148 phosphorylation was predicted to impair the interaction of AMPK with other known glycogen-binding proteins. To this end, the interaction of muscle-specific AMPK β 2 with protein phosphatase 1-targeting subunit R6 was investigated (**Chapter 3**). To advance our understanding of AMPK activity at specific subcellular localizations, *i.e.*, glycogen *versus* cytoplasm, FLIM/FRET was employed as a novel, alternative approach to monitor intracellular AMPK activity (**Chapter 4**). Furthermore, a drug-targeting approach to identify and characterize novel compounds that compete with binding of AMPK to glycogen was carried out. The disruption of the AMPK–glycogen binding may have beneficial impact on metabolic diseases such as type 2 diabetes (**Chapter 5**). To conclude, in **chapter 6** the findings described in this thesis in relation to the dynamic interplay of AMPK and glycogen are being discussed.

Chapter 1

General Introduction – Tour d'AMPK: *Grand Départ*

The aim of this chapter is to give a general overview of the two key players of this doctoral thesis: AMP-activated protein kinase (AMPK), a key metabolic enzyme coordinating cellular and whole-body energy metabolism, and glycogen, the storage form of glucose. This chapter presents the route from myocellular glucose uptake to glycogen, and provides information about AMPK, *i.e.*, its structure, tissue-specific roles, activation, and subcellular localization. Clarification of the AMPK–glycogen binding and known AMPK-binding to proteins, as well as the AMPK involvement in glycogen metabolism, supports the understanding of the following experimental chapters of this thesis.

Coordination of myocellular glucose uptake and glycogen metabolism

Glucose is an essential substrate for cellular energy homeostasis, in which skeletal muscle is one of the major organs for glucose deposition into glycogen, and is therefore essential for proper regulation of blood glucose (1,2). Similar to insulin, an increased rate of muscle contraction, for instance as elicited by exercise, facilitates the uptake of glucose by increasing GLUT4 translocation to the plasma membrane; importantly, this pathway occurs in an insulin-independent manner (2). Besides glucose entering the glycolytic pathway for the production of ATP, myocellular glucose can be stored into glycogen. This process requires coordinated regulation, as glycogen serves as an immediate source of glucose for cellular use, *e.g.*, under conditions of metabolic stress. This paragraph presents current knowledge on the structure (3), regulation (4), and subcellular localization of glycogen (5), and on enzymes involved in glycogen metabolism (6), which encompasses the regulated release and storage of glucose. AMPK facilitates glucose import, binds to glycogen and phosphorylates glycogen-bound proteins (7). Thus, expanding our knowledge on the role of AMPK can contribute to a better understanding of specific mechanisms involved in regulating this dynamic process.

Glycogen is an essential macromolecule that consists of a highly branched network containing maximally 12 tiers (macroglycogen) (8). The molecule *per se* has a spherical shape, but only the four most external tiers are usually involved in the turnover process of synthesis and degradation (9). Glycogen synthesis and degradation are biochemical processes, which each require the interplay of various, but different enzymes. Glycogen synthesis takes place upon excess of intracellular glucose. The initiation of glycogen synthesis depends on the dimeric protein glycogenin (10), a glycosyltransferase. Glycogenin catalyzes the addition of glucosyl units to form short α -1,4-glucose chains, which are covalently bound to a specific tyrosine residue of glycogenin, by self-glucosylation (10), and then serves as a substrate for both glycogen synthase (GS) and glycogen branching enzyme (GBE) (6). GS is the key regulatory protein in glycogen synthesis and catalyzes the synthesis of α -1,4 linkages to the glycogen chain. In general, glycogen contains α -1,4-linkages that have an average chain length of 10–18 glucose residues, and each chain can have two branching points at least four glucose residues away from each other. A branch, and

thus a branching point, originates from interruption of an α -1,4-glycosidic bond and formation of an α -1,6-glycosidic bond. When a glycogen chain reaches its maximal length, GBE cuts the distal end of the chain, preferentially with six or seven glucose residues, and transfers it at an α -1,6-position of an existing chain, creating such a new branching point. Subsequently, this allows GS to promote further growth of the glycogen chain (11) (**Fig. 1**).

Figure 1. Branched structure of glycogen. Glycogen has a spherical shape and consists of several tiers. The initiation of glycogen synthesis is mediated by the core protein glycogenin (G). Glycogen is composed of glucose chains linked by α -1,4-glycosidic bonds, and driven by the enzyme glycogen synthase (GS). Upon reaching a length of approximately 12 glucose molecules, glycogen-branching enzyme (GBE) transfers part of the glucose chain to another position creating a new branching point. Per chain, maximally two branching points exist (adapted from Melendez *et al.* (8)).

Glycogen breakdown is a multi-step process, in which the key enzyme glycogen phosphorylase (GP) is involved. GP catalyzes the degradation of glycogen and release of glucose-1-phosphate (G1P), which can be converted into glucose-6-phosphate (G6P), and stops degrading the outer chains when four glucose units remain on the side chain (12). Because glucose-6-phosphatase is absent in heart and skeletal muscle, G6P will enter the glycolytic pathway for ATP production. Although GP is a rate-limiting enzyme in glycogenolysis, glycogen debranching enzyme (GDE) is responsible for complete degradation of glycogen by resolving the remaining branches (12).

The enzyme activities of both GS and GP are dependent on phosphorylation and allosteric activation (13,14). GS is known to be phosphorylated at multiple residues by a variety of protein kinases, such as protein kinase A (PKA) and glycogen synthase kinase-3 (GSK-3), casein kinase 1/2 (CK1/2), and AMPK, leading to inactivation of the enzyme (15), whereas phosphorylation of GP results in increased enzymatic activity. It has been shown that G6P can allosterically activate GS, whereas ATP inhibits it (16,17). On the contrary, GP comprises an allosteric AMP binding site in muscle isoforms that stimulates its activity, while G6P allosterically inhibits it (18). Studies using transgenic knockin mice bearing a constitutively active GSK3 α / β mutation provided evidence that G6P is involved in glycogen storage. In these mice, insulin failed to induce activation of GS, whereas glycogen content was not impaired (19). They showed that insulin-induced glycogen synthesis mainly depends on G6P,

which allosterically activates GS, thereby promoting glycogen synthesis (20). Furthermore, these researchers observed a strong reduction in insulin-induced muscle glycogen synthesis and glycogen content using knockin mice overexpressing a G6P-insensitive mutant. Together, these data provided evidence that allosteric activation of GS by G6P contributes to glycogen synthesis *in vivo* (21). Interestingly, it has also been shown that the phosphorylation status of skeletal muscle GS is not only involved in the regulation of GS activity, but also determines its subcellular localization (22). For instance, Prats *et al.* reported that phosphorylation of GS at Ser-7/Ser-10 targets the enzyme to subsarcolemmal or intermyofibrillar glycogen particles, not only in rabbit but also in human skeletal muscle (22). Therefore, it could be speculated that also allosteric activation might affect the glycogen-binding affinity or subcellular localization of GS.

Also fundamental for the regulation of glycogen metabolism is the involvement of protein phosphatases (PP). These enzymes decrease glycogenolysis by dephosphorylating and inactivating key metabolic enzymes such as GP, but also accelerate glycogen synthesis by dephosphorylating GS and converting it to its active form.

Although one might expect that glycogen is equally distributed throughout the cell, transmission electron microscopy revealed that glycogen localizes to three main cellular areas in type I (slow-twitch) and type II (fast-twitch) fibres of skeletal muscle, *i.e.*, subsarcolemmal, intermyofibrillar, and intramyofibrillar (5,23). It has even been shown that the depletion of glycogen is dependent both on subcellular localization and fibre type in response to exercise (24); for instance, submaximal exercise results in degradation of intermyofibrillar and subsarcolemmal glycogen (25). Furthermore, cardiac glycogen is found to be either cytosolic or vacuolar, *i.e.*, enclosed in double-membrane bound structures, presumably autophagosomes (26), suggesting coordinated regulation of glycogen turnover by various mechanisms. The next paragraph provides insight into how proteins are capable of binding to glycogen.

Glycogen-binding via the Carbohydrate-Binding Module

Many of the enzymes involved in cellular glycogen metabolism have been shown to contain a specific subdomain termed the carbohydrate-binding module (CBM), also known as glycogen-binding domain. Over the past years, structural and biochemical insight has led to a better understanding of specific carbohydrate recognition and the mechanisms involved in carbohydrate binding (27,28). Originally, CBM was used as a term to describe all non-catalytic sugar-binding modules derived from glycosidases (29). Besides being part of enzymes active in carbohydrate metabolism (29), *i.e.*, carbohydrate-active enzymes (28), CBM domains can also be found in non-hydrolytic enzymes (30) or as single domains (31). To date, roughly 64 families of CBM domains have been defined in the CAZY database (<http://www.cazy.org>) based on their amino acid similarities. Of these, many are known to bind to cell wall carbohydrates of the plant, whereas others are known to target starch or glycogen (32), indicating that the CBM domain promotes targeting of the enzyme to its substrate in order to accelerate its degradation (27,28). Although not generally accepted, it has even been reported that some cellulose-binding proteins are capable of non-catalytically disrupting the crystalline structure of cellulose (28). Furthermore, despite being part of a same CBM family, it has been shown that the ligand-recognition by the CBM is correlated with its biological function. For instance, three of the family 35 CBMs appended to plant cell wall degrading enzymes, rather than directly targeting the substrate of the catalytic enzyme, whereas the fourth one acts as an anchoring module for bacterial cell wall surfaces by binding to uronic acid sugars (33). Moreover, CBMs have been shown to be involved in pathogenesis (34) and, more importantly for this thesis, in glycogen metabolism (35).

Although CBM domains differ in its three-dimensional structures, in which the β -sheet is the most important structural element (28), the properties of protein-carbohydrate interactions are fairly similar in all CBM-containing proteins. The CBM families have been subdivided, for instance based on their function, *i.e.*, CBM Type A (surface-binding), CBM Type B (glycan-chain-binding) and CBM Type C (small-sugar-binding) (28) (**Fig. 2**).

Figure 2. CBM types. Schematic overview of three different CBM types bound to polysaccharides (cazypedia.org).

For example, Type A class includes families of CBM domains that bind to the surface of crystalline polysaccharides such as cellulose or chitin, a derivative of glucose, as for example found in CBM family 1, 2a, 3a, and 10, respectively (36). Type B class allows CBM domains to bind internally to glycan chains, and therefore is referred as to ‘chain binders’, which is characteristic for CBM families 2b, 4, 6, 15, 17, 20, 22, 27–29, 34, and 36, respectively (36). Type C class CBM domains optimally bind the ends of glycans, such as mono-, di or tri-saccharides, therefore called small sugar-binding CBM, such as CBM families 9, 13, 14, 18, and 32 (37).

In a recent review, CBMs that have an affinity for starch have been referred to as starch binding domains (SBD), and nine families (CBM family 20, 21, 25, 26, 34, 41, 45, 48, and 53, respectively) have been reported to contain SBDs (38). To better characterize CBM families, Carvalho *et al.* recently placed 27 of the CBM families into nine tribes, which encompass CBM families whose members have very similar tertiary structures and common binding mechanisms (37), showing that, for instance, tribe CBM-A contains the CBM20 family, *e.g.*, laforin, whereas CBM families 21 and 48, including the PP1-glycogen-targeting subunits and AMPK (39), respectively, have been assigned into tribe CBM-B.

Protein-carbohydrate interactions are mainly stabilized by hydrogen bonds and van der Waals’s interactions. Additionally, exact regulation of these interactions is often mediated by several residues involved in forming the binding pocket, and a conformational change is enforced to modulate the accessibility to and from the binding site (40). Therefore, in addition to structural data and NMR, many studies used mutagenesis tools in order to understand the residues involved in protein interactions at the level of the glycogen–protein complex (28,40). As already mentioned, the CBM domain is also crucial for regulating glycogen metabolism in a coordinated fashion, as it allows enzymes to bind to glycogen with high affinity (32), thus guiding the catalytic domain towards its substrate. An important protein phosphatase involved in glycogen metabolism is protein phosphatase 1 (PP1) (41), a protein that is known to dephosphorylate AMPK (42). PP1 has seven glycogen-targeting subunits that mediate its recruitment to sites of glycogen formation via a conserved CBM domain (43–45). Although these subunits (*i.e.*, GM, GL, R5, R6, R3E, R3F and R3G) are differentially regulated (46), they all enable PP1 to dephosphorylate its substrates (47), *e.g.*, GS or GP (48), via a PP1-binding motif (49). Consequently, these actions facilitate glycogen synthesis, while preventing glycogen degradation (35). For instance, the targeting subunit R5 is a scaffold protein, as it binds to glycogen particles as well as to PP1 or other relevant proteins involved in regulating glycogen metabolism, such as GS, GP and laforin (50,51).

Laforin is a ubiquitous protein that is most highly expressed in brain, skeletal muscle, heart and liver (52,53). Laforin, a glycogen-associated protein (54), is the only dual specificity phosphatase in eukaryotes that contains a carbohydrate-binding module type 20 (CBM20) (35). The N-terminal CBM of laforin localizes it to glycogen, where it can dephosphorylate glycogen (50) or other complex phosphorylated carbohydrates (55), suggesting that the phosphatase activity may be necessary for the maintenance of normal cellular glycogen. Thus, laforin, forming a complex together with malin, is known to regulate glycogen metabolism (56). Mutations in the CBM domain of laforin (*e.g.*, W32G) disrupt the binding pocket, thereby inducing mis-targeting of the enzyme (57). Such mutations are associated with Lafora Disease, a fatal autosomal recessive neurodegenerative disorder that is related to intraneuronal accumulation of so-called Lafora bodies, which consequently result in progressive myoclonus epilepsy. As mentioned, another protein interacting with laforin is malin. Although malin does not contain a CBM domain, this protein is capable of directly binding to and subsequently ubiquitinating laforin, thus targeting laforin for degradation. Also mutations in malin have been associated with Lafora Disease.

Another protein that has been shown to bind glycogen, *in vitro*, is starch-binding domain-containing protein 1 (STBD1), a protein highly expressed in muscle and liver (58). Via its C-terminal CBM belonging to family 20 (35), Stbd1 preferentially binds to less branched but more phosphorylated carbohydrates (58), and interacts with other glycogen-binding proteins, such as GS, GP and laforin (59). Besides playing a role in glycogen-binding and glycogen metabolism, Stbd1 is also associated with autophagy (58). Stbd1 mediates membrane anchorage through interaction with the autophagy protein GABA(A) receptor-associated protein-like 1 (GABARAPL1), suggesting that particular autophagic pathways exist for the breakdown of glycogen (60).

The enzymatic duo GBE and GDE also each carry a CBM domain (61) (*e.g.*, CBM48), which serves as a glycogen-binding domain. Interestingly, Diaz *et al.* reported that GS, and likely GP, contains a functionally conserved non-catalytic high affinity glycogen-binding site located away from its active center, which appears to be different from the classical CBM (62). Using GS mutants encompassing reduced carbohydrate-binding affinity, they found impaired intracellular distribution and lowered glycogenic capacity, indicating that the glycogen-binding site is required for the catalytic properties of GS (62).

Altogether, glycogen is not only a target for proteins that contribute to its metabolism, but also serves as a scaffold compartment to which the enzymes involved in its metabolism can associate and eventually interact. Therefore, such glycogen-protein complex is termed the glycosome (11). Another key metabolic enzyme that may be considered as being part of the glycosome is AMPK (3) (**Fig. 3**), as it is capable of targeting glycogen via its CBM domain (63,64). Before highlighting the role of the

CBM domain of AMPK, first, the physiological function, mode of activation and tissue-specific distribution of AMPK will be reviewed in the next paragraphs.

Figure 3. Representation of the glycosome. The glycosome, also known as glycogen–protein complex, comprises intracellular glycogen associated with key enzymes involved in its metabolism, such as R6, PP1, GS and AMPK (11).

AMPK, a key metabolic enzyme involved in preserving energy homeostasis

AMPK is an $\alpha\beta\gamma$ -trimeric protein complex, consisting of one catalytic subunit (α) and two regulatory subunits (β and γ). The α -subunit ($\alpha 1$ and $\alpha 2$ are the two alternative isoforms) contains an N-terminal serine/threonine kinase domain that carries the crucial threonine-172 (Thr-172) phosphorylation site within the activation loop (65), and a C-terminal domain involved in the heterotrimeric complex formation. The β -subunit exists in two different isoforms, ubiquitously expressed $\beta 1$ and muscle-specific $\beta 2$. In addition to its role in glycogen-binding, due to a specific mid-molecule CBM domain, the β -subunit is also involved in tethering the α - and γ -subunits together via its C-terminal α/γ -interacting domain (66). The γ -subunit, ($\gamma 1$, $\gamma 2$, and $\gamma 3$ being the three isoforms) contains four repeats of cystathionine- β -synthase (CBS) domains that are essential for binding of adenine nucleotides (67).

Under physiological conditions, AMPK activity is relatively low. Thus, in cells that do not undergo any kind of metabolic stress, the ATP: AMP: ADP ratio is around 10: 0.1: 1, driving the adenylate kinase reaction towards ADP synthesis ($\text{ATP} + \text{AMP} \rightarrow 2\text{ADP}$), thereby maintaining very low concentrations of AMP. However, under conditions of metabolic stress, in which the aforementioned reaction is reversed, increases in AMP levels are more pronounced than increases in ADP or ATP. Nowadays, it has been generally accepted that binding of AMP (and to a lesser extend ADP (68,69)) to the γ -subunit induces a conformational change that results in allosteric activation of AMPK up to 10-fold, and protects AMPK from dephosphorylation of Thr-172 by phosphatases (70-72). Simultaneously, allosteric activation further triggers phosphorylation of Thr-172 by AMPK upstream kinases.

Mammalian AMPK is activated by AMP via three mechanisms: *i*) promotion of Thr-172 phosphorylation; *ii*) inhibition of Thr-172 dephosphorylation; and *iii*) allosteric activation. The second effect can be mediated by ADP (73), whereas all three mechanisms are antagonized by ATP. Upon allosteric activation via an increase in AMP level, LKB1 (liver kinase B1), the major upstream kinase of AMPK (74), has been demonstrated to promote Thr-172 phosphorylation and activation of AMPK (**Fig. 4**).

In addition to LKB1, CaMKK2/CaMKK β (Ca²⁺/calmodulin-dependent protein kinase kinase β) was revealed as an alternative upstream kinase that acts independently of changes in adenine nucleotides (75-77), but only phosphorylates Thr-172 upon increases in intracellular Ca²⁺ levels. The third recognized, and debated, upstream kinase of AMPK is TAK1 (transforming growth factor (TGF)- β -activated kinase 1) (78), a mediator of TGF- β signaling in mammalian cells (79). TAK1, in combination with its binding partner TAB1, phosphorylates AMPK at Thr-172 (80,81).

As earlier mentioned (**Preface**), upon activation, AMPK controls cellular and whole-body energy homeostasis by switching off ATP-requiring anabolic pathways (*e.g.*, glycogen synthesis, fatty acid synthesis, cholesterol synthesis, and hepatic gluconeogenesis), whereas switching on ATP-producing catabolic pathways (*e.g.*, muscle glucose and fatty acid transport, glycolysis, fatty acid oxidation, mitochondrial biogenesis). An overview of the role of AMPK is displayed in **Figure 4**.

Figure 4. Regulation of AMPK. AMPK is a heterotrimeric complex consisting of an α -, β -, and γ -subunit. Upon cellular stress (*i*), a rise in AMP/ADT:ATP ratio causes allosteric activation of AMPK (*ii*). This further triggers upstream kinases to activate AMPK via phosphorylation of the Thr-172 residue (*iii*). AMPK restores energy balance by activating processes that produce energy, while inhibiting those that consume energy (*iv*). Once energy homeostasis is restored, phosphatases are capable of dephosphorylating and inactivating AMPK (*v*) (adapted from O'Neill *et al.* (82)).

Control of AMPK by pharmacological activators

Besides the canonical regulation of AMPK by phosphorylation and physiological changes in adenine nucleotide levels, AMPK can also be pharmacologically activated by a variety of compounds. A widely used compound that is intracellularly converted into ZMP, an analog of AMP, is 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which is able of indirectly activating AMPK without changing adenine nucleotide ratios (83). In skeletal muscle, AICAR has been shown to stimulate glucose uptake (84), whereas in heart it only stimulated fatty acid uptake (85). Furthermore, it has been shown that AICAR increases phosphorylation of TBC1D1 and TBC1D4 in rodent skeletal muscle (7), thus involving GLUT4 trafficking. In hepatocytes, it has been shown that AICAR inactivates GS via Ser-7 phosphorylation (86), thereby regulating glycogen synthesis. Most other pharmacological AMPK activators such as oligomycin, hydrogen peroxide and the biguanide drug metformin are metabolic poisons that inhibit ATP production, thus increasing cellular AMP levels and subsequently activating AMPK indirectly. Also, thiazolidinediones (TZDs) are insulin-sensitizing agents that can indirectly, via increased expression and production of adiponectin, or directly activate AMPK in tissues (87). More direct activators of AMPK have been derived from high-throughput screens targeting AMPK, such as 991 (88), MT 63-78 (89), Compound 2 (90) and A-769662, known as Abbott compound (91-93). These compounds preferentially activate β 1-containing AMPK complexes, and bind at a site between the α -kinase and β -CBM domain, which is distinct from the AMP binding site.

Targeting of AMPK with A-769662 has been shown to be beneficial for the treatment of metabolic disorders (91). Similar to AMP, A-769662 inhibits Thr-172 dephosphorylation (93), although this is not the primary mechanism of action. Instead, depending on the phosphorylation status of β 1-Ser-108, A-769662 allosterically activates AMPK in the absence of Thr-172 phosphorylation. Also salicylate, a natural plant product, has been shown to activate AMPK and binds at the A-769662 site to allosterically activate AMPK and inhibit dephosphorylation of Thr-172 (94). Other natural products, such as resveratrol (red grapes), quercetin (fruits and vegetables), curcumin (*Curcuma longa*), berberine (*Coptis chinensis*), epigallocatechin gallate (EGCG, green tea) and theaflavin (black tea) have also been reported to activate AMPK, presumably via indirect inhibition of ATP synthesis and thereby resulting in an increased AMP:ATP ratio (95).

Tissue-specific expression and function of AMPK

Despite being ubiquitously expressed, AMPK has a multifaceted role throughout the body, the significance of each individual subunit should not be underestimated. All three subunits, including its different isoforms, are differentially expressed in a wide variety of tissues (see **Table 1**).

Table 1. Tissue-specific distribution of the individual AMPK subunits (adapted from Hardie *et al.* (96)).

Subunit	Tissue Expression	Function
$\alpha 1$	Ubiquitous	Catalytic subunit
$\alpha 2$	Cardiac / skeletal muscle	
$\beta 1$	Ubiquitous	Carbohydrate binding, tethering α and γ
$\beta 2$	Cardiac / skeletal muscle	
$\gamma 1$	Ubiquitous	Binding of adenine nucleotides
$\gamma 2$	Cardiac / skeletal muscle	
$\gamma 3$	Skeletal muscle	

Taking a closer look at each of the subunits of AMPK (**Fig. 5**), in particular at the α -subunit, it has been reported that both cardiac and skeletal muscles predominantly express AMPK $\alpha 2$ complexes. In adipose tissue, AMPK $\alpha 1$ is most abundantly present, whereas the liver expresses both $\alpha 1$ and $\alpha 2$ -containing complexes (97). Importantly, it is widely known that AMPK plays a central role in a wide variety of metabolic processes, and therefore can be found at different subcellular localizations, such as the cytosol or the nucleus. This nuclear–cytoplasmic shuttling is mediated via the nuclear export sequence in the α -subunit (98), and implies direct phosphorylation of transcription factors or other essential downstream targets. Hence, earlier studies showed that AMPK $\alpha 1$ and $\alpha 2$ complexes have a different dependence on AMP and that AMPK $\alpha 2$ is preferentially located in the nucleus, whereas $\alpha 1$ is found in the cytoplasm (99,100). Also, it has been reported that AMPK $\alpha 2$ subcellular redistribution is regulated by the β -subunit isoform, as activated $\alpha 2\beta 1$ -containing complex were situated in the cytoplasm, whereas activated $\alpha 2\beta 2$ -containing complex localized to the nucleus (101). In human skeletal muscle, it has been shown that AMPK $\alpha 2$ nuclear translocation is further induced upon exercise (102). Also, not only various forms of stress, potentially mediated via the extracellular signal-regulated kinase 1/2 (ERK1/2)-mitogen-activated protein kinase (MAPK) pathway (103), but also leptin (101) has been shown to induce nuclear AMPK accumulation. Moreover, other studies demonstrated that the nuclear localization of AMPK $\alpha 1$ is regulated in a circadian rhythm (76,77). Altogether, these results indicate that AMPK subcellular localization is well-coordinated, which is required for proper response to local metabolic stimuli.

The expression of $\gamma 1$ is ubiquitous, whereas the expression of $\gamma 2$ and $\gamma 3$ is highly expressed in cardiac (104) and skeletal muscle (105), respectively. It has been reported that $\gamma 1$, similar to $\alpha 1$, is almost exclusively present in the cytosol, and that $\gamma 2$, equal to $\alpha 2$, is mainly locating to subcellular organelles such as the nucleus (106). Furthermore, AMPK $\gamma 3$ -containing complexes are only weakly activated by AMP (107), and highest activation is found in $\alpha 2\gamma 2$ -containing AMPK complexes. In skeletal muscle, AMPK $\alpha 1\beta 2\gamma 1$ (15%), $\alpha 2\beta 2\gamma 1$ (65%) and $\alpha 2\beta 2\gamma 3$ (20%) complexes are significantly present, but not expressed to the same extent (108,109). Wojtaszewski *et al.* reported that AMPK $\alpha 2\beta 2$ complexes preferentially form a complex with $\gamma 3$, whereas the remaining $\alpha 1\beta 2$ and $\alpha 2\beta 2$ associated with $\gamma 1$ (108). Furthermore, it was shown that the AMPK $\alpha 2\beta 2\gamma 3$ complex appears to be the predominant isoform activated in skeletal muscle in response to exercise (105,109).

Besides α - and γ -subunits, also the β -subunit displays some variation in tissue distribution. AMPK $\beta 1$ is ubiquitously expressed, although most abundantly found in brain and liver. AMPK $\beta 2$ -subunit is primarily expressed in heart and skeletal muscles (110,111), but more recently it was also found in human liver (112). Interestingly, also the AMPK $\beta 1$ subunit has been shown to translocate to various subcellular localizations, which is dependent on its post-translational modifications (113). For instance, Dasgupta *et al.* showed that $\beta 2$ is predominantly cytoplasmic in neuronal stem cells (114), whereas Turnley *et al.* reported that $\beta 1$ is predominantly located in the nucleus, in both neuronal stem cells and neurons (100).

Figure 5. Subunit structure of AMPK. Schematic representation of each subunit of the heterotrimeric enzyme. AID, auto-inhibitory domain; CBM, carbohydrate-binding module; CBS, cystathionine beta synthase; CD, catalytic domain; CTD, C-terminal domain.

Relevance of AMPK in cardiac and skeletal muscle

AMPK plays a prominent role in maintaining energy homeostasis in key metabolically relevant tissues such as heart, skeletal muscle, adipose tissue, liver, and hypothalamus. Although the main focus is on the role of AMPK in cardiac and skeletal muscle, the other tissues will also be shortly discussed.

Both in heart and skeletal muscle, highly metabolically flexible organs (115,116), activation of AMPK increases essential metabolic processes such as glucose uptake, fatty acid uptake and oxidation and mitochondrial biogenesis, by regulating gene expression, substrate transporter translocation and activity of several key enzymes (117). Acute muscle AMPK activation increases fatty acid oxidation by decreasing malonyl-CoA levels through inhibition of acetyl-CoA carboxylase (ACC) and activation of malonyl-CoA decarboxylase (MCD), changes that also have been found in liver and adipose tissue, for instance due to the release of exercise-induced hormones such as interleukin-6 (IL-6) (118). AMPK activation has also been shown to increase long-term fatty acid oxidation via enhanced expression of PPAR α -target genes, suggesting that AMPK regulates many genes involved in skeletal muscle metabolism (119). Activated AMPK increases the TBC1D1-mediated myocellular translocation of the glucose transporter GLUT4 from intracellular storage vesicles to the plasma membrane to stimulate glucose uptake (120,121), and it is known to enhance GLUT4 expression and glycolysis. AMPK also coordinates skeletal muscle energy production by regulating mitochondrial biogenesis, mediated by transcription factors such as nuclear respiratory factor-1 (NRF-1), mitochondrial transcription factor A (MTFA) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1 α). AMPK can directly phosphorylate PGC1 α and it is known to drive the expression of PGC1 α upon exercise or pharmacological AMPK activation (122). In parallel, AMPK increases cellular NAD $^{+}$ levels and enhances SIRT1 activity, resulting in deacetylation and activation of PGC1 α (123). During fasting and after exercise, skeletal muscle can efficiently switch from carbohydrates to lipids in order to preserve glycogen and blood glucose levels for glucose-dependent tissues. In this respect, AMPK can function as an initial sensor, as a deficiency in AMPK resulted in improper regulation of SIRT1 activity and its downstream transcriptional regulators PGC1 α and FOXO1 (123).

In adipocytes, AMPK activation inhibits insulin-stimulated glucose metabolism, but also increases the production of anti-inflammatory cytokines such as IL-10 in macrophages and promotes macrophage polarization to an anti-inflammatory functional phenotype (124). AMPK also has been shown to increase fatty acid oxidation by inactivating ACC, and possibly inhibits lipolysis by phosphorylation of hormone sensitive lipase (HSL) and adipose triacylglycerol lipase (ATGL) (125-127). Liver AMPK is involved in controlling glucose homeostasis mainly through inhibition of genes and proteins involved in hepatic glucose output and gluconeogenesis. For instance, AMPK inhibits gluconeogenesis by disrupting CREB-CRTC2 interaction and binding to phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase gene promoters (128). AMPK also decreases hepatic lipogenesis via phosphorylation and inactivation

of ACC1/2 (129). Hypothalamic AMPK has been shown to regulate appetite, probably indirectly via ACC, in response to circulating nutrient, (*e.g.*, glucose and fatty acids), as well as hormonal signals derived from the pancreas, adipose tissue, and gut (130). Leptin, an adipocyte hormone, inhibits AMPK activity in the hypothalamus to mediate the anorexic effect of leptin (131), whereas ghrelin, a peptide hormone released from the stomach, activates AMPK in the hypothalamus (132), which may contribute to its orexigenic effect (133).

It is obvious that AMPK plays a key physiological role in cellular and whole-body metabolism (96). Detailed insight into the physiological roles of the various subunits and isoforms of AMPK has been gained from extensive knockout and transgenic mice studies. For instance, the generation of catalytic α -subunit knockout animals has highlighted the role for AMPK α 1 and AMPK α 2 in the regulation of energy metabolism (97). Although AMPK α 1 α 2 double-knockout mice have been found embryonically lethal, studies on AMPK α 1 or AMPK α 2 tissue-specific or whole-body knockout mice provided insight. Whereas AMPK α 1 or α 2 whole-body knockout mice did not show any effect on appetite or body mass, Jørgensen *et al.* observed lowered AMPK activity both at rest and during exercise in AMPK α 2 knockout muscles (134). The generation of transgenic mice overexpressing a dominant-negative form of the catalytic α -subunit (135) or transient overexpression of constitutively active forms of AMPK (136) have provided information on the role of AMPK in different tissues. The generation of transgenic mice harboring naturally occurring mutations in the human γ 2 (137-140) and pig γ 3 (141) subunit advanced our understanding of AMPK regulation in heart and skeletal muscle, respectively. Genetic models with deletion of the AMPK β subunit provided evidence that this regulatory subunit is of great importance for AMPK activity and thus for regulating metabolic homeostasis. Steinberg *et al.* reported that AMPK β 2 null mice, despite overcompensation of β 1, had reduced muscle AMPK activity and exercise tolerance (142), and showed that AICAR-stimulated glucose uptake was impaired in these mice (142). Also, these mice appeared to have a similar phenotype to the mice lacking the α 2 (143) or γ 3 (144) subunit (109). Mice bearing a germline deletion of AMPK β 1 displayed reduced hepatic AMPK activity due to lowered AMPK α -subunit expression, and had lowered appetite, adiposity and total body mass (145). Further, young mice lacking muscle-specific β 1 β 2 showed that AMPK plays a role in maintaining mitochondrial capacity and contraction-stimulated glucose uptake into skeletal muscle (109). In summary, these data provide evidence that AMPK plays a pivotal role in heart and skeletal muscle. Nevertheless, the explicit function of AMPK with respect to glycogen, and glycogen metabolism, has not been sufficiently addressed as yet. Identification of a CBM domain in the β -subunit of AMPK suggests the involvement of AMPK in key processes related to glycogen metabolism. To better understand how AMPK is capable of binding to glycogen, the next paragraph provides detailed insight into the role of the β -CBM domain. Furthermore, a compact overview of AMPK's function regarding glycogen metabolism will be given.

Role of the AMPK β -CBM domain

Both using cell free and cellular assays (63,146), it has been shown that the CBM of AMPK is involved in glycogen binding. The sequence of the CBM domain is conserved in the yeast homologue *SNF1*, referred to as glycogen-binding domain of the regulatory subunits Gal83 and Sip2 (146), as well as in plant homologue SnRK1, referred to as SBD of AKIN β 2 and a γ -type subunit AKIN β γ (147), respectively. To better understand the process of AMPK attaching to glycogen, several groups have taken a closer look at the structure and function of the CBM domain by means of mutagenesis studies. Structural analysis of AMPK β 1-CBM in complex with β -cyclodextrin (β -CD) (**Fig. 6**), a cyclic model sugar mimicking glycogen, revealed certain well-conserved key residues within the AMPK-CBM (*i.e.*, W100, W133, N150 and K126 and L146) that are essential for AMPK-carbohydrate interaction, and mutagenesis studies on these residues indeed revealed either partial or complete loss of carbohydrate-binding ability (63,64). In contrast with the C-terminal $\alpha\gamma$ -interaction domain (148,149), the CBM domain appears not to be essential for the formation of stable (active) heterotrimeric complexes (150).

Although the ubiquitously expressed β 1 and muscle-specific β 2 subunits are almost 100% identical concerning the residues involved in binding to β -CD, critical examination of the binding of AMPK β 1- and β 2-containing complexes to carbohydrates revealed that β 2 is distinct from β 1. Koay *et al.* reported that the major difference between the two β -isoforms is a threonine-101 insertion (corresponding to Thr-102 in β 2 subunit) (**Fig. 6**). This particular insertion differentiates the carbohydrate-binding affinity between the two isoforms (151), whereas other differences (*e.g.*, F82I, Y92F, T106I, R107K, N11D, T134V, and Q154H) did not appear to have impact on the structure or biological function of the CBM domain.

Figure 6. Overlay of β 1-CBM and β 2-CBM in complex with β -cyclodextrin (151). β 1-CBM is displayed in light blue and purple, whereas β 2-CBM, including a Thr-101 insertion, is presented in green and yellow (151). The cyclic sugar β -CD is shown in blue.

Although AMPK carries a CBM domain within the β subunit, not all AMPK is localized to glycogen (99,103), which implies that AMPK presumably acts in a coordinated localization-dependent manner. Several studies extensively investigated the role and activity of AMPK at glycogen. Consistent with *in vitro* data of Carling and Hardie (152,153) showing that AMPK is capable of phosphorylating and inhibiting myocellular GS at Ser-7 (site 2), it has been shown that glycogen-localized AMPK physically interacts with GS, GP and GDE in mammalian cells (110,150,154), and AMPK is capable of phosphorylating the glycogen phosphatase laforin (155). Regarding the involvement of AMPK in myocellular glycogen metabolism, several studies reported that acute treatment of isolated rat muscles with AICAR impaired insulin-stimulated glycogen synthesis (156). In AMPK- $\alpha 2$ knockout mice, AICAR-mediated phosphorylation of GS was prevented (154), suggesting a significant role for AMPK in the regulation of glycogen metabolism. In contrast, chronic AMPK activation in rodents rather promoted glycogen storage both in cardiac and skeletal muscle (157), which in some cases even may be related to cardiac dysfunction. For instance, patients with McArdle's Disease (carrying an inherited mutation on the glycogen phosphorylase gene) have increased glycogen accumulation, although AMPK is hyper-activated. Also, transgenic mice bearing a naturally occurring $\gamma 2$ mutation, which mimics the Wolff-Parkinson-White (WPW) syndrome in humans, display an unusual type of cardiomyopathy due to abnormal accumulation of glycogen. This mutation disrupts the binding site for AMP, which is causal for the aberrant AMPK activity observed (137,140,158).

Altogether, it is obvious that AMPK is involved in controlling glycogen metabolism, but the consequence of AMPK activity on glycogen storage seems to be conflicting. On one hand, it has been established that AMPK phosphorylates GS, indicating that AMPK might be a partner of the glycosome. On the other hand, using knock-in mice bearing a dominant-negative mutation on GS, it was shown that AICAR-stimulated glycogen synthesis is regulated by G6P-mediated allosteric activation, suggesting that G6P overcomes the primary inhibitory function of AMPK on GS and consequently glycogen synthesis (159). Regarding the level of AMPK activity at subcellular localizations such as glycogen, McBride *et al.* reported that the CBM domain of AMPK is not only involved in localizing AMPK to glycogen, but also regulates allosteric inhibition of AMPK activity when bound to highly branched glycogen (160). Consistently, Momcilovic *et al.* reported that the glycogen-binding domain of the regulatory Gal83 subunit is involved in glucose-mediated inhibition of SNF1 activity (161), whereas others failed to observe such effects on AMPK activity (63,162). Furthermore, glycogen loading has been shown to prevent AICAR-mediated AMPK activation in rat skeletal muscles (151, 152) or exercise-mediated AMPK activation in humans (155). Conversely, AMPK is activated in muscle by exercise/contraction (163), suggesting that AMPK-mediated glucose import might be enhanced as a consequence of glycogen degradation.

Because this thesis has a main focus on the interplay of AMPK with glycogen, in particular the AMPK β subunit, this chapter also introduces the various post-translational modifications known to occur on the β -subunit and elucidates how these post-translational modifications regulate AMPK.

Regulation of CBM and full-length AMPK β by post-translational modifications

Ubiquitination

Ubiquitination is a multistep process in which specific proteins are recognized by E3 ubiquitin ligases. First, the E1 ubiquitin-activating enzyme interacts with ubiquitin in an ATP-dependent process to form a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1 sulfhydryl moiety. Second, ubiquitin is transferred to the active cysteine of the E2 ubiquitin-conjugating enzyme. Finally, E3 ubiquitin ligases recognize specific substrates and transfer ubiquitin to a lysine on its target protein. Qi *et al.* demonstrated that cell death-inducing DFFA-like effector a (Cidea) interacts specifically with the AMPK β subunit to form a complex that can be targeted for ubiquitination in order to promote AMPK degradation (164). Conversely, ubiquitination of AMPK β introduced by E3-ubiquitination ligase malin does not target the enzyme for proteasomal degradation, but promotes accumulation of AMPK into inclusion bodies, likely affecting subcellular distribution (165).

Sumoylation

Sumoylation is the addition of sumo-conjugates to enzymes, carried out by an E3-small ubiquitin-like modifier (SUMO) ligase. It has been shown that protein inhibitor of activated STAT (PIASy) adds SUMO2- but not SUMO1-conjugates to AMPK β 2, which results in enhanced AMPK activity. Interestingly, these researchers also found that sumoylation can simultaneously compete for ubiquitination, a condition that is known to inactivate AMPK (166). Also the yeast homolog Snf1 has been shown to undergo sumoylation, a modification that inhibits SNF1 activity (167).

Acetylation

Recently, Lu *et al.* showed significant effects upon acetylation of Sip2 (168). Sip2 is a regulatory β subunit of SNF1 protein kinase, which is mainly involved in metabolism and other cellular processes in response to energy supply. They demonstrated that Sip2 acetylation increases physical interaction with Snf1, thereby antagonizing its catalytic activity. This further results in decreased phosphorylation of downstream targets involved in cell aging (168), indicating that acetylation of the AMPK β subunit regulates various cellular processes in yeast, which needs to be verified in mammalian cells.

Myristoylation

The β -subunit is post-translationally modified by N-terminal myristoylation, which targets AMPK β 1-containing complexes to the plasma membrane (113,169). Oakhill *et al.* showed that the muscle-specific AMPK β 2 subunit can also be myristoylated, and that myristoylation is required for allosteric stimulation of Thr-172 phosphorylation, whereas it is not required for protection from dephosphorylation or allosteric stimulation of kinase activity. Additionally, glucose deprivation has been shown to promote membrane association of myristoylated AMPK (170), indicating that there is a myristoyl-switch mechanism upon cellular stress. Removal of the myristoylation site using site-directed mutagenesis (G2A mutant) provided insight into the regulation of enzyme activity, heterotrimeric assembly and subcellular localization, showing that myristoylation inhibits AMPK activity (113). Also, Lin *et al.* reported that N-myristoylation of Sip2a, the Snf1 kinase β -subunit, perturbs cellular histone kinase activity, thereby regulating aging (171).

(Auto)Phosphorylation

Nowadays, protein phosphorylation and autophosphorylation are well-established post-translational modifications known to play a role in the regulation of subcellular localization (172,173). Indeed, AMPK can undergo extensive phosphorylation on α - and β -subunits (113,174), and in particular, AMPK can be autophosphorylated at multiple sites as a result of AMPK activation (174,175). Focusing on the β -subunit, it has been shown that mutations on known phosphorylation residues serine-182, serine-24 and serine-25 within the β 1 subunit, the latter two being identified as autophosphorylation sites, are associated with nuclear redistribution but not with altering enzyme activity (174,175). Also, autophosphorylation of the β -serine-108 residue situated in the CBM (174), appears to be required in activating AMPK by A-769662 without affecting cellular localization (92,176). Interestingly, we recently identified a novel autophosphorylation site in the AMPK β subunit, involved in coordinating AMPK recruitment to glycogen, a topic that is highlighted in chapter 2.

AMPK as a therapeutic target to combat muscle insulin resistance

Type 2 diabetes is a growing epidemic, thus increasing the demand for safe and effective medications. It is widely known that activation of AMPK, a key metabolic master switch, is impaired in rodents and humans with the metabolic syndrome. Because AMPK has beneficial impact on a wide variety of peripheral organs, by increasing catabolic processes while turning off anabolic processes, and can decrease blood glucose levels independent of insulin, activation of AMPK is considered as a therapeutic target for treating metabolic diseases, such as type 2 diabetes. Skeletal muscle is the major site of impaired glucose disposal in insulin resistance (2). Both exercise and pharmacological activation of AMPK have been shown to improve insulin sensitivity in skeletal muscle of obese (177) or insulin resistant rodents and humans (178). Notably, one key feature of type 2 diabetes is impaired stimulation of insulin-induced glycogen synthesis (179,180), causing a reduction in muscular glycogen content. Although increased AMPK activity lowers blood glucose levels by increasing the intracellular glucose uptake, AMPK is simultaneously known to phosphorylate and inhibit glycogen synthase, thereby channeling glucose into glycolysis. Therefore, the subcellular localization of activated AMPK has significant physiological impact on glucose and glycogen metabolism, as AMPK will likely encounter a different subset of downstream targets in its surrounding (*i.e.*, cytoplasmic free *versus* glycogen-bound).

Conclusion

In summary, AMPK extensively undergoes post-translational modifications, which in turn has metabolic consequences on a wide variety of tissues. In skeletal muscle, activation of AMPK promotes glucose uptake, fatty acid oxidation, and mitochondrial biogenesis, whereas it inhibits protein and glycogen synthesis. Similarly, in cardiac muscle, AMPK activation stimulates glucose uptake, fatty acid oxidation and glycolysis. On the other hand, 150 years after the discovery of glycogen granules, there is still more to learn about the regulation of glycogen metabolism. Although AMPK is a well-established metabolic master switch adapting to changes in energy balance by controlling glucose homeostasis, surprisingly, there is a lack of understanding regarding the role of AMPK in coordinating myocellular glycogen metabolism. Additionally, the physiological impact of post-translational modifications of AMPK with respect to glycogen metabolism is relatively understudied. With this in mind, this thesis aims at advancing the current knowledge of the complex interplay between AMPK and glycogen, and the role of modulators involved in glycogen metabolism.

REFERENCES

1. Defronzo, R. A., Simonson, D., Ferrannini, E., and Barrett, E. (1981) Insulin resistance: a universal finding in diabetic states. *Bull Schweiz Akad Med Wiss*, 223-238
2. Merry, T. L., and McConnell, G. K. (2009) Skeletal muscle glucose uptake during exercise: a focus on reactive oxygen species and nitric oxide signaling. *IUBMB Life* **61**, 479-484
3. Rybicka, K. K. (1996) Glycosomes--the organelles of glycogen metabolism. *Tissue Cell* **28**, 253-265
4. Shearer, J., and Graham, T. E. (2004) Novel aspects of skeletal muscle glycogen and its regulation during rest and exercise. *Exerc Sport Sci Rev* **32**, 120-126
5. Nielsen, J., Suetta, C., Hvid, L. G., Schroder, H. D., Aagaard, P., and Ortenblad, N. (2010) Subcellular localization-dependent decrements in skeletal muscle glycogen and mitochondria content following short-term disuse in young and old men. *Am J Physiol Endocrinol Metab* **299**, E1053-1060
6. Graham, T. E., Yuan, Z., Hill, A. K., and Wilson, R. J. (2010) The regulation of muscle glycogen: the granule and its proteins. *Acta Physiol (Oxf)* **199**, 489-498
7. O'Neill, H. M. (2013) AMPK and Exercise: Glucose Uptake and Insulin Sensitivity. *Diabetes Metab J* **37**, 1-21
8. Melendez, R., Melendez-Hevia, E., and Canela, E. I. (1999) The fractal structure of glycogen: A clever solution to optimize cell metabolism. *Biophys J* **77**, 1327-1332
9. Lomako, J., Lomako, W. M., and Whelan, W. J. (1991) Proglycogen: a low-molecular-weight form of muscle glycogen. *FEBS Lett* **279**, 223-228
10. Lin, A., Mu, J., Yang, J., and Roach, P. J. (1999) Self-glucosylation of glycogenin, the initiator of glycogen biosynthesis, involves an inter-subunit reaction. *Arch Biochem Biophys* **363**, 163-170
11. Rybicka, K. K. (1996) Glycosomes the organelles of glycogen metabolism. *Tissue&Cell*
12. Walker, G. J., and Whelan, W. J. (1960) The mechanism of carbohydase action. 8. Structures of the muscle-phosphorylase limit dextrins of glycogen and amylopectin. *Biochem J* **76**, 264-268
13. Johnson, L. N. (1992) Glycogen phosphorylase: control by phosphorylation and allosteric effectors. *FASEB J* **6**, 2274-2282
14. Ferrer, J. C., Favre, C., Gomis, R. R., Fernandez-Novell, J. M., Garcia-Rocha, M., de la Iglesia, N., Cid, E., and Guinovart, J. J. (2003) Control of glycogen deposition. *FEBS Lett* **546**, 127-132
15. Poulter, L., Ang, S. G., Gibson, B. W., Williams, D. H., Holmes, C. F., Caudwell, F. B., Pitcher, J., and Cohen, P. (1988) Analysis of the in vivo phosphorylation state of rabbit skeletal muscle glycogen synthase by fast-atom-bombardment mass spectrometry. *Eur J Biochem* **175**, 497-510
16. Palm, D. C., Rohwer, J. M., and Hofmeyr, J. H. (2013) Regulation of glycogen synthase from mammalian skeletal muscle--a unifying view of allosteric and covalent regulation. *FEBS J* **280**, 2-27
17. Okubo, M., Bogardus, C., Lillioja, S., and Mott, D. M. (1988) Glucose-6-phosphate stimulation of human muscle glycogen synthase phosphatase. *Metabolism* **37**, 1171-1176
18. Johnson, L. N., Snape, P., Martin, J. L., Acharya, K. R., Barford, D., and Oikonomakos, N. G. (1993) Crystallographic binding studies on the allosteric inhibitor glucose-6-phosphate to T state glycogen phosphorylase b. *J Mol Biol* **232**, 253-267
19. McManus, E. J., Sakamoto, K., Armit, L. J., Ronaldson, L., Shpiro, N., Marquez, R., and Alessi, D. R. (2005) Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. *EMBO J* **24**, 1571-1583
20. Bouskila, M., Hirshman, M. F., Jensen, J., Goodyear, L. J., and Sakamoto, K. (2008) Insulin promotes glycogen synthesis in the absence of GSK3 phosphorylation in skeletal muscle. *Am J Physiol Endocrinol Metab* **294**, E28-35
21. Bouskila, M., Hunter, R. W., Ibrahim, A. F., Delattre, L., Pegg, M., van Diepen, J. A., Voshol, P. J., Jensen, J., and Sakamoto, K. (2010) Allosteric regulation of glycogen synthase controls glycogen synthesis in muscle. *Cell Metab* **12**, 456-466
22. Prats, C., Helge, J. W., Nordby, P., Qvortrup, K., Ploug, T., Dela, F., and Wojtaszewski, J. F. (2009) Dual regulation of muscle glycogen synthase during exercise by activation and compartmentalization. *J Biol Chem* **284**, 15692-15700
23. Marchand, I., Chorneyko, K., Tarnopolsky, M., Hamilton, S., Shearer, J., Potvin, J., and Graham, T. E. (2002) Quantification of subcellular glycogen in resting human muscle: granule size, number, and location. *J Appl Physiol* (1985) **93**, 1598-1607
24. Nielsen, J., Holmberg, H. C., Schroder, H. D., Saltin, B., and Ortenblad, N. (2011) Human skeletal muscle glycogen utilization in exhaustive exercise: role of subcellular localization and fibre type. *J Physiol* **589**, 2871-2885
25. Sjostrom, M., Friden, J., and Ekblom, B. (1982) Fine structural details of human muscle fibres after fibre type specific glycogen depletion. *Histochemistry* **76**, 425-438
26. Kotoulas, O. B., Kalamidas, S. A., and Kondomerkos, D. J. (2006) Glycogen autophagy in glucose homeostasis. *Pathol Res Pract* **202**, 631-638
27. Shoseyov, O., Shani, Z., and Levy, I. (2006) Carbohydrate binding modules: biochemical properties and novel applications. *Microbiol Mol Biol Rev* **70**, 283-295
28. Boraston, A. B., Bolam, D. N., Gilbert, H. J., and Davies, G. J. (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochem J* **382**, 769-781
29. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res* **37**, D233-238
30. Vaaje-Kolstad, G., Horn, S. J., van Aalten, D. M., Synstad, B., and Eijsink, V. G. (2005) The non-catalytic chitin-binding protein CBP21 from *Serratia marcescens* is essential for chitin degradation. *J Biol Chem* **280**, 28492-28497

31. Barral, P., Suarez, C., Batanero, E., Alfonso, C., Alche Jde, D., Rodriguez-Garcia, M. I., Villalba, M., Rivas, G., and Rodriguez, R. (2005) An olive pollen protein with allergenic activity, Ole e 10, defines a novel family of carbohydrate-binding modules and is potentially implicated in pollen germination. *Biochem J* **390**, 77-84
32. Guillen, D., Sanchez, S., and Rodriguez-Sanoja, R. (2010) Carbohydrate-binding domains: multiplicity of biological roles. *Appl Microbiol Biotechnol* **85**, 1241-1249
33. Montanier, C., van Bueren, A. L., Dumon, C., Flint, J. E., Correia, M. A., Prates, J. A., Firbank, S. J., Lewis, R. J., Grondin, G. G., Ghinet, M. G., Gloster, T. M., Herve, C., Knox, J. P., Talbot, B. G., Turkenburg, J. P., Kerovuo, J., Brzezinski, R., Fontes, C. M., Davies, G. J., Boraston, A. B., and Gilbert, H. J. (2009) Evidence that family 35 carbohydrate binding modules display conserved specificity but divergent function. *Proc Natl Acad Sci U S A* **106**, 3065-3070
34. Xu, G., Potter, J. A., Russell, R. J., Oggioni, M. R., Andrew, P. W., and Taylor, G. L. (2008) Crystal structure of the NanB sialidase from *Streptococcus pneumoniae*. *J Mol Biol* **384**, 436-449
35. Roach, P. J., Depaoli-Roach, A. A., Hurley, T. D., and Tagliabracci, V. S. (2012) Glycogen and its metabolism: some new developments and old themes. *Biochem J* **441**, 763-787
36. Blake, A. W., McCartney, L., Flint, J. E., Bolam, D. N., Boraston, A. B., Gilbert, H. J., and Knox, J. P. (2006) Understanding the biological rationale for the diversity of cellulose-directed carbohydrate-binding modules in prokaryotic enzymes. *J Biol Chem* **281**, 29321-29329
37. Carvalho, C. C., Phan, N. N., Chen, Y., and Reilly, P. J. (2015) Carbohydrate-binding module tribes. *Biopolymers* **103**, 203-214
38. Christiansen, C., Abou Hachem, M., Janecek, S., Vikso-Nielsen, A., Blennow, A., and Svensson, B. (2009) The carbohydrate-binding module family 20--diversity, structure, and function. *FEBS J* **276**, 5006-5029
39. Machovič, M., and Janeček, Š. (2008) Domain evolution in the GH13 pullulanase subfamily with focus on the carbohydrate-binding module family 48. *Biologia* **63**, 1057-1068
40. Quiocho, F. A. (1986) Carbohydrate-binding proteins: tertiary structures and protein-sugar interactions. *Annu Rev Biochem* **55**, 287-315
41. Cohen, P. T. (2002) Protein phosphatase 1--targeted in many directions. *J Cell Sci* **115**, 241-256
42. Garcia-Haro, L., Garcia-Gimeno, M. A., Neumann, D., Beullens, M., Bollen, M., and Sanz, P. (2012) Glucose-dependent regulation of AMP-activated protein kinase in MIN6 beta cells is not affected by the protein kinase A pathway. *FEBS Lett* **586**, 4241-4247
43. Armstrong, C. G., Doherty, M. J., and Cohen, P. T. (1998) Identification of the separate domains in the hepatic glycogen-targeting subunit of protein phosphatase 1 that interact with phosphorylase a, glycogen and protein phosphatase 1. *Biochem J* **336 (Pt 3)**, 699-704
44. Wu, J., Liu, J., Thompson, I., Oliver, C. J., Shenolikar, S., and Brautigan, D. L. (1998) A conserved domain for glycogen binding in protein phosphatase-1 targeting subunits. *FEBS Lett* **439**, 185-191
45. Heroes, E., Lesage, B., Gornemann, J., Beullens, M., Van Meervelt, L., and Bollen, M. (2013) The PP1 binding code: a molecular-lego strategy that governs specificity. *FEBS J* **280**, 584-595
46. Rubio-Villena, C., Garcia-Gimeno, M. A., and Sanz, P. (2013) Glycogenic activity of R6, a protein phosphatase 1 regulatory subunit, is modulated by the laforin-malin complex. *Int J Biochem Cell Biol* **45**, 1479-1488
47. Kelsall, I. R., Voss, M., Munro, S., Cuthbertson, D. J., and Cohen, P. T. (2011) R3F, a novel membrane-associated glycogen targeting subunit of protein phosphatase 1 regulates glycogen synthase in astrocytoma cells in response to glucose and extracellular signals. *J Neurochem* **118**, 596-610
48. Fong, N. M., Jensen, T. C., Shah, A. S., Parekh, N. N., Saltiel, A. R., and Brady, M. J. (2000) Identification of binding sites on protein targeting to glycogen for enzymes of glycogen metabolism. *J Biol Chem* **275**, 35034-35039
49. Peti, W., Nairn, A. C., and Page, R. (2013) Structural basis for protein phosphatase 1 regulation and specificity. *FEBS J* **280**, 596-611
50. Worby, C. A., Gentry, M. S., and Dixon, J. E. (2008) Malin decreases glycogen accumulation by promoting the degradation of protein targeting to glycogen (PTG). *J Biol Chem* **283**, 4069-4076
51. Fernandez-Sanchez, M. E., Criado-Garcia, O., Heath, K. E., Garcia-Fojeda, B., Medrano-Fernandez, I., Gomez-Garre, P., Sanz, P., Serratos, J. M., and Rodriguez de Cordoba, S. (2003) Laforin, the dual-phosphatase responsible for Lafora disease, interacts with R5 (PTG), a regulatory subunit of protein phosphatase-1 that enhances glycogen accumulation. *Hum Mol Genet* **12**, 3161-3171
52. Serratos, J. M., Gomez-Garre, P., Gallardo, M. E., Anta, B., de Bernabe, D. B., Lindhout, D., Augustijn, P. B., Tassinari, C. A., Malafosse, R. M., Topcu, M., Grid, D., Dravet, C., Berkovic, S. F., and de Cordoba, S. R. (1999) A novel protein tyrosine phosphatase gene is mutated in progressive myoclonus epilepsy of the Lafora type (EPM2). *Hum Mol Genet* **8**, 345-352
53. Gentry, M. S., Roma-Mateo, C., and Sanz, P. (2013) Laforin, a protein with many faces: glucan phosphatase, adapter protein, et alii. *FEBS J* **280**, 525-537
54. Tagliabracci, V. S., Turnbull, J. J., Wang, W., Girard, J. M., Zhao, X., Skurat, A. V., Delgado-Escueta, A. V., Minassian, B. A., Depaoli-Roach, A. A., and Roach, P. J. (2007) Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen in vivo. *Proc Natl Acad Sci U S A* **104**, 19262-19266
55. Worby, C. A., Gentry, M. S., and Dixon, J. E. (2006) Laforin, a dual specificity phosphatase that dephosphorylates complex carbohydrates. *J Biol Chem* **281**, 30412-30418
56. Singh, P. K., Singh, S., and Ganesh, S. (2012) The laforin-malin complex negatively regulates glycogen synthesis by modulating cellular glucose uptake via glucose transporters. *Mol Cell Biol* **32**, 652-663
57. Wang, J., Stuckey, J. A., Wishart, M. J., and Dixon, J. E. (2002) A unique carbohydrate binding domain targets the lafora disease phosphatase to glycogen. *J Biol Chem* **277**, 2377-2380
58. Jiang, S., Heller, B., Tagliabracci, V. S., Zhai, L., Irimia, J. M., DePaoli-Roach, A. A., Wells, C. D., Skurat, A. V., and Roach, P. J. (2010) Starch binding domain-containing protein 1/genethonin 1 is a novel participant in glycogen metabolism. *J Biol Chem* **285**, 34960-34971

59. Zhu, Y., Zhang, M., Kelly, A. R., and Cheng, A. (2014) The carbohydrate-binding domain of overexpressed STBD1 is important for its stability and protein-protein interactions. *Biosci Rep* **34**
60. Chandramouli, C., Varma, U., Stevens, E. M., Xiao, R. P., Stapleton, D. I., Mellor, K. M., and Delbridge, L. M. (2015) Myocardial glycogen dynamics: New perspectives on disease mechanisms. *Clin Exp Pharmacol Physiol* **42**, 415-425
61. Zmasek, C. M., and Godzik, A. (2014) Phylogenomic analysis of glycogen branching and debranching enzymatic duo. *BMC Evol Biol* **14**, 183
62. Diaz, A., Martinez-Pons, C., Fita, I., Ferrer, J. C., and Guinovart, J. J. (2011) Processivity and subcellular localization of glycogen synthase depend on a non-catalytic high affinity glycogen-binding site. *J Biol Chem* **286**, 18505-18514
63. Polekhina, G., Gupta, A., Michell, B. J., van Denderen, B., Murthy, S., Feil, S. C., Jennings, I. G., Campbell, D. J., Witters, L. A., Parker, M. W., Kemp, B. E., and Stapleton, D. (2003) AMPK β Subunit Targets Metabolic Stress Sensing to Glycogen. *Current Biology* **13**, 867-871
64. Polekhina, G., Gupta, A., van Denderen, B. J., Feil, S. C., Kemp, B. E., Stapleton, D., and Parker, M. W. (2005) Structural basis for glycogen recognition by AMP-activated protein kinase. *Structure* **13**, 1453-1462
65. Hardie, D. G., Hawley, S. A., and Scott, J. W. (2006) AMP-activated protein kinase--development of the energy sensor concept. *J Physiol* **574**, 7-15
66. Woods, A., Cheung, P. C., Smith, F. C., Davison, M. D., Scott, J., Beri, R. K., and Carling, D. (1996) Characterization of AMP-activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro. *J Biol Chem* **271**, 10282-10290
67. Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G., and Hardie, D. G. (2004) CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *Journal of Clinical Investigation* **113**, 274-284
68. Gowans, G. J., Hawley, S. A., Ross, F. A., and Hardie, D. G. (2013) AMP is a true physiological regulator of AMP-activated protein kinase by both allosteric activation and enhancing net phosphorylation. *Cell Metab* **18**, 556-566
69. Zhang, Y. L., Guo, H., Zhang, C. S., Lin, S. Y., Yin, Z., Peng, Y., Luo, H., Shi, Y., Lian, G., Zhang, C., Li, M., Ye, Z., Ye, J., Han, J., Li, P., Wu, J. W., and Lin, S. C. (2013) AMP as a low-energy charge signal autonomously initiates assembly of AXIN-AMPK-LKB1 complex for AMPK activation. *Cell Metab* **18**, 546-555
70. Riek, U., Scholz, R., Konarev, P., Rufer, A., Suter, M., Nazabal, A., Ringler, P., Chami, M., Muller, S. A., Neumann, D., Forstner, M., Hennig, M., Zenobi, R., Engel, A., Svergun, D., Schlattner, U., and Wallimann, T. (2008) Structural properties of AMP-activated protein kinase: dimerization, molecular shape, and changes upon ligand binding. *J Biol Chem* **283**, 18331-18343
71. Sanders, M. J., Grondin, P. O., Hegarty, B. D., Snowden, M. A., and Carling, D. (2007) Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochem J* **403**, 139-148
72. Suter, M., Riek, U., Tuerk, R., Schlattner, U., Wallimann, T., and Neumann, D. (2006) Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. *J Biol Chem* **281**, 32207-32216
73. Xiao, B., Sanders, M. J., Underwood, E., Heath, R., Mayer, F. V., Carmena, D., Jing, C., Walker, P. A., Eccleston, J. F., Haire, L. F., Saiu, P., Howell, S. A., Aasland, R., Martin, S. R., Carling, D., and Gamblin, S. J. (2011) Structure of mammalian AMPK and its regulation by ADP. *Nature* **472**, 230-233
74. Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R., and Hardie, D. G. (2003) Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol* **2**, 28
75. Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G., and Hardie, D. G. (2005) Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* **2**, 9-19
76. Woods, A., Dickerson, K., Heath, R., Hong, S. P., Momcilovic, M., Johnstone, S. R., Carlson, M., and Carling, D. (2005) Ca²⁺/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab* **2**, 21-33
77. Lamia, K. A., Sachdeva, U. M., DiTacchio, L., Williams, E. C., Alvarez, J. G., Egan, D. F., Vazquez, D. S., Juguilon, H., Panda, S., Shaw, R. J., Thompson, C. B., and Evans, R. M. (2009) AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science* **326**, 437-440
78. Xie, M., Zhang, D., Dyck, J. R., Li, Y., Zhang, H., Morishima, M., Mann, D. L., Taffet, G. E., Baldini, A., Khoury, D. S., and Schneider, M. D. (2006) A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *Proc Natl Acad Sci U S A* **103**, 17378-17383
79. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995) Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* **270**, 2008-2011
80. Momcilovic, M., Hong, S. P., and Carlson, M. (2006) Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. *J Biol Chem* **281**, 25336-25343
81. Scholz, R., Sidler, C. L., Thali, R. F., Winssinger, N., Cheung, P. C., and Neumann, D. (2010) Autoactivation of transforming growth factor beta-activated kinase 1 is a sequential bimolecular process. *J Biol Chem* **285**, 25753-25766
82. O'Neill, L. A., and Hardie, D. G. (2013) Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature* **493**, 346-355
83. Zhang, L., Frederich, M., He, H., and Balschi, J. A. (2006) Relationship between 5-aminoimidazole-4-carboxamide-ribotide and AMP-activated protein kinase activity in the perfused mouse heart. *Am J Physiol Heart Circ Physiol* **290**, H1235-1243

84. Merrill, G. F., Kurth, E. J., Hardie, D. G., and Winder, W. W. (1997) AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* **273**, E1107-1112
85. Dirx, E., Schwenk, R. W., Coumans, W. A., Hoebers, N., Angin, Y., Viollet, B., Bonen, A., van Eys, G. J., Glatz, J. F., and Luiken, J. J. (2012) Protein kinase D1 is essential for contraction-induced glucose uptake but is not involved in fatty acid uptake into cardiomyocytes. *J Biol Chem* **287**, 5871-5881
86. Bulut, L., Guigas, B., Von Wilamowitz-Moellendorff, A., Maisin, L., Vertommen, D., Hussain, N., Beullens, M., Guinovart, J. J., Foretz, M., Viollet, B., Sakamoto, K., Hue, L., and Rider, M. H. (2012) AMP-activated protein kinase phosphorylates and inactivates liver glycogen synthase. *Biochem J* **443**, 193-203
87. LeBrasseur, N. K., Kelly, M., Tsao, T. S., Farmer, S. R., Saha, A. K., Ruderman, N. B., and Tomas, E. (2006) Thiazolidinediones can rapidly activate AMP-activated protein kinase in mammalian tissues. *Am J Physiol Endocrinol Metab* **291**, E175-181
88. Xiao, B., Sanders, M. J., Carmena, D., Bright, N. J., Haire, L. F., Underwood, E., Patel, B. R., Heath, R. B., Walker, P. A., Hallen, S., Giordanetto, F., Martin, S. R., Carling, D., and Gamblin, S. J. (2013) Structural basis of AMPK regulation by small molecule activators. *Nat Commun* **4**, 3017
89. Zadra, G., Photopoulos, C., Tyekucheva, S., Heidari, P., Weng, Q. P., Fedele, G., Liu, H., Scaglia, N., Priolo, C., Sciscna, G., Mahmood, U., Signoretti, S., Birnberg, N., and Loda, M. (2014) A novel direct activator of AMPK inhibits prostate cancer growth by blocking lipogenesis. *EMBO Mol Med* **6**, 519-538
90. Gomez-Galeno, J. E., Dang, Q., Nguyen, T. H., Boyer, S. H., Grote, M. P., Sun, Z., Chen, M., Craig, W. A., van Poelje, P. D., MacKenna, D. A., Cable, E. E., Rolzin, P. A., Finn, P. D., Chi, B., Linemeyer, D. L., Hecker, S. J., and Erion, M. D. (2010) A Potent and Selective AMPK Activator That Inhibits de Novo Lipogenesis. *ACS Med Chem Lett* **1**, 478-482
91. Cool, B., Zinker, B., Chiou, W., Kifle, L., Cao, N., Perham, M., Dickinson, R., Adler, A., Gagne, G., Iyengar, R., Zhao, G., Marsh, K., Kym, P., Jung, P., Camp, H. S., and Frevet, E. (2006) Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab* **3**, 403-416
92. Scott, J. W., Ling, N., Issa, S. M., Dite, T. A., O'Brien, M. T., Chen, Z. P., Galic, S., Langendorf, C. G., Steinberg, G. R., Kemp, B. E., and Oakhill, J. S. (2014) Small molecule drug A-769662 and AMP synergistically activate naive AMPK independent of upstream kinase signaling. *Chem Biol* **21**, 619-627
93. Sanders, M. J., Ali, Z. S., Hegarty, B. D., Heath, R., Snowden, M. A., and Carling, D. (2007) Defining the mechanism of activation of AMP-activated protein kinase by the small molecule A-769662, a member of the thienopyridone family. *J Biol Chem* **282**, 32539-32548
94. Hawley, S. A., Fullerton, M. D., Ross, F. A., Schertzer, J. D., Chevtzoff, C., Walker, K. J., Pegg, M. W., Zibrova, D., Green, K. A., Mustard, K. J., Kemp, B. E., Sakamoto, K., Steinberg, G. R., and Hardie, D. G. (2012) The ancient drug salicylate directly activates AMP-activated protein kinase. *Science* **336**, 918-922
95. Hawley, S. A., Ross, F. A., Chevtzoff, C., Green, K. A., Evans, A., Fogarty, S., Towler, M. C., Brown, L. J., Ogunbayo, O. A., Evans, A. M., and Hardie, D. G. (2010) Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab* **11**, 554-565
96. Hardie, D. G. (2013) AMPK: a target for drugs and natural products with effects on both diabetes and cancer. *Diabetes* **62**, 2164-2172
97. Viollet, B., Athea, Y., Mounier, R., Guigas, B., Zarrinpashneh, E., Horman, S., Lantier, L., Hebrard, S., Devin-Leclerc, J., Beauloye, C., Foretz, M., Andreelli, F., Ventura-Clapier, R., and Bertrand, L. (2009) AMPK: Lessons from transgenic and knockout animals. *Front Biosci (Landmark Ed)* **14**, 19-44
98. Kazgan, N., Williams, T., Forsberg, L. J., and Brenman, J. E. (2010) Identification of a nuclear export signal in the catalytic subunit of AMP-activated protein kinase. *Mol Biol Cell* **21**, 3433-3442
99. Salt, I., Celler, J. W., Hawley, S. A., Prescott, A., Woods, A., Carling, D., and Hardie, D. G. (1998) AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. *Biochem J* **334** (Pt 1), 177-187
100. Turnley, A. M., Stapleton, D., Mann, R. J., Witters, L. A., Kemp, B. E., and Bartlett, P. F. (1999) Cellular distribution and developmental expression of AMP-activated protein kinase isoforms in mouse central nervous system. *J Neurochem* **72**, 1707-1716
101. Suzuki, A., Okamoto, S., Lee, S., Saito, K., Shiuchi, T., and Minokoshi, Y. (2007) Leptin stimulates fatty acid oxidation and peroxisome proliferator-activated receptor alpha gene expression in mouse C2C12 myoblasts by changing the subcellular localization of the alpha2 form of AMP-activated protein kinase. *Mol Cell Biol* **27**, 4317-4327
102. McGee, S. L., Howlett, K. F., Starkie, R. L., Cameron-Smith, D., Kemp, B. E., and Hargreaves, M. (2003) Exercise increases nuclear AMPK alpha2 in human skeletal muscle. *Diabetes* **52**, 926-928
103. Kodiha, M., Rassi, J. G., Brown, C. M., and Stochaj, U. (2007) Localization of AMP kinase is regulated by stress, cell density, and signaling through the MEK->ERK1/2 pathway. *Am J Physiol Cell Physiol* **293**, C1427-1436
104. Pinter, K., Grignani, R. T., Watkins, H., and Redwood, C. (2013) Localisation of AMPK gamma subunits in cardiac and skeletal muscles. *J Muscle Res Cell Motil* **34**, 369-378
105. Birk, J. B., and Wojtaszewski, J. F. (2006) Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. *J Physiol* **577**, 1021-1032
106. Pinter, K., Jefferson, A., Czibik, G., Watkins, H., and Redwood, C. (2012) Subunit composition of AMPK trimers present in the cytoskeletal apparatus: Implications for drug target identification. *Cell Cycle* **11**, 917-921
107. Cheung, P. C., Salt, I. P., Davies, S. P., Hardie, D. G., and Carling, D. (2000) Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. *Biochem J* **346** Pt 3, 659-669
108. Wojtaszewski, J. F., Birk, J. B., Frosig, C., Holten, M., Pilegaard, H., and Dela, F. (2005) 5'AMP activated protein kinase expression in human skeletal muscle: effects of strength training and type 2 diabetes. *J Physiol* **564**, 563-573

109. O'Neill, H. M., Maarbjerg, S. J., Crane, J. D., Jeppesen, J., Jorgensen, S. B., Schertzer, J. D., Shyroka, O., Kiens, B., van Denderen, B. J., Tarnopolsky, M. A., Kemp, B. E., Richter, E. A., and Steinberg, G. R. (2011) AMP-activated protein kinase (AMPK) beta1beta2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. *Proc Natl Acad Sci U S A* **108**, 16092-16097
110. Chen, Z., Heierhorst, J., Mann, R. J., Mitchelhill, K. I., Michell, B. J., Witters, L. A., Lynch, G. S., Kemp, B. E., and Stapleton, D. (1999) Expression of the AMP-activated protein kinase beta1 and beta2 subunits in skeletal muscle. *FEBS Lett* **460**, 343-348
111. Thornton, C., Snowden, M. A., and Carling, D. (1998) Identification of a novel AMP-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle. *J Biol Chem* **273**, 12443-12450
112. Wu, J., Puppala, D., Feng, X., Monetti, M., Lapworth, A. L., and Geoghegan, K. F. (2013) Chemoproteomic analysis of intertissue and interspecies isoform diversity of AMP-activated protein kinase (AMPK). *J Biol Chem* **288**, 35904-35912
113. Warden, S. M., Richardson, C., O'Donnell, J., Jr., Stapleton, D., Kemp, B. E., and Witters, L. A. (2001) Post-translational modifications of the beta-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization. *Biochem J* **354**, 275-283
114. Dasgupta, B., and Milbrandt, J. (2009) AMP-activated protein kinase phosphorylates retinoblastoma protein to control mammalian brain development. *Dev Cell* **16**, 256-270
115. Kolwicz, S. C., Jr., Purohit, S., and Tian, R. (2013) Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes. *Circ Res* **113**, 603-616
116. Kelley, D. E., Reilly, J. P., Veneman, T., and Mandarino, L. J. (1990) Effects of insulin on skeletal muscle glucose storage, oxidation, and glycolysis in humans. *Am J Physiol* **258**, E923-929
117. Winder, W. W. (2001) Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. *J Appl Physiol* (1985) **91**, 1017-1028
118. Ruderman, N. B., Keller, C., Richard, A. M., Saha, A. K., Luo, Z., Xiang, X., Giralt, M., Ritov, V. B., Menshikova, E. V., Kelley, D. E., Hidalgo, J., Pedersen, B. K., and Kelly, M. (2006) Interleukin-6 Regulation of AMP-Activated Protein Kinase: Potential Role in the Systemic Response to Exercise and Prevention of the Metabolic Syndrome. *Diabetes* **55**, S48-S54
119. Lee, W. J., Kim, M., Park, H. S., Kim, H. S., Jeon, M. J., Oh, K. S., Koh, E. H., Won, J. C., Kim, M. S., Oh, G. T., Yoon, M., Lee, K. U., and Park, J. Y. (2006) AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARalpha and PGC-1. *Biochem Biophys Res Commun* **340**, 291-295
120. McGee, S. L., van Denderen, B. J., Howlett, K. F., Mollica, J., Schertzer, J. D., Kemp, B. E., and Hargreaves, M. (2008) AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. *Diabetes* **57**, 860-867
121. Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J., and Winder, W. W. (1999) 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* **48**, 1667-1671
122. Jager, S., Handschin, C., St-Pierre, J., and Spiegelman, B. M. (2007) AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Proc Natl Acad Sci U S A* **104**, 12017-12022
123. Canto, C., Jiang, L. Q., Deshmukh, A. S., Matak, C., Coste, A., Lagouge, M., Zierath, J. R., and Auwerx, J. (2010) Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle. *Cell Metab* **11**, 213-219
124. Sag, D., Carling, D., Stout, R. D., and Suttles, J. (2008) Adenosine 5'-Monophosphate-Activated Protein Kinase Promotes Macrophage Polarization to an Anti-Inflammatory Functional Phenotype. *The Journal of Immunology* **181**, 8633-8641
125. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kadowaki, T. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* **8**, 1288-1295
126. Liu, Q., Gauthier, M. S., Sun, L., Ruderman, N., and Lodish, H. (2010) Activation of AMP-activated protein kinase signaling pathway by adiponectin and insulin in mouse adipocytes: requirement of acyl-CoA synthetases FATP1 and Acs1 and association with an elevation in AMP/ATP ratio. *FASEB J* **24**, 4229-4239
127. Bijland, S., Mancini, S. J., and Salt, I. P. (2013) Role of AMP-activated protein kinase in adipose tissue metabolism and inflammation. *Clin Sci (Lond)* **124**, 491-507
128. Lee, J. M., Seo, W. Y., Song, K. H., Chanda, D., Kim, Y. D., Kim, D. K., Lee, M. W., Ryu, D., Kim, Y. H., Noh, J. R., Lee, C. H., Chiang, J. Y., Koo, S. H., and Choi, H. S. (2010) AMPK-dependent repression of hepatic gluconeogenesis via disruption of CREB-CRTC2 complex by orphan nuclear receptor small heterodimer partner. *J Biol Chem* **285**, 32182-32191
129. Fullerton, M. D., Galic, S., Marcinko, K., Sikkema, S., Pulinilkunnil, T., Chen, Z. P., O'Neill, H. M., Ford, R. J., Palanivel, R., O'Brien, M., Hardie, D. G., Macaulay, S. L., Schertzer, J. D., Dyck, J. R., van Denderen, B. J., Kemp, B. E., and Steinberg, G. R. (2013) Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. *Nat Med* **19**, 1649-1654
130. Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1**, 15-25
131. Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y. B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferre, P., Birnbaum, M. J., Stuck, B. J., and Kahn, B. B. (2004) AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* **428**, 569-574
132. Andersson, U., Filipsson, K., Abbott, C. R., Woods, A., Smith, K., Bloom, S. R., Carling, D., and Small, C. J. (2004) AMP-activated protein kinase plays a role in the control of food intake. *J Biol Chem* **279**, 12005-12008

133. Kola, B., Hubina, E., Tucci, S. A., Kirkham, T. C., Garcia, E. A., Mitchell, S. E., Williams, L. M., Hawley, S. A., Hardie, D. G., Grossman, A. B., and Korbonits, M. (2005) Cannabinoids and ghrelin have both central and peripheral metabolic and cardiac effects via AMP-activated protein kinase. *J Biol Chem* **280**, 25196-25201
134. Jorgensen, S. B., Wojtaszewski, J. F., Viollet, B., Andreelli, F., Birk, J. B., Hellsten, Y., Schjerling, P., Vaulont, S., Neufer, P. D., Richter, E. A., and Pilegaard, H. (2005) Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. *FASEB J* **19**, 1146-1148
135. Fujii, N., Seifert, M. M., Kane, E. M., Peter, L. E., Ho, R. C., Winstead, S., Hirshman, M. F., and Goodyear, L. J. (2007) Role of AMP-activated protein kinase in exercise capacity, whole body glucose homeostasis, and glucose transport in skeletal muscle -insight from analysis of a transgenic mouse model. *Diabetes Res Clin Pract* **77 Suppl 1**, S92-98
136. Foretz, M., Ancellin, N., Andreelli, F., Saintillan, Y., Grondin, P., Kahn, A., Thorens, B., Vaulont, S., and Viollet, B. (2005) Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. *Diabetes* **54**, 1331-1339
137. Arad, M., Moskowitz, I. P., Patel, V. V., Ahmad, F., Perez-Atayde, A. R., Sawyer, D. B., Walter, M., Li, G. H., Burgon, P. G., Maguire, C. T., Stapleton, D., Schmitt, J. P., Guo, X. X., Pizard, A., Kupersmidt, S., Roden, D. M., Berul, C. I., Seidman, C. E., and Seidman, J. G. (2003) Transgenic mice overexpressing mutant PRKAG2 define the cause of Wolff-Parkinson-White syndrome in glycogen storage cardiomyopathy. *Circulation* **107**, 2850-2856
138. Luptak, I., Shen, M., He, H., Hirshman, M. F., Musi, N., Goodyear, L. J., Yan, J., Wakimoto, H., Morita, H., Arad, M., Seidman, C. E., Seidman, J. G., Ingwall, J. S., Balschi, J. A., and Tian, R. (2007) Aberrant activation of AMP-activated protein kinase remodels metabolic network in favor of cardiac glycogen storage. *J Clin Invest* **117**, 1432-1439
139. Kim, M., Hunter, R. W., Garcia-Menendez, L., Gong, G., Yang, Y. Y., Kolwicz, S. C., Jr., Xu, J., Sakamoto, K., Wang, W., and Tian, R. (2014) Mutation in the gamma2-subunit of AMP-activated protein kinase stimulates cardiomyocyte proliferation and hypertrophy independent of glycogen storage. *Circ Res* **114**, 966-975
140. Folmes, K. D., Chan, A. Y., Koonen, D. P., Pullinilkunnil, T. C., Baczkowski, I., Hunter, B. E., Thorn, S., Allard, M. F., Roberts, R., Gollob, M. H., Light, P. E., and Dyck, J. R. (2009) Distinct early signaling events resulting from the expression of the PRKAG2 R302Q mutant of AMPK contribute to increased myocardial glycogen. *Circ Cardiovasc Genet* **2**, 457-466
141. Nilsson, E. C., Long, Y. C., Martinsson, S., Glund, S., Garcia-Roves, P., Svensson, L. T., Andersson, L., Zierath, J. R., and Mahlapuu, M. (2006) Opposite transcriptional regulation in skeletal muscle of AMP-activated protein kinase gamma3 R225Q transgenic versus knock-out mice. *J Biol Chem* **281**, 7244-7252
142. Steinberg, G. R., O'Neill, H. M., Dzamko, N. L., Galic, S., Naim, T., Koopman, R., Jorgensen, S. B., Honeymann, J., Hewitt, K., Chen, Z. P., Schertzer, J. D., Scott, J. W., Koentgen, F., Lynch, G. S., Watt, M. J., van Denderen, B. J., Campbell, D. J., and Kemp, B. E. (2010) Whole body deletion of AMP-activated protein kinase {beta}2 reduces muscle AMPK activity and exercise capacity. *J Biol Chem* **285**, 37198-37209
143. Jorgensen, S. B., Viollet, B., Andreelli, F., Frosig, C., Birk, J. B., Schjerling, P., Vaulont, S., Richter, E. A., and Wojtaszewski, J. F. (2004) Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* **279**, 1070-1079
144. Barnes, B. R., Marklund, S., Steiler, T. L., Walter, M., Hjalmar, G., Amarger, V., Mahlapuu, M., Leng, Y., Johansson, C., Galuska, D., Lindgren, K., Abrink, M., Stapleton, D., Zierath, J. R., and Andersson, L. (2004) The 5'-AMP-activated protein kinase gamma3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. *J Biol Chem* **279**, 38441-38447
145. Dzamko, N., van Denderen, B. J., Hevener, A. L., Jorgensen, S. B., Honeymann, J., Galic, S., Chen, Z. P., Watt, M. J., Campbell, D. J., Steinberg, G. R., and Kemp, B. E. (2010) AMPK beta1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. *J Biol Chem* **285**, 115-122
146. Wiatrowski, H. A., van Denderen, B. J. W., Berkeley, C. D., Kemp, B. E., Stapleton, D., and Carlson, M. (2003) Mutations in the Gal83 Glycogen-Binding Domain Activate the Snf1/Gal83 Kinase Pathway by a Glycogen-Independent Mechanism. *Molecular and Cellular Biology* **24**, 352-361
147. Avila-Castaneda, A., Gutierrez-Granados, N., Ruiz-Gayosso, A., Sosa-Peinado, A., Martinez-Barajas, E., and Coello, P. (2014) Structural and functional basis for starch binding in the SnRK1 subunits AKINbeta2 and AKINbetagama. *Front Plant Sci* **5**, 199
148. Chen, L., Wang, J., Zhang, Y. Y., Yan, S. F., Neumann, D., Schlattner, U., Wang, Z. X., and Wu, J. W. (2012) AMP-activated protein kinase undergoes nucleotide-dependent conformational changes. *Nat Struct Mol Biol* **19**, 716-718
149. Xiao, B., Heath, R., Saiu, P., Leiper, F. C., Leone, P., Jing, C., Walker, P. A., Haire, L., Eccleston, J. F., Davis, C. T., Martin, S. R., Carling, D., and Gamblin, S. J. (2007) Structural basis for AMP binding to mammalian AMP-activated protein kinase. *Nature* **449**, 496-500
150. Hudson, E. R., Pan, D. A., James, J., Lucocq, J. M., Hawley, S. A., Green, K. A., Baba, O., Terashima, T., and Hardie, D. G. (2003) A Novel Domain in AMP-Activated Protein Kinase Causes Glycogen Storage Bodies Similar to Those Seen in Hereditary Cardiac Arrhythmias. *Current Biology* **13**, 861-866
151. Koay, A., Woodcroft, B., Petrie, E. J., Yue, H., Emanuelle, S., Bieri, M., Bailey, M. F., Hargreaves, M., Park, J. T., Park, K. H., Ralph, S., Neumann, D., Stapleton, D., and Gooley, P. R. (2010) AMPK beta subunits display isoform specific affinities for carbohydrates. *FEBS Lett* **584**, 3499-3503
152. Carling, D., and Hardie, D. G. (1989) The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta* **1012**, 81-86
153. Skurat, A. V., Wang, Y., and Roach, P. J. (1994) Rabbit skeletal muscle glycogen synthase expressed in COS cells. Identification of regulatory phosphorylation sites. *J Biol Chem* **269**, 25534-25542

154. Jorgensen, S. B., Nielsen, J. N., Birk, J. B., Olsen, G. S., Viollet, B., Andreelli, F., Schjerling, P., Vaulont, S., Hardie, D. G., Hansen, B. F., Richter, E. A., and Wojtaszewski, J. F. (2004) The $\alpha 2$ -5'-AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* **53**, 3074-3081
155. Roma-Mateo, C., Solaz-Fuster Mdol, C., Gimeno-Alcaniz, J. V., Dukhande, V. V., Donderis, J., Worby, C. A., Marina, A., Criado, O., Koller, A., Rodriguez De Cordoba, S., Gentry, M. S., and Sanz, P. (2011) Laforin, a dual-specificity phosphatase involved in Lafora disease, is phosphorylated at Ser25 by AMP-activated protein kinase. *Biochem J* **439**, 265-275
156. Fediciu, S., Gaidhu, M. P., and Ceddia, R. B. (2006) Inhibition of insulin-stimulated glycogen synthesis by 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside-induced adenosine 5'-monophosphate-activated protein kinase activation: interactions with Akt, glycogen synthase kinase 3- α /beta, and glycogen synthase in isolated rat soleus muscle. *Endocrinology* **147**, 5170-5177
157. Holmes, B. F., Kurth-Kraczek, E. J., and Winder, W. W. (1999) Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. *J Appl Physiol* (1985) **87**, 1990-1995
158. Arad, M., Benson, D. W., Perez-Atayde, A. R., McKenna, W. J., Sparks, E. A., Kanter, R. J., McGarry, K., Seidman, J. G., and Seidman, C. E. (2002) Constitutively active AMP kinase mutations cause glycogen storage disease mimicking hypertrophic cardiomyopathy. *Journal of Clinical Investigation* **109**, 357-362
159. Hunter, R. W., Treebak, J. T., Wojtaszewski, J. F., and Sakamoto, K. (2011) Molecular mechanism by which AMP-activated protein kinase activation promotes glycogen accumulation in muscle. *Diabetes* **60**, 766-774
160. McBride, A., Ghilagaber, S., Nikolaev, A., and Hardie, D. G. (2009) The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. *Cell Metab* **9**, 23-34
161. Momcilovic, M., Iram, S. H., Liu, Y., and Carlson, M. (2008) Roles of the glycogen-binding domain and Snf4 in glucose inhibition of SNF1 protein kinase. *J Biol Chem* **283**, 19521-19529
162. Li, X., Wang, L., Zhou, X. E., Ke, J., de Waal, P. W., Gu, X., Tan, M. H., Wang, D., Wu, D., Xu, H. E., and Melcher, K. (2015) Structural basis of AMPK regulation by adenine nucleotides and glycogen. *Cell Res* **25**, 50-66
163. Jorgensen, S. B., Richter, E. A., and Wojtaszewski, J. F. (2006) Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise. *J Physiol* **574**, 17-31
164. Qi J, G. J., Zhao T, Zhao J, Lam P, Ye J, Zhong Li J, Wu J, Zhou H-M, Li P. (2008) Downregulation of AMP-activated protein kinase by Cidea-mediated ubiquitination and degradation in brown adipose tissue. *EMBO J*
165. Moreno, D., Towler, M. C., Hardie, D. G., Knecht, E., and Sanz, P. (2010) The laforin-malin complex, involved in Lafora disease, promotes the incorporation of K63-linked ubiquitin chains into AMP-activated protein kinase beta subunits. *Mol Biol Cell* **21**, 2578-2588
166. Rubio, T., Vernia, S., and Sanz, P. (2013) Sumoylation of AMPKbeta2 subunit enhances AMP-activated protein kinase activity. *Mol Biol Cell* **24**, 1801-1811, S1801-1804
167. Simpson-Lavy, K. J., and Johnston, M. (2013) SUMOylation regulates the SNF1 protein kinase. *Proc Natl Acad Sci U S A* **110**, 17432-17437
168. Lu, J. Y., Lin, Y. Y., Sheu, J. C., Wu, J. T., Lee, F. J., Chen, Y., Lin, M. I., Chiang, F. T., Tai, T. Y., Berger, S. L., Zhao, Y., Tsai, K. S., Zhu, H., Chuang, L. M., and Boeke, J. D. (2011) Acetylation of yeast AMPK controls intrinsic aging independently of caloric restriction. *Cell* **146**, 969-979
169. Resh, M. D. (1999) Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta* **1451**, 1-16
170. Oakhill, J. S., Chen, Z. P., Scott, J. W., Steel, R., Castelli, L. A., Ling, N., Macaulay, S. L., and Kemp, B. E. (2010) beta-Subunit myristoylation is the gatekeeper for initiating metabolic stress sensing by AMP-activated protein kinase (AMPK). *Proc Natl Acad Sci U S A* **107**, 19237-19241
171. Lin, S. S., Manchester, J. K., and Gordon, J. I. (2003) Sip2, an N-myristoylated beta subunit of Snf1 kinase, regulates aging in *Saccharomyces cerevisiae* by affecting cellular histone kinase activity, recombination at rDNA loci, and silencing. *J Biol Chem* **278**, 13390-13397
172. Feng, X., and Hannun, Y. A. (1998) An essential role for autophosphorylation in the dissociation of activated protein kinase C from the plasma membrane. *J Biol Chem* **273**, 26870-26874
173. Lorenz, K., Schmitt, J. P., Schmitteckert, E. M., and Lohse, M. J. (2009) A new type of ERK1/2 autophosphorylation causes cardiac hypertrophy. *Nat Med* **15**, 75-83
174. Mitchellhill, K. I., Michell, B. J., House, C. M., Stapleton, D., Dyck, J., Gamble, J., Ullrich, C., Witters, L. A., and Kemp, B. E. (1997) Posttranslational modifications of the 5'-AMP-activated protein kinase beta1 subunit. *J Biol Chem* **272**, 24475-24479
175. Tuerk, R. D., Auchli, Y., Thali, R. F., Scholz, R., Wallimann, T., Brunisholz, R. A., and Neumann, D. (2009) Tracking and quantification of 32P-labeled phosphopeptides in liquid chromatography matrix-assisted laser desorption/ionization mass spectrometry. *Anal Biochem* **390**, 141-148
176. Woods, A., Vertommen, D., Neumann, D., Turk, R., Bayliss, J., Schlattner, U., Wallimann, T., Carling, D., and Rider, M. H. (2003) Identification of phosphorylation sites in AMP-activated protein kinase (AMPK) for upstream AMPK kinases and study of their roles by site-directed mutagenesis. *J Biol Chem* **278**, 28434-28442
177. Bergeron, R., Previs, S. F., Cline, G. W., Perret, P., Russell, R. R., 3rd, Young, L. H., and Shulman, G. I. (2001) Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. *Diabetes* **50**, 1076-1082
178. Steinberg, G. R., and Kemp, B. E. (2009) AMPK in Health and Disease. *Physiol Rev* **89**, 1025-1078
179. Halse, R., Bonavaud, S. M., Armstrong, J. L., McCormack, J. G., and Yeaman, S. J. (2001) Control of glycogen synthesis by glucose, glycogen, and insulin in cultured human muscle cells. *Diabetes* **50**, 720-726
180. Shulman, G. I., Rothman, D. L., Jue, T., Stein, P., DeFronzo, R. A., and Shulman, R. G. (1990) Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by ¹³C nuclear magnetic resonance spectroscopy. *N Engl J Med* **322**, 223-228

Chapter 2

The recruitment of AMP-activated protein kinase to glycogen is regulated by autophosphorylation

Yvonne Oligschlaeger*, Marie Miglianico*, Dipanjan Chanda, Roland Scholz, Ramon F. Thali, Roland Tuerk, David I. Stapleton, Paul R. Gooley, Dietbert Neumann

J Biol Chem. 2015 May 1;290(18):11715-28

*Authors equally contributed

ABSTRACT

The mammalian AMP-activated protein kinase (AMPK) is an obligatory $\alpha\beta\gamma$ heterotrimeric complex carrying a carbohydrate-binding module (CBM) in the β -subunit (AMPK β) capable of attaching AMPK to glycogen. Nonetheless, AMPK localizes at many different cellular compartments, implying the existence of mechanisms that prevent AMPK from glycogen binding. Cell-free carbohydrate-binding assays revealed that AMPK autophosphorylation abolished its carbohydrate-binding capacity. X-ray structural data of the CBM displays the central positioning of threonine-148 residue (T148) within the binding pocket. Substitution of T148 for a phospho-mimicking aspartate (T148D) prevents AMPK from binding to carbohydrate. Overexpression of isolated CBM or β 1-containing AMPK in cellular models revealed that wild-type (WT) localizes to glycogen particles, whereas T148D shows a diffuse pattern. Pharmacological AMPK activation and glycogen degradation by glucose deprivation but not Forskolin enhanced cellular T148 phosphorylation. Cellular glycogen content was higher if pharmacological AMPK activation was combined with overexpression of T148D mutant relative to WT-AMPK. In summary, these data show that glycogen-binding capacity of AMPK β is regulated by T148 autophosphorylation with likely implications in regulation of glycogen turnover. The findings further raise the possibility of regulated carbohydrate-binding function in a wider variety of CBM-containing proteins.

INTRODUCTION

Many enzymes involved in the synthesis and degradation of complex carbohydrate molecules, such as cellulose or glycogen, contain a domain called the carbohydrate-binding module (CBM) specialized in the binding of complex carbohydrates. CBMs are present in various taxa and protein types (1,2). Despite their lack of direct catalytic activity, CBMs may enhance specific enzymatic activity by targeting the enzyme to its substrates and increasing its effective concentration (3,4). Other proteins that are not directly carbohydrate-active but are involved in glucose metabolism regulation are also known to contain a CBM: this is notably the case for the mammalian energy sensor AMP-activated protein kinase (AMPK) (5,6), as well as its fungal homolog SNF1 (7) and its plant homolog SnRK1 (8).

AMPK is a heterotrimer consisting of (i) a catalytic subunit α ($\alpha 1$ or $\alpha 2$) carrying the kinase domain, (ii) a regulatory subunit β ($\beta 1$ or $\beta 2$) with the CBM and the C-terminal region tethering α and γ together, and (iii) a regulatory subunit γ ($\gamma 1$, $\gamma 2$ or $\gamma 3$) responsible for the adenine nucleotide sensing (9,10). AMPK is a well-known, highly conserved metabolic enzyme central for coordinating cellular and whole body energy homeostasis. Upon cellular stress, such as contraction, exercise or hypoxia, AMPK is activated by several mechanisms. Briefly, the increased AMP level induces a conformational change in AMPK facilitating the phosphorylation of threonine-172 (T172) on the activation loop of the α -subunit by upstream kinases such as liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase 2 (CaMKK2) resulting in roughly 1000-fold activation of the enzyme (11-13). The AMP-binding to AMPK γ also reduces the rate of dephosphorylation at T172, thus keeping AMPK in its active state (11,14). As a result, AMPK phosphorylates a number of downstream targets to restore energy balance by facilitating glucose uptake, glycolysis, and fatty acid oxidation, thus increasing the energy production, and simultaneously switching off ATP-consuming pathways, such as glycogen, fatty acid and cholesterol synthesis (9,12).

Considering the micromolar affinity of recombinant AMPK complexes and CBM for small cyclic and linear oligosaccharides (15,16), the interaction between AMPK and glycogen in cells may be relatively strong. Since some AMPK targets, such as glycogen synthase (17), are associated with glycogen (18), the presence of a CBM might be beneficial in juxtaposing the kinase next to its substrates (5). However, in the case of targets not located at glycogen, such as the acetyl-CoA carboxylase 2 (ACC2), which is associated with the mitochondria (19), the retention of AMPK to glycogen by the CBM could be detrimental for a quick cellular response to energy stress. Besides, AMPK has been detected in different subcellular areas, for example at the cell membrane (20,21) or in the nucleus (22,23). This variety of localizations and actions implies that the AMPK-carbohydrate binding ability may be regulated in cells.

In this study, using cell-free and cellular systems, we show that AMPK loses its carbohydrate-binding ability upon activation due to a bimolecular autophosphorylation event that modifies the AMPK β -CBM at threonine-148 (T148).

EXPERIMENTAL PROCEDURES

Plasmids - Bacterial expression plasmids encoding for hexahistidine-tagged wild-type (WT) AMPK ($\alpha 1\beta 1\gamma 1$, $\alpha 1\beta 2\gamma 1$, $\alpha 2\beta 1\gamma 1$, $\alpha 2\beta 2\gamma 1$) (24,25), non-tagged LKB1-MO25-STRAD α complex (26), and GST-tagged CaMKK2 (11) have been described. The isolated $\beta 1$ - and $\beta 2$ -CBMs were bacterially expressed as described (16). The cDNA of $\beta 1$ lacking the CBM (aa 182-270) was amplified by PCR and initially subcloned into the NcoI and SpeI restriction sites of the pET3dx vector (24). Subsequently, the bacterial expression plasmid encoding for the heterotrimeric hexahistidine-tagged AMPK lacking the CBM ($\alpha 1\beta 1\Delta\text{CBM}\gamma 1$) was generated using the published procedure (24). The kinase-deficient AMPK $\alpha 1$ plasmids with a non-phosphorylatable alanine (D157A) ($\alpha 1\beta 1\gamma 1$ -KD or $\alpha 1\beta 2\gamma 1$ -KD, respectively) were constructed as published earlier (27). The coding sequence for T148 in the AMPK $\beta 1$ subunits was changed to encode a non-phosphorylatable alanine (T148A) or a phosphorylation-mimicking aspartate (T148D) by site-directed mutagenesis following the manufacturer's instructions (QuikChange, Stratagene).

To obtain activated AMPK phosphorylated at T172, each of the tricistronic WT and kinase-deficient coding sequences were combined with the LKB1-MO25-STRAD α coding sequence in a single hexacistronic plasmid, allowing for co-expression of both protein complexes and the subsequent purification of the hexahistidine-tagged AMPK alone (referred to as $\alpha 1\beta 1\Delta\text{CBM}\gamma 1$ active, $\alpha 1\beta 1\gamma 1$ and $\alpha 1\beta 2\gamma 1$ active, $\alpha 1\beta 1\gamma 1$ -KD pT172, and T148A active, respectively) as also published recently (28).

For expression in mammalian cells, the cDNA of wild-type (WT) $\beta 1$ -CBM (aa 68-163) or $\beta 2$ -CBM (aa 67-163) was amplified by PCR and ligated in frame into the pAcGFP expression vector (Clontech) via the EcoRI and SalI restriction sites, resulting in expression constructs for CBM $\beta 1$ -WT-GFP or CBM $\beta 2$ -WT-GFP, respectively. The cDNA of full-length $\beta 1$ was amplified by PCR and subcloned either into the pmCherry expression vector (Clontech) via EcoRI and SalI restriction sites, or into the pCMV5-3xHA expression vector (29) via HindIII and SalI restriction sites. GFP-tagged CBMs or mCherry-tagged $\beta 1$ bearing a threonine-to-aspartate mutation on residue 148 (T148D) were generated using the Quick-change site-directed mutagenesis kit (Stratagene). The pcDNA3 constructs for expression of AMPK $\alpha 1$ -myc and $\gamma 1$ -subunit were kindly provided by Dr. D. Carling (Imperial College London, London, United Kingdom).

For retroviral overexpression, GFP-tagged CBM $\beta 1$ or CBM $\beta 2$ (either WT or T148D) was subcloned, using an oligonucleotide linker, into the EcoRI and SalI restriction sites of the pBMZ-ires-neo retroviral backbone (kindly provided by Dr. G. Nolan, Stanford University, CA). Primer sequences are available from the author upon request. All of the constructs were verified by sequencing.

Bacterial Expression and Purification - Proteins were expressed in Rosetta 2 (DE3) *Escherichia coli* cells (Novagen). Bacteria were grown in auto-induction medium and proteins were purified as described with few modifications (30). Briefly, the bacterial pellet was collected by centrifugation, resuspended in lysis buffer (for hexahistidine-tagged proteins: 50 mM NaH₂PO₄, 30 % Glycerol, 0.5 M sucrose, 10 mM imidazole, pH 8; for GST-tagged proteins: PBS, pH 7.3) and lysed using a high-pressure homogenizer. After centrifugation, the supernatant was incubated for 1 h at 4°C on a roller bank in presence of either 1 mL of nickel-sepharose HP (GE Healthcare) for the hexahistidine-tagged proteins or with 1 mL of glutathione sepharose 4B (GE Healthcare) for the GST-tagged proteins. After centrifugation, the resin was washed 3 times with wash buffer (for hexahistidine-tagged proteins: 50 mM NaH₂PO₄, 30 % Glycerol, 0.5 M sucrose, 20 mM imidazole, pH 8; for GST-tagged proteins: PBS, pH 7.3) then the protein was eluted in elution buffer (for hexahistidine-tagged proteins: 50 mM NaH₂PO₄, 30 % Glycerol, 0.5 M sucrose, 250 mM imidazole, pH 8; for GST-tagged proteins: Tris-HCl 50 mM, 10 mM reduced glutathione, pH 8), frozen in liquid nitrogen and kept at -80°C until use.

β -cyclodextrin Binding Assay - β -cyclodextrin was immobilized on epoxy-activated sepharose 6B (GE Healthcare) according to the manufacturer's protocol. Briefly, Ni²⁺-affinity purified AMPK was rebuffed into assay buffer (10 mM Tris-HCl pH 7.2) using PD-10 columns (GE Healthcare) and 500 μ l (25 μ g/ml) of this AMPK solution (I-fraction) was incubated with 50 μ l of pre-equilibrated β -cyclodextrin resin in a 0.7 ml reaction tube (LoBind, Eppendorf) with gentle agitation for 15-20 minutes. The supernatant containing the non-bound material (S-fraction) was removed after centrifugation (2000 $\times$ g, 1 min) and the resin was washed twice in assay buffer. The wash fractions were discarded. Bound AMPK was eluted by incubation in 500 μ l of elution buffer (5 mM β -cyclodextrin in assay buffer) for 5 min with gentle agitation. After centrifugation the supernatant (P-fraction) was collected, the resin was washed twice with elution buffer and supernatants were discarded. Finally, the resin was incubated with SDS-sample buffer at 95°C to obtain the precipitated fraction (L-fraction). For analyses, 5 μ l of each fraction were subjected to SDS-PAGE and Western blotting, and further processed with AMPK α and phospho-AMPK (pT172) antibodies (Cell Signaling Technologies).

Phosphorylation Assay of Recombinant Proteins – AMPK was *in vitro* activated by upstream kinases as previously described (30). Briefly, recombinant AMPK ($\alpha 1\beta 1\gamma 1$ or $\alpha 1\beta 2\gamma 1$, 25 $\mu\text{g/ml}$) was activated in kinase buffer by recombinant GST-CamKK2 (15 $\mu\text{g/ml}$) for 30 min at 37 °C, and subsequently incubated with β -cyclodextrin resin for 10 min with gentle agitation to allow for binding. Alternatively, recombinant AMPK was first incubated with β -cyclodextrin resin, followed by activation with upstream kinases. As described above, the assay was continued with three washing steps, then three times β -cyclodextrin elution buffer, and finally SDS-sample buffer. Subsequently, the presence of (phosphorylated) AMPK in the various fractions (I, S, P, L) was probed by Western blot analysis.

Cell Culture - The human hepatocyte HepG2 and human embryonic kidney 293T cell line (HEK293T) were cultured in DMEM with high glucose (25 mM) (Gibco), supplemented with 10 % (v/v) heat-inactivated fetal calf serum (Bodinco BV, Alkmaar, The Netherlands) and penicillin/streptomycin (Invitrogen), unless otherwise stated. HL-1 cardiomyocyte cell line was kindly provided by Dr. W. Claycomb (Louisiana State University, New Orleans, LA, USA), cultured on fibronectin (5 $\mu\text{g/ml}$, Sigma)/gelatin (0.01 %, Merck)-coating in Claycomb medium (supplemented with 10 % heat-inactivated fetal calf serum (iFCS), 0.1 mmol/l noradrenaline [norepinephrine], 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin) at 37 °C and 5 % CO_2 .

For transient transfections, HEK293T cells were seeded to 30 % confluence in 6-well plates (Greiner Bio-One) 24 h before transfection. Cells were co-transfected with plasmid DNA ($\alpha 1$ -myc, mCherry-tagged $\beta 1$ -WT/ $\beta 1$ -T148D and $\gamma 1$ for immunoprecipitation and Western blotting, or $\alpha 1$ -myc, HA-tagged $\beta 1$, $\gamma 1$ and mCherry-tagged $\beta 1$ -WT/ $\beta 1$ -T148D for localization studies of the holoenzyme) using Lipofectamine 2000 (Invitrogen) in antibiotic-free culture medium. Six to eight hours after transfection, transfection medium was replaced by normal growth medium. At 24 - 48h after transfection, cells were either harvested or fixed.

For the glycogen depletion experiments, cells were either maintained in high glucose medium (DMEM with 25 mM glucose and 10 % iFCS) or treated with forskolin (100 μM , Sigma) in the same medium, or glucose-deprived (glucose-free DMEM, 10 % iFCS), for 16 hours.

In order to activate cellular AMPK, cells were serum-starved (plain DMEM, 5.5 mM glucose) for 16h and subsequently treated with 5-aminoimidazole-4-carboxamide riboside (AICAR, 1 or 1.5 mM, Sigma), oligomycin (3 or 5 μM , Sigma), A769662 (100 μM) or phenformin (1.5 mM), vehicle (DMSO) or high glucose medium for 45 – 60 min. Insulin (100 nM, Sigma) treatment for 15 min was done in order to stimulate glycogen synthesis.

Immunoprecipitation and Immunoblotting - Cells were lysed in immunoprecipitation lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10 % glycerol, 1 % Triton X-100, 2 mM EDTA), supplemented with protease and phosphatase inhibitor cocktails (Roche). Endogenous AMPK was immunoprecipitated using a combination of AMPK α 1 and AMPK α 2 antibodies raised in sheep (kindly provided by G. Hardie). Myc-tagged AMPK was immunoprecipitated using a myc-tag antibody (Cell Signaling Technology, Beverly, MA). The primary antibody was incubated top-over-top with 350 μ g of protein lysate at 4 °C for 16 h, followed by incubation with protein G Sepharose beads for 3-4 h at 4 °C. The immune complexes were then collected by centrifugation. The immunoprecipitated proteins were electrophoresed by SDS-PAGE and analyzed by Western blot analysis. Immunoblot analysis was carried out with the following primary antibodies: myc-tag, AMPK α , phospho-AMPK-T172, Akt, phospho-Akt-S473 and GS (all from Cell Signaling). GS-pS7 antibodies were a kind gift from G. Hardie. In order to detect changes in T148 phosphorylation, a phospho-specific AMPK β -T148 antibody was produced (peptide sequence AMPK β 2 (142-154) pT148; VTSQLGINNLI) (5). Detection was performed using anti-rabbit or anti-mouse horse-radish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology or Dako, respectively), followed by chemiluminescence.

Biochemical Cellular Glycogen Measurement - Extraction of glycogen from 293T cells was adapted from a method described by McMahon and Frost (31). Briefly, cells were lysed in potassium hydroxide (30 %) and boiled at 70°C for 30 min. Subsequently, samples were cooled to 25°C before sodium sulfate (6 % w/v) and EtOH (99.5 % v/v) were added at the ratio 1:1:3. After thorough mixing, samples were rotated top-over-top at 4°C for 30-60 min. The precipitate was collected by centrifugation at 5000 rpm for 5 min at 4°C. To hydrolyze, pellets were dissolved in 1M HCl and boiled at 100°C for 2h. Samples were cooled before neutralization using 2M NaOH. Hydrolysates were used for glucose determination using a Glucose (GO) Assay Kit (Sigma), according to the manufacturer's instructions.

Retroviral Vectors and Infections - Retroviral systems and Phoenix helper-free retrovirus producer cell lines were used as published before (32-34). Amphotropic retroviral supernatants were produced following calcium phosphate/DNA transfection of producer cells; 24-48 hours post-transfection, the supernatants were harvested, filtered (0.45 micron filters; Corning, Germany) and used for infection of HepG2, HEK293T and HL-1 cells in presence of 4 μ g/ml polybrene (Sigma). For infections, cells were incubated with virus particles for 6-8 hours and then allowed to recover for 48 hours on fresh medium before selection pressure was applied. Infected cells were selected with 200 to 500 μ g/ml G418 (PAA Laboratories GmbH) for two weeks preceding experiments.

Immunocytochemistry - Cells were grown in 12-wells plates (Greiner Bio-One) on coverslips (Ø 20 mm; Thermo Scientific) to 60-80 % confluence. Cells were washed twice with PBS and fixed with 4 % formaldehyde in PBS for 10 min at room temperature. Fixed cells were stored at 4°C in PBS- NaN_3 (0.03 %) or washed three times with PBS+/+ (Gibco) and directly used for immunocytochemistry (IC). Subsequently, cells were permeabilized (0.1 % Triton X-100 and 0.2 % BSA in PBS) for 15 min and blocked (2 % BSA-PBS) for 30 min at room temperature. Primary and secondary antibodies (2 % BSA-PBS) were incubated for 1h at room temperature. Coverslips were washed and mounted onto glass slides using DABCO-glycerol medium (Sigma-Aldrich) containing diamidino-2-phenylindole (DAPI, 1:10000, Sigma-Aldrich) in order to counterstain nuclei. The anti-glycogen antibody (1:500) was a courtesy of Dr. O. Baba (Tokyo Medical and Dental University, Tokyo, Japan), the secondary antibody was goat anti-mouse IgM Alexa647 (1:200, Invitrogen) or goat anti-mouse IgM Alexa488 (1:200, Invitrogen).

Microscopy and Image Capturing - Fixed cells were imaged using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems GmbH) equipped with an air-cooled Argon-Krypton mixed gas laser, using oil-immersion objectives (63×, NA=1.4). Optical sections were recorded 3 scans for each image. Image J software was used to process and analyse the images. Image brightness and contrast was adjusted to the same settings were needed.

Statistical Analysis - All bar graph data are presented as means $\pm$ S.E.M. Statistical analysis was performed by using Student's *t* test and statistical analysis software Prism 4 (GraphPad Software, Inc.). A *p* value of <0.05 was considered statistically significant.

RESULTS

The carbohydrate-binding ability of AMPK is lost upon activation

To verify the carbohydrate-binding ability of AMPK, β -cyclodextrin was coupled to sepharose beads, and recombinant AMPK ($\alpha 1\beta 1\gamma 1$) was expressed in bacteria, purified and loaded onto the packed column. As shown in **Figure 1A**, immobilized β -cyclodextrin retains the AMPK complex in the column and could therefore be used to affinity-purify recombinant AMPK. On the contrary, when no β -cyclodextrin was immobilized to the sepharose, the overexpressed AMPK was not retained confirming that AMPK retention is dependent on β -cyclodextrin. In further experiments, this procedure was simplified by allowing the recombinant AMPK to bind to the β -cyclodextrin resin in a batch set-up (**Fig. 1B**) instead of using a column. Fractions of the protein that could not bind to the resin stayed in the supernatant (S), whereas fractions that bound to the resin were eluted from this initial pellet fraction (P) by inclusion of soluble β -cyclodextrin in the buffer. The remaining resin bound proteins were solubilized by extraction with Laemmli buffer (L) and considered as unspecific precipitates. All fractions were collected and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue Stain or Western blotting. This assay was performed both with the isolated CBM ($\beta 1$ -CBM or $\beta 2$ -CBM, respectively) and a kinase-dead (KD) mutant of AMPK ($\alpha 1\beta 1\gamma 1$ -KD or $\alpha 1\beta 2\gamma 1$ -KD, respectively) (**Fig. 1C**). As expected, both CBM isoforms as well as both catalytically inactive AMPK complexes were able to bind to the resin and were consequently found in the pellet fraction.

To obtain T172-phosphorylated active AMPK, the tricistronic plasmid for AMPK expression (24,25) was combined with the LKB1-Mo25 α -Strada α tricistron resulting in a hexacistronic AMPK-LKB1 co-expression construct (**Fig. 1D**). AMPK α is the only protein of the plasmid constructs carrying a His-tag, which resulted in purified AMPK complexes when using the His-tag purification protocol. Heterotrimeric $\alpha 1\beta 1\gamma 1$ and $\alpha 1\beta 2\gamma 1$ AMPK complexes, as well as the AMPK $\alpha 1\beta 1\gamma 1$ complex lacking the N-terminal CBM ($\alpha 1\beta 1$ - Δ CBM $\gamma 1$), were expressed and purified in its inactive and active state (**Fig. 1E**). As shown in **Figure 1F**, the $\alpha 1\beta 1$ - Δ CBM $\gamma 1$ complex was expectedly found in the supernatant fraction and therefore did not retain the ability to bind to carbohydrates (**Fig. 1F**, upper panel). As seen before with the KD mutants, the inactive forms of the AMPK complexes containing full-length $\beta 1$ or $\beta 2$ normally bound to β -cyclodextrin, since these were found in the pellet fraction. In contrast, the active AMPK isoforms showed a loss of binding-affinity to the β -cyclodextrin resin similar to the truncated AMPK complex (**Fig. 1F**, lower panel). These findings suggest that activation of AMPK prevents its later binding to carbohydrates such as β -cyclodextrin.

Figure 1. The carbohydrate-binding ability of AMPK is lost upon activation. (A) Application of immobilized β -cyclodextrin for purification of AMPK. Epoxy-activated Sepharose was allowed to react with soluble β -cyclodextrin or was inactivated (control). The column resin was equilibrated in 100 mM Tris-HCl pH 7.2 (binding buffer). Bacterially expressed AMPK heterotrimers ($\alpha 1\beta 1\gamma 1$) were Ni^{2+} -affinity purified. 4.6 mg of the eluate was subsequently rebuffed to binding buffer. The AMPK sample was split into halves and either loaded to control resin or resin with immobilized β -cyclodextrin (500 μl bed volume each). The flow through (FT) was collected, and both columns were washed repeatedly with binding buffer (W1-W3) and then eluted twice with binding buffer containing 5 mM β -cyclodextrin. 10 μl of each fraction was subjected to SDS-PAGE and gel was stained by Coomassie Brilliant Blue.

Figure 1 continued. (B) Schematic model illustrating the β -cyclodextrin binding assay protocol. (C) His-tagged CBM (CBM β 1 or CBM β 2) and kinase-deficient AMPK (α 1 β 1 γ 1-KD or α 1 β 2 γ 1-KD) were subjected to the β -cyclodextrin binding assay and visualized by Coomassie Brilliant Blue Stain. (D) Schematic representation of the hexacistronic expression vector containing both AMPK (e.g., α 1 β 1 γ 1) and its upstream kinase LKB1-MO25 α -Strad α , allowing for 'in vivo' activation of AMPK in the bacterial cytosol and subsequent purification of active hexahistidine-tagged AMPK (28). (E) Detection of purified inactive and active heterotrimeric AMPK (α 1 β 1 γ 1 or α 1 β 2 γ 1) and AMPK complexes lacking the CBM (α 1 β - Δ CBM γ 1) by Coomassie Brilliant Blue Stain. Recombinant active proteins were bacterially expressed by means of hexacistronic plasmids. (F) Immunoblot analysis of inactive and active (pT172) WT α 1 β 1 γ 1/ α 1 β 2 γ 1 or truncated AMPK complexes (α 1 β 1 Δ CBM γ 1). AMPK binding to β -cyclodextrin was assessed using the total AMPK α antibody. Activation of AMPK was assessed using the pT172 antibody. Data are representative of three experiments. I, input; S, supernatant/non-bound protein; P, pellet/bound protein, L, Laemmli buffer/precipitated protein.

AMPK activation by upstream kinases is not directly responsible for the loss of binding ability to carbohydrates

To test whether phosphorylation of T172 in AMPK α was sufficient to cause the loss of carbohydrate binding, a KD mutant of AMPK α 1 β 1 γ 1 was bacterially expressed in the presence or absence of the LKB1-Mo25 α -Strad α complex and subsequently purified. In contrast to WT-AMPK, both T172-phosphorylated and non-phosphorylated KD complexes were found in the pellet fraction, indicating that catalytically inactive AMPK did not lose its ability to bind to the β -cyclodextrin when co-expressed with the LKB1-Mo25 α -Strad α complex (**Fig. 2A**). Thus, phosphorylation of AMPK at T172 by an upstream kinase is insufficient to trigger the loss of carbohydrate binding, suggesting that AMPK enzyme activity is required.

To confirm this, AMPK α 1 β 1 γ 1, α 1 β 2 γ 1 and α 1 β 1- Δ CBM γ 1 were expressed as inactive kinases (i.e., without co-expression of the LKB1-Mo25-Strad α complex) for subsequent activation *in vitro* before or during the β -cyclodextrin binding assay. Bacterial expression of recombinant CaMKK2 yields higher purity than LKB1 complex and was therefore used as an alternative upstream kinase of AMPK for these assays. As shown by the phosphorylation of T172, all AMPK isoforms were activated and, as expected, mostly lost their binding ability to the resin material (**Fig. 2B**), indicating that activation of AMPK leads to loss of its carbohydrate-binding ability irrespective of the identity of the upstream kinase used, CaMKK2 or LKB1. However, when inactive AMPK was initially allowed to bind to the β -cyclodextrin resin and was subsequently incubated with CaMKK2, both AMPK isoforms retained their ability to bind to the resin, as the proteins were predominantly found in the pellet fraction, despite phosphorylation at T172 (**Fig. 2C**). The β 2-isoform showed stronger retention upon activation compared to the β 1 complex, which corresponds to a higher binding affinity of the β 2-CBM for β -cyclodextrin (16). In this experimental setup the α 1 β 1- Δ CBM γ 1 only appears in the input and not in the S or P fraction due to its inability to bind the β -cyclodextrin resin, and therefore the protein is lost upon removal of the supernatant in step 1. Similar results were obtained using α 2-containing (α 2 β 1 γ 1 and α 2 β 2 γ 1) AMPK complexes (**Fig. 2D-F**).

The data, together with the retention of carbohydrate binding for the phosphorylated KD mutant, underline that phosphorylation of AMPK at T172 is compatible with its binding capacity to carbohydrates. Further, the binding of AMPK to β -cyclodextrin prior to activation is protective against the loss of binding ability.

We speculated that autophosphorylation could be responsible for the loss of binding to carbohydrates, because autophosphorylation is a consequence of the initial activation of kinases. Moreover, autophosphorylation at multiple sites has been demonstrated for AMPK upon activation (35,36). Experimentally it is difficult to initiate autophosphorylation without kinase activation. However, presuming a bimolecular mechanism it can be tested if the phosphorylation of inactive AMPK by its active counterpart would result in the loss of carbohydrate-binding ability. Hence, inactive recombinant AMPK ($\alpha 1\beta 1\gamma 1$, $\alpha 1\beta 2\gamma 1$ or $\alpha 1\beta 1\text{-}\Delta\text{CBM}\gamma 1$, respectively) was incubated with a very low amount of active AMPK (ratio 100:1). In the absence of ATP, both full-length AMPK proteins retained their ability to bind to the β -cyclodextrin resin, whereas the heterotrimer lacking the CBM was not capable of binding (**Fig. 2G**). However, in the presence of ATP, both AMPK isoforms were found in the supernatant fraction, indicating their loss of binding ability to the β -cyclodextrin resin despite the lack of T172 phosphorylation by upstream kinases. The presence of the small fraction of active AMPK was confirmed by prolonged exposure (**Fig. 2G**, lower panel). These results show that a catalytic amount of enzymatically active AMPK is sufficient to prevent inactive AMPK from binding to β -cyclodextrin in the presence of ATP, suggesting a bimolecular autophosphorylation event precludes AMPK from β -cyclodextrin binding.

Figure 2. AMPK activation by upstream kinases is not directly responsible for the loss of binding ability to carbohydrates. (A) Immunoblot of non-phosphorylated and phosphorylated (pT172) recombinant kinase-deficient AMPK ($\alpha 1\beta 1\gamma 1$ -KD) (as in Fig. 1) subjected to the β -cyclodextrin binding assay.

Figure 2 continued. (B, C) Immunoblot of WT ($\alpha 1\beta 1\gamma 1$ or $\alpha 1\beta 2\gamma 1$) or truncated recombinant AMPK complexes ($\alpha 1\beta 1\Delta\text{CBM}\gamma 1$) (as in **Fig. 1**) phosphorylated *in-vitro* by the upstream kinase CaMKK2 (B) prior to exposure to β -cyclodextrin, or (C) after initial binding to β -cyclodextrin. (D-F) Four different isoforms of AMPK heterotrimers were subjected to the β -cyclodextrin binding assay. Immunoblot analysis of (D) inactive AMPK complexes, (E) AMPK complexes that were *in-vitro* phosphorylated by the upstream kinase CaMKK2 prior to β -cyclodextrin exposure, or (F) AMPK complexes that were *in-vitro* phosphorylated after initial binding to β -cyclodextrin. (G) Inactive and active AMPK complexes were mixed (ratio 100:1) and incubated in the presence or absence of ATP and subjected to the β -cyclodextrin binding assay. In all experiments, the effect on AMPK binding to the model carbohydrate was evaluated. AMPK binding and activation status of AMPK was assessed using the total AMPK α and pT172 antibodies, respectively. Data are representative of three experiments. I.e., long exposure; β -CD, β -cyclodextrin.

Autophosphorylation of AMPK at β -T148 causes a loss of binding ability to carbohydrate

Considering that the binding of β -cyclodextrin prior to AMPK activation is protective from loss of binding, we speculated that the autophosphorylation event takes place directly in the carbohydrate-binding pocket of the CBM. Inspection of the X-ray structure of the $\beta 1$ -CBM identified the threonine-148 (T148) residue as centrally located in the carbohydrate-binding pocket (**Fig. 3A**). Using point mutagenesis, this site was mutated to an alanine (phosphorylation-resistant mutant T148A) in both inactive and active $\alpha 1\beta 1\gamma 1$ complexes. In the β -cyclodextrin binding assay, the inactive mutant conserved its binding capacity (**Fig. 3B**). However, in contrast to the active WT (*cf.* **Fig. 1F**), the active T148A mutant partially conserved its carbohydrate-binding capacity upon co-expression of AMPK with the LKB1-Mo25-Strad α complex (**Fig. 3B**), implying a protective role of the T148A mutation.

To further establish the involvement of T148 phosphorylation in the carbohydrate-binding ability of AMPK $\alpha 1\beta 1\gamma 1$, a phosphorylation-mimicking T148D mutant was incubated with the β -cyclodextrin resin. Although not being activated, the T148D mutant was unable to bind to the β -cyclodextrin resin (**Fig. 3C**). In addition, a phosphorylation site-specific T148 (pT148) antibody was developed allowing for detection of the modification in AMPK by Western blotting. The recombinant activated WT-AMPK $\alpha 1\beta 1\gamma 1$ and $\alpha 1\beta 2\gamma 1$ complexes showed a T148 phospho-specific signal, whereas neither the inactive counterparts nor inactive/active $\alpha 1\beta 1\Delta\text{CBM}\gamma 1$ showed detectable signals (**Fig. 3D**). Moreover, the T172 phosphorylated KD and T148A mutants of AMPK $\alpha 1\beta 1\gamma 1$ did not show a pT148 signal either (**Fig. 3E**), thus indicating the specificity of the pT148 antibody.

Therefore, the T148 site is indeed autophosphorylated and this modification directly correlates with the inability of AMPK to bind to the β -cyclodextrin resin. Taken together, these results indicate that autophosphorylation of AMPK at T148 mediates its loss of carbohydrate-binding capacity.

Figure 3. Autophosphorylation of AMPK at β -T148 causes a loss of carbohydrate-binding ability. (A) Representation of AMPK β 1-CBM co-crystallized with β -cyclodextrin (PDB code: 1Z0M). The secondary structure of the protein is represented in light grey ribbon and molecular surface, the β -cyclodextrin in dark sticks and the side chain of T148 within the carbohydrate-binding pocket is highlighted in sticks. This image was generated using the software YASARA View (37). (B) Binding capacity of inactive and active AMPK bearing a non-phosphorylatable mutation at β 1-T148 (T148A). (C) Effect of a phospho-mimicking modification of β 1-T148 (T148D) on AMPK-carbohydrate binding. (D) Determination of T148 phosphorylation on inactive and active recombinant AMPK complexes using a phospho-specific β -T148 antibody. (E) Immunoblot analysis of inactive and active recombinant AMPK. Wild-type (WT) AMPK or AMPK complexes bearing either a kinase-deficient ($\alpha 1\beta 1\gamma 1\text{-KD}$) (as in Fig. 1) or a non-phosphorylatable mutation at residue T148 (T148A) were analyzed for T148 phosphorylation using the phospho-specific T148 antibody. AMPK presence was assessed using the total AMPK α antibody. Activation was determined by the pT172 antibody. Data are representative of three experiments.

The phosphorylation-mimicking T148D mutation prevents AMPK from binding to glycogen

Next, we investigated whether the T148 phospho-mimicking mutation (T148D) triggers a loss of binding of AMPK to glycogen, its natural ligand, both in the β 1- and β 2-isoforms. Our initial analysis concentrated on the isolated WT and T148D β -CBMs. Stable cell lines were established that expressed GFP-tagged CBM variants in the human hepatic cell line HepG2 and cultured mouse HL-1 cardiomyocytes. Immunofluorescence showed a marked speckled pattern of the WT GFP-tagged constructs, β 1-CBM in HepG2 (**Fig. 4A**) and β 2-CBM in HL-1 (**Fig. 4B**) both co-localizing with glycogen. In contrast, the β 1- and β 2-CBM T148D presented a diffuse pattern despite the presence of glycogen, as shown by a glycogen-specific antibody. Similar results were obtained using the HEK293T cell line stably expressing the β 1-CBM (**Fig. 4C**): the speckled pattern of the WT-CBM co-localized with glycogen, whereas the diffuse pattern of the mutant was incongruent with the observed glycogen staining. We further assessed the glycogen localization of mCherry-tagged full-length AMPK β 1-WT and T148D mutant after transient transfection of AMPK subunits in HEK293T cells. Results shown in **Figure 4D** again revealed co-localization with glycogen for the WT, while the T148D mutant was diffusely located in the cytosol. Altogether, these findings indicate that the CBM of AMPK, expressed as an isolated domain or as full-length protein forming part of the AMPK heterotrimer, naturally binds to glycogen, whereas the phospho-mimicking T148D mutation prevents this binding from occurring.

Figure 4. The phosphorylation-mimicking AMPK β -T148D mutation prevents AMPK from binding to cellular glycogen. (A) HepG2, (B) HL-1, (C) HEK293T cells stably overexpressing the WT and T148D mutant GFP-tagged CBM β 1 or CBM β 2 isoforms (in green) as indicated. Cells were fixed and stained with an anti-glycogen antibody that was detected by an Alexa647-labeled secondary antibody (in red) and with DAPI for nuclei (in blue), after which co-localization was assessed by confocal imaging. (D) Co-localization of WT or T148D mCherry-tagged β 1-AMPK complexes (in red) was assessed as in A and B, but using Alexa488-labeled secondary antibody for glycogen detection (in green). Data are representative of three to five experiments. Data have been adjusted for brightness and contrast to obtain best quality fluorescent images.

Endogenous AMPK β -T148 phosphorylation is a dynamic and regulated process

In order to investigate the occurrence of T148 phosphorylation in cells and its detection with the pT148 antibody, we immuno-precipitated endogenous AMPK from treated and untreated HEK293T or HepG2 cells (**Fig. 5A** and **B**). Importantly, phosphorylation of T148 could be detected in both cell lines, although under different conditions. In HEK293T, only the treatment with AICAR plus insulin led to the detection of the T148 phosphorylation (**Fig. 5A**) and this treatment also showed the highest level of T172 phosphorylation. In HepG2, however, T148 phosphorylation occurred independently of AMPK activation changes (**Fig. 5B**). Hence, the data verifies T148 phosphorylation as an endogenous posttranslational modification. In addition, the differences of T148 phosphorylation between cell types and conditions underline the control and dynamics of this post-translational modification.

Figure 5. Endogenous AMPK β -T148 phosphorylation is a dynamic and regulated process.

Immunoprecipitation of endogenous AMPK with AMPK $\alpha 1/\alpha 2$ antibodies from **(A)** HEK293T or **(B)** HepG2 cells. Immunoblot analyses using total AMPK α , AMPK α -pT172, AMPK β -pT148, total Akt and Akt-pS473 antibodies. Phosphorylation of AMPK β -T148 was detected following immunoprecipitation upon single treatment of insulin (100 nM, 15 min) or AICAR (1 mM, 45 min), or a combined treatment (Ins + AICAR). All immunocomplexes were assessed for activation of AMPK and β -T148 phosphorylation using the pT172 and pT148 antibody, respectively. Data are representative of two experiments.

Glucose deprivation triggers AMPK β -T148 phosphorylation

To investigate the signals leading to AMPK T148-autophosphorylation, we tested whether glycogen depletion induced AMPK β -T148 phosphorylation (**Fig. 6A**) and compared β 1-WT with β 1-T148D mutant AMPK expressing cells (**Fig. 6B**). HEK293T cells were either triple-transfected (as before) with AMPK γ 1, myc-tagged AMPK α 1 and mCherry-tagged AMPK β 1 (WT or T148D) or left untransfected, and then treated for 16 hours with a high glucose medium with or without forskolin, a drug which promotes glycogen degradation (38), or with a medium without glucose. As expected, in forskolin-treated or glucose deprived cells, the glycogen content was diminished if compared to control condition in high glucose medium, although statistical significance was only reached in untransfected cells (**Fig. 6B**). In all of the three culturing conditions the glycogen content was similar in untransfected, β 1-WT and β 1-T148D mutant expressing cells. Notably, T148 phosphorylation was specifically induced by glucose deprivation but not forskolin, although both treatments depleted from glycogen (**Fig. 6A**). The pT148 signal was absent in immuno-precipitates of T148D cells, reassuring that the antibody specifically recognizes the phosphorylation modification on the T148 residue. Given the fact that increased AMPK activation by glucose deprivation is correlated with increased phosphorylation of T148, these data support the cellular AMPK-mediated T148 autophosphorylation mechanism.

Figure 6. Glucose deprivation triggers AMPK β -T148 phosphorylation. (A, B) HEK293T cells transiently overexpressing myc-tagged α 1, γ 1 and WT or T148D mCherry-tagged β 1, were either treated for 16 hours with high glucose medium containing serum (HiG), with the same medium supplemented with forskolin (100 μ M; HiG-Forsk) or with medium containing serum but no glucose (NoG). (A) AMPK α was immunoprecipitated using the myc-tag antibody. Immunoblot using myc-tag antibody following precipitation shows the immunoprecipitation efficiency. Immunoblot with AMPK α -pT172 and AMPK β -pT148 show AMPK activity and autophosphorylation status. (B) Glycogen was quantified biochemically, corrected for protein concentration and normalized to the level of WT cells under HiG. Statistical test: Student's *t*-test HiG vs. treatment within one cell type (*: $p < 0.05$, #: $p = 0.06$); $n = 2$

β -T148 phosphorylation plays a role in regulation of glycogen metabolism

To further explore the physiological significance of T148 phosphorylation we treated cells with different AMPK activators and measured the resulting glycogen content. To allow for comparison of β 1-WT with β 1-T148D mutant AMPK, we again employed the HEK293T triple-transfection model. As expected, in serum-starved cells the treatment with oligomycin, A769662 and phenformin resulted in activation of AMPK in β 1-WT as well as in β 1-T148D cells, as observed by an increase in phosphorylation of T172 (**Fig. 7A**). Further, the pT172 levels were paralleled by similar increases in T148 phosphorylation with the pT148 signal virtually absent in high-glucose treated cells and highest upon phenformin treatment. Cellular glycogen content generally decreased upon AMPK activation in accordance with the expected AMPK-induced shift towards catabolism (**Fig. 7C**). Statistically significant reductions were observed between vehicle and AICAR or oligomycin-treated WT-transfected cells. Interestingly, in A769662 or AICAR-treated cells, glycogen content was significantly higher in T148D vs. WT-transfected cells, which may relate to AMPK β 1-T148D being absent from glycogen and thus unable to inhibit glycogen synthesis. Indeed, the AMPK-dependent inhibition of glycogen synthase (GS) by phosphorylation at serine 7 (pS7) was strongly increased in WT-transfected cells upon oligomycin and A769662, whereas the respective pS7 signals were fainter in the T148D-transfected cells (**Fig. 7B**). Moreover, the glycogen content of WT-transfected cells treated with phenformin showed neither a difference with the vehicle-treated WT cells, nor with the phenformin-treated T148D cells (**Fig. 7C**). Interestingly, this absence of difference correlated with the highest T148 phosphorylation (**Fig. 7A**), suggesting that the autophosphorylation was sufficient to mimic the effect of the mutation. Taken together, T148 is phosphorylated upon AMPK activation and thus prevents AMPK from binding to glycogen, which affects glycogen metabolism.

Figure 7. AMPK β -T148 phosphorylation plays a role in regulation of glycogen metabolism. (A-C) HEK293T cells transiently overexpressing myc-tagged $\alpha 1$, $\gamma 1$, and $\beta 1$ -mCherry, either wild-type (WT) or $\beta 1$ carrying the phospho-mimicking T148D (T148D) were serum-starved for 16h prior to treatment with either high glucose medium (HiG) or DMSO, AICAR (1.5 mM), oligomycin (3 μ M), A769662 (100 μ M) or Phenformin (1.5 mM) for 1 h. **(A)** AMPK α was immunoprecipitated using the myc-tag antibody. Immunoblot using myc-tag antibody following precipitation shows the immunoprecipitation efficiency. Immunoblot with AMPK α -pT172 and AMPK β -pT148 show AMPK activity and autophosphorylation status. **(B)** Immunoblot of the associated input samples shows the expression levels of overexpressed myc-protein and of glycogen synthase (tGS) and the phosphorylation status of glycogen synthase (GS-pS7). **(C)** Glycogen was quantified biochemically, corrected for protein concentration and normalized to the level of WT cells under HiG. Statistical test: Student's *t*-test (*: $p < 0.05$)

DISCUSSION

In this study, we investigated the molecular mechanism causing AMPK to refrain from binding to glycogen thereby affecting glycogen turnover. Our results demonstrate that bimolecular AMPK autophosphorylation at β -subunit T148 interferes with its carbohydrate-binding capacity. The T148 site is located within the center of the carbohydrate-binding pocket, thereby controlling the attachment to glycogen. Accordingly, autophosphorylation at T148 is prevented by carbohydrate occupancy. We also provide evidence that WT isolated CBMs and full-length β 1-subunit as part of the AMPK heterotrimer localizes to glycogen particles, whereas the respective T148D mutants do not bind cellular glycogen. In addition, we show that activation of AMPK enhances cellular T148 phosphorylation and provide first evidence for involvement of β 1-T148 phosphorylation in preventing AMPK from glycogen metabolism regulation.

It is established that many protein kinases catalyze their own activation by autophosphorylation (39). Autophosphorylation of AMPK has been described previously (11,35,36) but, at present, little is known about the biological functions of AMPK autophosphorylation. Autophosphorylation of S108 is required for activation of AMPK by the small molecule A-769662 independently of α T172 phosphorylation (40), but the physiological function of this modification has remained elusive so far. The phosphorylation of S24/25 in the β 1 subunit leads to nuclear exclusion (20) and S24/25 were identified as autophosphorylation sites (35,36). Here, we identify T148 as a new autophosphorylation site and also ascribe its function to regulation of subcellular localization. Likewise, Feng and Hannun (41) reported a dissociation of protein kinase C (PKC) from the plasma membrane to the cytosol upon autophosphorylation. Also, the kinase ERK1/2 translocates to the nucleus upon autophosphorylation induced by extracellular signal, where it phosphorylates nuclear targets that are known to initiate cardiac hypertrophy (42). Hence, autophosphorylation, which more commonly has been regarded as a 'mistake' of active kinases, rather emerges as a self-regulatory mechanism with possible consequences in subcellular targeting, and thus relevant for health and disease.

Our investigation revealed that little amounts of active AMPK were sufficient to prevent catalytically inactive AMPK from binding to glycogen, suggesting that a bimolecular autophosphorylation event is involved in this process. Therefore, it seems possible that few activated AMPK molecules are able to prevent glycogen binding of the entire pool of AMPK molecules, which would explain why the correlation of T172 and T148 phosphorylation was not always seen in cells. However, we can expect that the cell will have preventive measures, such as dephosphorylation of T148 in order to restore glycogen binding. The phosphatase responsible for dephosphorylation therefore awaits identification.

Although some proteins can indirectly associate to glycogen via interacting with other glycogen-binding proteins (43), most proteins capable of directly attaching to glycogen or other polysaccharides possess a CBM. Regulation of glycogen binding by post-translational modifications has been previously reported. Notably, the protein

phosphatase 1 (PP1) was found to detach from glycogen upon phosphorylation of its CBM-containing regulatory subunit G (44). Later work showed that this phosphorylation led to dissociation of the PP1 from the G-subunit and its translocation to cytosol, while the G-subunit stayed bound to glycogen (45). Glycogen synthase, which does not carry a CBM, has also been reported to change cellular localization upon phosphorylation: when phosphorylated at site 1b, it associates with intramyofibrillar glycogen particles, whereas the site 2+2a phosphorylated enzyme binds to intermyofibrillar particles (46). Nevertheless, both of these phosphorylation events are unrelated to the glycogen-binding site of the glycogen synthase (18). AMPK carries a β -subunit CBM that is known to target proteins to glycogen (5,6), but regulation of its glycogen-binding function is unknown as AMPK is found in various subcellular compartments including those devoid of glycogen. In this current study, we provide evidence for a direct modification within the CBM, which we believe is the first example of such modification within a CBM resulting in loss of carbohydrate-binding affinity. We report the blocking of AMPK glycogen binding by T148 autophosphorylation. Indeed, X-ray structural data indicated that T148 of the AMPK- β subunit is positioned in the center of the carbohydrate-binding pocket and thus could be predicted to cause loss of binding upon phosphorylation. In support, our results revealed that the phosphorylation mimicking AMPK-T148D mutant was indeed incapable of binding to carbohydrates, suggesting that T148 phosphorylation plays a role in localizing AMPK away from carbohydrates. This novel mechanism of AMPK regulation is furthermore consistent with studies using large-scale phosphoproteome analyses and reporting T148 as a β 2 phosphorylation site (47,48). Notably, in our study we find the β 1 and β 2-subunits are both modified by autophosphorylation at the T148 site. Although the antibody was raised against the β 2-sequence, it recognizes the modification in both isoforms (**Fig. 3E**). Therefore, based on our data we cannot draw conclusions on isoform-specific differences of cellular T148 phosphorylation.

To further evaluate the significance of T148, we addressed the question whether β -T148 is being phosphorylated in cellular models. Our data showed that the levels of T148 phosphorylation were mostly correlated with AMPK activity. In fact, based on our findings, we can assume that the molecular T148 autophosphorylation mechanism we describe here and binding to glycogen occurs in a mutually exclusive manner. In other words, AMPK-carbohydrate binding results in masking of the T148 residue, which subsequently prevents AMPK from autophosphorylation. In line with this, the binding of β -cyclodextrin prior to activation was protective for the loss of carbohydrate-binding ability. Moreover, insulin treatment prevented further increases of AICAR-mediated T148 phosphorylation in HepG2 (**Fig. 5B**). This observation could be related to the fact that insulin promotes glycogen synthesis (49), thereby stimulating AMPK binding to glycogen and preventing it from autophosphorylation at T148. As a possible interpretation of our results, even if AMPK in response to stresses would get very highly activated, T148-phosphorylated and,

thus, prevented from interacting with glycogen, the glycogen-residing part of the AMPK pool would remain bound to carry on with AMPK activities at glycogen.

The precise roles of AMPK associated with glycogen are still subject to debate. On the one hand, AMPK stimulates GLUT4-mediated myocellular glucose uptake (50,51) and co-immunoprecipitates with glycogen-binding proteins, such as glycogen phosphorylase (52), glycogen debranching enzyme (53) and glycogen synthase (54), suggesting AMPK plays a role in the disposal of glucose into glycogen. In support of this notion, the phosphorylation of glycogen synthase by AMPK switches off the synthesis of glycogen (17,55) and that of laforin reduces phosphatase activity and regulates its interaction with malin, thereby playing a major role in glycogen metabolism (56). However, on the other hand, Hunter et al. (54) delimited the supposed role of AMPK on glycogen synthesis by showing that the AMPK-mediated inactivation of glycogen synthase can be overridden by increased glucose-6-phosphate levels. Partly in line with this, McBride et al. (57) showed a clear inhibition of AMPK when bound to certain branch points of glycogen, raising a doubt on the ability of AMPK to quickly phosphorylate its glycogen-bound downstream targets. Recently, Li et al. showed that binding of the CBM to carbohydrates destabilizes the CBM-kinase domain interaction thereby increasing the accessibility of the kinase domain for T172 dephosphorylation (58), which is consistent with an inhibitory effect of glycogen-binding on AMPK kinase activity. Our findings suggest irrelevance of AMPK glycogen-localization for the process of glycogen degradation (**Fig. 6**). In contrast, the increased glycogen content in β 1-T148D mutant vs. β 1-WT expressing cells upon AMPK activation (**Fig. 7C**) could be interpreted as augmented rate of glycogenesis due to loss of AMPK glycogen localization and thus exclusion from glycogen synthesis inhibition. In any case, the unraveling of a new mechanism allowing for AMPK to stay unbound in the cytosol has consequences for our understanding of the kinase function, especially in the myocellular cell types where glycogen is a major fuel resource (59). Hence, future efforts will be focused on determining the biological roles of β -T148 phosphorylation in cardiac or skeletal muscle.

In summary, using cell-free systems and cellular models, we shed light on a novel molecular mechanism that is responsible for the loss of AMPK-glycogen interaction. T148 autophosphorylation blocks the carbohydrate-binding pocket of the β -CBM, thus adding a new layer of complexity and identifying an unexpected switch regulating AMPK subcellular localization with relevance for glycogen metabolism.

REFERENCES

1. Janacek, S., Svensson, B., and MacGregor, E. A. (2011) Structural and evolutionary aspects of two families of non-catalytic domains present in starch and glycogen binding proteins from microbes, plants and animals. *Enzyme Microb Technol* **49**, 429-440
2. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res* **37**, D233-238
3. Shoseyov, O., Shani, Z., and Levy, I. (2006) Carbohydrate binding modules: biochemical properties and novel applications. *Microbiol Mol Biol Rev* **70**, 283-295
4. Guillen, D., Sanchez, S., and Rodriguez-Sanoja, R. (2010) Carbohydrate-binding domains: multiplicity of biological roles. *Appl Microbiol Biotechnol* **85**, 1241-1249
5. Polekhina, G., Gupta, A., Michell, B. J., van Denderen, B., Murthy, S., Feil, S. C., Jennings, I. G., Campbell, D. J., Witters, L. A., Parker, M. W., Kemp, B. E., and Stapleton, D. (2003) AMPK beta subunit targets metabolic stress sensing to glycogen. *Curr Biol* **13**, 867-871
6. Hudson, E. R., Pan, D. A., James, J., Lucocq, J. M., Hawley, S. A., Green, K. A., Baba, O., Terashima, T., and Hardie, D. G. (2003) A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. *Curr Biol* **13**, 861-866
7. Wiatrowski, H. A., Van Denderen, B. J., Berkey, C. D., Kemp, B. E., Stapleton, D., and Carlson, M. (2004) Mutations in the gal83 glycogen-binding domain activate the snf1/gal83 kinase pathway by a glycogen-independent mechanism. *Mol Cell Biol* **24**, 352-361
8. Avila-Castaneda, A., Gutierrez-Granados, N., Ruiz-Gayosso, A., Sosa-Peinado, A., Martinez-Barajas, E., and Coello, P. (2014) Structural and functional basis for starch binding in the SnRK1 subunits AKINbeta2 and AKINbetagamma. *Front Plant Sci* **5**, 199
9. Fogarty, S., and Hardie, D. G. (2010) Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. *Biochim Biophys Acta* **1804**, 581-591
10. Hardie, D. G., Ross, F. A., and Hawley, S. A. (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol* **13**, 251-262
11. Suter, M., Riek, U., Tuerk, R., Schlattner, U., Wallimann, T., and Neumann, D. (2006) Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. *J Biol Chem* **281**, 32207-32216
12. Hegarty, B. D., Turner, N., Cooney, G. J., and Kraegen, E. W. (2009) Insulin resistance and fuel homeostasis: the role of AMP-activated protein kinase. *Acta Physiol (Oxf)* **196**, 129-145
13. Mihaylova, M. M., and Shaw, R. J. (2011) The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* **13**, 1016-1023
14. Sanders, M. J., Grondin, P. O., Hegarty, B. D., Snowden, M. A., and Carling, D. (2007) Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochem J* **403**, 139-148
15. Bieri, M., Mobbs, J. I., Koay, A., Louey, G., Mok, Y. F., Hatters, D. M., Park, J. T., Park, K. H., Neumann, D., Stapleton, D., and Gooley, P. R. (2012) AMP-activated protein kinase beta-subunit requires internal motion for optimal carbohydrate binding. *Biophys J* **102**, 305-314
16. Koay, A., Woodcroft, B., Petrie, E. J., Yue, H., Emanuelle, S., Bieri, M., Bailey, M. F., Hargreaves, M., Park, J. T., Park, K. H., Ralph, S., Neumann, D., Stapleton, D., and Gooley, P. R. (2010) AMPK beta subunits display isoform specific affinities for carbohydrates. *FEBS Lett* **584**, 3499-3503
17. Jorgensen, S. B., Nielsen, J. N., Birk, J. B., Olsen, G. S., Viollet, B., Andreelli, F., Schjerling, P., Vaulont, S., Hardie, D. G., Hansen, B. F., Richter, E. A., and Wojtaszewski, J. F. (2004) The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* **53**, 3074-3081
18. Diaz, A., Martinez-Pons, C., Fita, I., Ferrer, J. C., and Guinovart, J. J. (2011) Processivity and subcellular localization of glycogen synthase depend on a non-catalytic high affinity glycogen-binding site. *J Biol Chem* **286**, 18505-18514
19. Abu-Elheiga, L., Brinkley, W. R., Zhong, L., Chirala, S. S., Woldegiorgis, G., and Wakil, S. J. (2000) The subcellular localization of acetyl-CoA carboxylase 2. *Proc Natl Acad Sci U S A* **97**, 1444-1449
20. Warden, S. M., Richardson, C., O'Donnell, J., Jr., Stapleton, D., Kemp, B. E., and Witters, L. A. (2001) Post-translational modifications of the beta-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization. *Biochem J* **354**, 275-283
21. Oakhill, J. S., Chen, Z. P., Scott, J. W., Steel, R., Castelli, L. A., Ling, N., Macaulay, S. L., and Kemp, B. E. (2010) beta-Subunit myristoylation is the gatekeeper for initiating metabolic stress sensing by AMP-activated protein kinase (AMPK). *Proc Natl Acad Sci U S A* **107**, 19237-19241
22. Kazgan, N., Williams, T., Forsberg, L. J., and Brenman, J. E. (2010) Identification of a nuclear export signal in the catalytic subunit of AMP-activated protein kinase. *Mol Biol Cell* **21**, 3433-3442
23. Kim, N., Lee, J. O., Lee, H. J., Lee, S. K., Moon, J. W., Kim, S. J., Park, S. H., and Kim, H. S. (2014) AMPKalpha2 translocates into the nucleus and interacts with hnRNP H: implications in metformin-mediated glucose uptake. *Cell Signal* **26**, 1800-1806
24. Neumann, D., Woods, A., Carling, D., Wallimann, T., and Schlattner, U. (2003) Mammalian AMP-activated protein kinase: functional, heterotrimeric complexes by co-expression of subunits in Escherichia coli. *Protein Expr Purif* **30**, 230-237
25. Riek, U., Scholz, R., Konarev, P., Rufer, A., Suter, M., Nazabal, A., Ringler, P., Chami, M., Muller, S. A., Neumann, D., Forstner, M., Hennig, M., Zenobi, R., Engel, A., Svergun, D., Schlattner, U., and Wallimann, T. (2008) Structural properties of AMP-activated protein kinase: dimerization, molecular shape, and changes upon ligand binding. *J Biol Chem* **283**, 18331-18343

26. Neumann, D., Suter, M., Tuerk, R., Riek, U., and Wallimann, T. (2007) Co-expression of LKB1, MO25alpha and STRADalpha in bacteria yield the functional and active heterotrimeric complex. *Mol Biotechnol* **36**, 220-231
27. Woods, A., Vertommen, D., Neumann, D., Turk, R., Bayliss, J., Schlattner, U., Wallimann, T., Carling, D., and Rider, M. H. (2003) Identification of phosphorylation sites in AMP-activated protein kinase (AMPK) for upstream AMPK kinases and study of their roles by site-directed mutagenesis. *J Biol Chem* **278**, 28434-28442
28. Bullen, J. W., Balsbaugh, J. L., Chanda, D., Shabanowitz, J., Hunt, D. F., Neumann, D., and Hart, G. W. (2014) Cross-talk between two essential nutrient-sensitive enzymes: O-GlcNAc transferase (OGT) and AMP-activated protein kinase (AMPK). *J Biol Chem* **289**, 10592-10606
29. Scholz, R., Sidler, C. L., Thali, R. F., Winssinger, N., Cheung, P. C., and Neumann, D. (2010) Autoactivation of transforming growth factor beta-activated kinase 1 is a sequential bimolecular process. *J Biol Chem* **285**, 25753-25766
30. Scholz, R., Suter, M., Weimann, T., Polge, C., Konarev, P. V., Thali, R. F., Tuerk, R. D., Viollet, B., Wallimann, T., Schlattner, U., and Neumann, D. (2009) Homo-oligomerization and activation of AMP-activated protein kinase are mediated by the kinase domain alphaG-helix. *J Biol Chem* **284**, 27425-27437
31. McMahon, R. J., and Frost, S. C. (1996) Glycogen: a carbohydrate source for GLUT-1 glycosylation during glucose deprivation of 3T3-L1 adipocytes. *Am J Physiol* **270**, E640-645
32. Kinsella, T. M., and Nolan, G. P. (1996) Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum Gene Ther* **7**, 1405-1413
33. Morgenstern, J. P., and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* **18**, 3587-3596
34. Voncken, J. W., Niessen, H., Neufeld, B., Rennefahrt, U., Dahlmans, V., Kubben, N., Holzer, B., Ludwig, S., and Rapp, U. R. (2005) MAPKAP kinase 3pK phosphorylates and regulates chromatin association of the polycomb group protein Bmi1. *J Biol Chem* **280**, 5178-5187
35. Mitchellhill, K. I., Mitchell, B. J., House, C. M., Stapleton, D., Dyck, J., Gamble, J., Ullrich, C., Witters, L. A., and Kemp, B. E. (1997) Posttranslational modifications of the 5'-AMP-activated protein kinase beta1 subunit. *J Biol Chem* **272**, 24475-24479
36. Tuerk, R. D., Auchli, Y., Thali, R. F., Scholz, R., Wallimann, T., Brunisholz, R. A., and Neumann, D. (2009) Tracking and quantification of 32P-labeled phosphopeptides in liquid chromatography matrix-assisted laser desorption/ionization mass spectrometry. *Anal Biochem* **390**, 141-148
37. Krieger, E., Koraimann, G., and Vriend, G. (2002) Increasing the precision of comparative models with YASARA NOVA--a self-parameterizing force field. *Proteins* **47**, 393-402
38. Singh, P. K., Singh, S., and Ganesh, S. (2012) The laforin-malin complex negatively regulates glycogen synthesis by modulating cellular glucose uptake via glucose transporters. *Mol Cell Biol* **32**, 652-663
39. Hashimoto, Y. K., Satoh, T., Okamoto, M., and Takemori, H. (2008) Importance of autophosphorylation at Ser186 in the A-loop of salt inducible kinase 1 for its sustained kinase activity. *J Cell Biochem* **104**, 1724-1739
40. Scott, J. W., Ling, N., Issa, S. M., Dite, T. A., O'Brien, M. T., Chen, Z. P., Galic, S., Langendorf, C. G., Steinberg, G. R., Kemp, B. E., and Oakhill, J. S. (2014) Small molecule drug A-769662 and AMP synergistically activate naive AMPK independent of upstream kinase signaling. *Chem Biol* **21**, 619-627
41. Feng, X., and Hannun, Y. A. (1998) An essential role for autophosphorylation in the dissociation of activated protein kinase C from the plasma membrane. *J Biol Chem* **273**, 26870-26874
42. Lorenz, K., Schmitt, J. P., Schmitteckert, E. M., and Lohse, M. J. (2009) A new type of ERK1/2 autophosphorylation causes cardiac hypertrophy. *Nat Med* **15**, 75-83
43. Vernia, S., Solaz-Fuster, M. C., Gimeno-Alcaniz, J. V., Rubio, T., Garcia-Haro, L., Foretz, M., de Cordoba, S. R., and Sanz, P. (2009) AMP-activated protein kinase phosphorylates R5/PTG, the glycogen targeting subunit of the R5/PTG-protein phosphatase 1 holoenzyme, and accelerates its down-regulation by the laforin-malin complex. *J Biol Chem* **284**, 8247-8255
44. Hiraga, A., and Cohen, P. (1986) Phosphorylation of the glycogen-binding subunit of protein phosphatase-1G by cyclic-AMP-dependent protein kinase promotes translocation of the phosphatase from glycogen to cytosol in rabbit skeletal muscle. *Eur J Biochem* **161**, 763-769
45. MacKintosh, C., Campbell, D. G., Hiraga, A., and Cohen, P. (1988) Phosphorylation of the glycogen-binding subunit of protein phosphatase-1G in response to adrenalin. *FEBS Lett* **234**, 189-194
46. Prats, C., Gomez-Cabello, A., and Hansen, A. V. (2011) Intracellular compartmentalization of skeletal muscle glycogen metabolism and insulin signalling. *Exp Physiol* **96**, 385-390
47. Han, G., Ye, M., Liu, H., Song, C., Sun, D., Wu, Y., Jiang, X., Chen, R., Wang, C., Wang, L., and Zou, H. (2010) Phosphoproteome analysis of human liver tissue by long-gradient nanoflow LC coupled with multiple stage MS analysis. *Electrophoresis* **31**, 1080-1089
48. Bian, Y., Song, C., Cheng, K., Dong, M., Wang, F., Huang, J., Sun, D., Wang, L., Ye, M., and Zou, H. (2014) An enzyme assisted RP-RPLC approach for in-depth analysis of human liver phosphoproteome. *J Proteomics* **96**, 253-262
49. Bouskila, M., Hirshman, M. F., Jensen, J., Goodyear, L. J., and Sakamoto, K. (2008) Insulin promotes glycogen synthesis in the absence of GSK3 phosphorylation in skeletal muscle. *Am J Physiol Endocrinol Metab* **294**, E28-35
50. Hayashi, T., Hirshman, M. F., Kurth, E. J., Winder, W. W., and Goodyear, L. J. (1998) Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* **47**, 1369-1373
51. Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J., and Winder, W. W. (1999) 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* **48**, 1667-1671

52. Chen, Z., Heierhorst, J., Mann, R. J., Mitchelhill, K. I., Michell, B. J., Witters, L. A., Lynch, G. S., Kemp, B. E., and Stapleton, D. (1999) Expression of the AMP-activated protein kinase beta1 and beta2 subunits in skeletal muscle. *FEBS Lett* **460**, 343-348
53. Sakoda, H., Fujishiro, M., Fujio, J., Shojima, N., Ogihara, T., Kushiya, A., Fukushima, Y., Anai, M., Ono, H., Kikuchi, M., Horike, N., Viana, A. Y., Uchijima, Y., Kurihara, H., and Asano, T. (2005) Glycogen debranching enzyme association with beta-subunit regulates AMP-activated protein kinase activity. *Am J Physiol Endocrinol Metab* **289**, E474-481
54. Hunter, R. W., Treebak, J. T., Wojtaszewski, J. F., and Sakamoto, K. (2011) Molecular mechanism by which AMP-activated protein kinase activation promotes glycogen accumulation in muscle. *Diabetes* **60**, 766-774
55. Carling, D., and Hardie, D. G. (1989) The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta* **1012**, 81-86
56. Roma-Mateo, C., Solaz-Fuster Mdel, C., Gimeno-Alcaniz, J. V., Dukhande, V. V., Donderis, J., Worby, C. A., Marina, A., Criado, O., Koller, A., Rodriguez De Cordoba, S., Gentry, M. S., and Sanz, P. (2011) Laforin, a dual-specificity phosphatase involved in Lafora disease, is phosphorylated at Ser25 by AMP-activated protein kinase. *Biochem J* **439**, 265-275
57. McBride, A., Ghilagaber, S., Nikolaev, A., and Hardie, D. G. (2009) The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. *Cell Metab* **9**, 23-34
58. Li, X., Wang, L., Zhou, X. E., Ke, J., de Waal, P. W., Gu, X., Tan, M. H., Wang, D., Wu, D., Xu, H. E., and Melcher, K. (2014) Structural basis of AMPK regulation by adenine nucleotides and glycogen. *Cell Res*
59. Jensen, J., Rustad, P. I., Kolnes, A. J., and Lai, Y. C. (2011) The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise. *Front Physiol* **2**, 112

Chapter 3

The interaction between AMPK β 2 and the PP1-targeting subunit R6 is dynamically regulated by intracellular glycogen content

Yvonne Oligschlaeger, Marie Miglianico, Vivian Dahlmans, Carla Rubio-Villena, Dipanjan Chanda, Maria Adelaida Garcia-Gimeno, Yilin Liu, J. Willem Voncken, Joost J.F.P. Luiken, Jan F.C. Glatz, Pascual Sanz, Dietbert Neumann

Submitted

ABSTRACT

AMP-activated protein kinase (AMPK) was suggested to coordinate muscular glucose uptake and glycogen metabolism in response to metabolic stress. We previously showed that glucose deprivation induces autophosphorylation of AMPK β at threonine-148 (Thr-148), which prevents the binding of AMPK to glycogen. Furthermore, in MIN6 cells AMPK β 1 binds to R6 (PPP1R3D), a glycogen-targeting subunit of protein phosphatase 1 (PP1), thereby regulating the glucose-induced inactivation of AMPK. Here, we further investigated the interaction of R6 with AMPK β and the possible dependency on Thr-148 phosphorylation status. Yeast two-hybrid analyses and co-immunoprecipitation of the overexpressed proteins in HEK293T cells revealed that both AMPK β 1 and β 2 wild-type (WT) isoforms bind to R6. The interaction with R6 was stronger with the muscle-specific β 2-WT and required association with the substrate-binding motif of R6. When HEK293T cells or C2C12 myotubes were cultured in high-glucose medium, AMPK β 2-WT and R6 weakly interacted. In contrast, glycogen depletion significantly enhanced this protein interaction. Mutation of AMPK β 2 Thr-148 prevented the interaction with R6 irrespective of the intracellular glycogen content. Treatment with the AMPK activator oligomycin enhanced AMPK β 2/R6 interaction in conjunction with increased Thr-148 phosphorylation in cells grown in low glucose medium. These data are in accordance with R6 binding directly to AMPK β 2 when both proteins detach from the diminishing glycogen particle, which is simultaneous to increased AMPK β 2 Thr-148 autophosphorylation. Such model points to a possible control of AMPK by PP1-R6 upon glycogen depletion in muscle.

INTRODUCTION

Muscular tissue, in particular skeletal muscle, is an important site for glucose storage. It has become evident that glucose disposal into glycogen is essential for coordinated glucose homeostasis, as conditions limiting glycogen synthesis are associated with, for instance, hyperglycemia and insulin resistance (1). AMP-activated protein kinase (AMPK) is a metabolic energy sensor that mediates insulin-independent GLUT4 translocation to the plasma membrane resulting in increased glucose uptake (2). Therefore, AMPK activation could normalize blood glucose levels in type 2 diabetic patients. In response to various cellular stresses (*e.g.*, contraction, nutrient-deprivation), AMPK is activated and modulates downstream targets to induce catabolic, ATP-producing processes and inhibit anabolic, ATP-consuming processes thereby restoring energy homeostasis. AMPK consists of three subunits, the catalytic α subunit, and two regulatory β and γ subunits; the latter are essential for regulating AMPK activity, as well as subcellular localization. AMPK subunits occur in different isoforms (α 1, α 2; β 1, β 2; γ 1, γ 2, γ 3) partly showing a tissue specific expression pattern. Namely, β 2 is the predominant isoform found in heart and skeletal muscle (3,4).

AMPK is known to shuttle between the nucleus (5,6) and the cytoplasm (7,8), suggesting that AMPK exerts compartment-specific effects in order to monitor and coordinate complex cell biological processes. The β -subunit carbohydrate-binding module (CBM) allows AMPK to associate with glycogen (9,10), where it interacts with other glycogen-targeting proteins including glycogen synthase (GS) (10) and glycogen phosphorylase (GP) (9). Exercise-induced AMPK activation inactivates GS via Ser7 phosphorylation in skeletal muscle, thereby preventing the glycogen synthesis process from further consuming ATP under conditions of cellular stress. Notably, β 2 shows higher affinity for glycogen compared to β 1 (11).

The regulation of the activity status of localized AMPK is dependent on allosteric activation/repression and the action of upstream kinases capable of phosphorylating Thr-172 (*e.g.*, LKB1, TAK1, CaMKK2). Several protein phosphatases (*e.g.*, PP1, PP2A, PP2C) dephosphorylate Thr-172 thus leading to AMPK inactivation. PP1 is an important protein phosphatase in mammalian cells that is involved in proper coordination of glycogen metabolism by dephosphorylating target enzymes such as GS and GP. Recruitment of PP1 to its target substrates and to glycogen occurs by means of its glycogen-targeting proteins, such as PTG/R5 (PPP1R3C) (12) or R6 (PPP1R3D) (13,14). Insight into PP1-mediated AMPK regulation came from our previous study (15), which showed that R6 physically interacts with the AMPK β 1-subunit resulting in glucose-induced AMPK dephosphorylation by the PP1-R6 complex. Furthermore, we found that the CBM domain within the AMPK β 1 subunit is required for interaction with R6, as substitution of glycine-147 for arginine (G147R) resulted in total loss of AMPK-R6 interaction (15). These results were consistent with parallel studies in yeast showing that Snf1 is regulated by glucose, and that Gal83 via

its CBM interacts with Reg1, the orthologs of AMPK β and R6, respectively (16), thus pointing to an evolutionary conserved mechanism.

We recently reported that autophosphorylation of Thr-148 interferes with the recruitment of AMPK to glycogen, suggesting that AMPK-glycogen localization is tightly regulated and linked to glycogen storage (17). Given the direct binding of AMPK β 1 to R6 and the observed loss of this interaction in the AMPK β 1-G147R mutant, we hypothesized a role for AMPK β Thr-148 phosphorylation. In this current study, we provide insight into the binding of AMPK β 2 to R6 in relation to glycogen content and Thr-148 phosphorylation. Our results indicate that the AMPK β 2-R6 interaction is dynamically controlled by glycogen content.

EXPERIMENTAL PROCEDURES

Plasmids - For expression in mammalian cells, the cDNA of full-length AMPK β 1 and AMPK β 2 was amplified by PCR and ligated in frame into the pmCherry expression vector (Clontech) via EcoRI and SalI restriction sites, as previously described (17). mCherry-AMPK β 2 constructs bearing a threonine-to-aspartate mutation on residue 148 (T148D) was generated using the Quick-change site-directed mutagenesis kit (Stratagene). The pcDNA3 constructs for expression of AMPK γ 1 and myc-AMPK α 1 were kindly provided by Dr. D. Carling (Imperial College London, London, United Kingdom). The corresponding ORF of AMPK β 2-T148D mutant was subcloned into pBTM116 to allow for expression in yeast (LexA-AMPK β 2-T148D). The pFLAG-R6 construct for mammalian expression of R6-WT and R6-mutants (R6-RARA, -RAHA, -WDNAD and -WANNA) were generated as previously described, and the pFLAG was used as empty vector (EV) control (15,18).

Other plasmids used for yeast two-hybrid analyses were pGADT7- Φ (GAD, empty plasmid), pGADT7-R6 (GAD-R6), pBTM-R6 (LexA-R6), pBTM-R6-RARA (LexA-R6-RARA), pBTM-R6-RAHA (LexA-R6-RAHA), pBTM-R6-WANNA (LexA-R6-WANNA), pBTM-R6-WDNAD (LexA-R6-WDNAD), pGADT7-AMPK β 1 (GAD-AMPK β 1), pGADT7-AMPK β 2 (GAD-AMPK β 2), pBTM-AMPK β 1 (LexA-AMPK β 1), pBTM-AMPK β 2 (LexA-AMPK β 2) (15,18-20).

For retroviral overexpression and optimal detection of exogenous β 2, full-length β 2-WT was amplified using PCR primers containing an optimized tetra-cysteine sequence (FlAsH-tag; FLNCCPGCCMEP). First, the corresponding ORF was subcloned into the SalI and NotI site of the mammalian pmCherry- β 2 construct, replacing mCherry- β 2-WT with FlAsH- β 2-WT. Second, FlAsH- β 2-WT was amplified and subcloned into the EcoRI and SalI site of the retroviral pBabe-puromycin retroviral backbone (kindly provided by Dr. G. Nolan, Stanford University, CA).

For retroviral co-expression of R6, the corresponding ORF (R6-WT) was amplified by PCR and subcloned into the BstxI and SalI restriction sites of the pBabe-hygromycin retroviral backbone (kindly provided by Dr. G. Nolan, Stanford University, CA). The pBabe-hygromycin EV was used as control. Primer sequences are available from the author upon request. All of the constructs were verified by DNA sequencing.

Cell culture - The human embryonic kidney 293T cell line (HEK293T) was cultured in DMEM with high glucose (25 mM) (Gibco), supplemented with 10 % (v/v) heat-inactivated fetal calf serum (iFCS, Bodinco BV, Alkmaar, The Netherlands) and penicillin/streptomycin (Invitrogen), unless otherwise stated. For transient transfections, HEK293T cells were seeded to 30 % confluence in 6-well plates (Greiner Bio-One) 24 h before transfection. For determination of protein-protein interaction, cells were co-transfected with AMPK (α 1-myc, γ 1, and mCherry- β 1-WT or mCherry- β 2-WT/ β 2-T148D), and/or FLAG-R6 (R6-WT/R6-RARA/R6-RAHA/R6-WANNA/ R6-WDNAD) or FLAG empty vector (EV) plasmid DNA using Lipofectamine 2000 (Invitrogen) in antibiotic-free culture medium. Six to eight hours after transfection, transfection medium was replaced by normal growth medium. At 48-72 h after transfection and continuous culturing (*i.e.*, without change of medium), cells were harvested for glycogen or subjected to immunoprecipitation and Western blotting.

In order to induce glycogen depletion, HEK293T cells growing in high glucose medium (DMEM 25 mM glucose, 10 % iFCS), were subjected to forskolin treatment (100 μ M, Sigma), low glucose medium (DMEM 3 mM glucose, 10 % iFCS), or glucose-deprived medium (DMEM 0 mM, 10 % iFCS) for 16 h. To activate AMPK, cells were serum-starved in high- (DMEM 25 mM) or low-glucose (3 mM) medium for 16 h, and subsequently treated with oligomycin (3 μ M, Sigma) for 1 h.

The mouse skeletal muscle cell line C2C12 was kindly provided by Dr. R.C. Langen (Maastricht University, The Netherlands). C2C12 cells were continuously cultured to approximately 80 % confluence in DMEM with high glucose (25 mM) (Gibco), supplemented with 10 % (v/v) iFCS (Bodinco BV, Alkmaar, The Netherlands) and 1 % penicillin/streptomycin (Invitrogen). For differentiation into myotubes, myocytes (75 - 85 % confluence) were further grown in differentiation medium (DMEM 25 mM glucose (Gibco), supplemented with 2 % heat-inactivated horse serum (Invitrogen, Life Technologies) and 1 % penicillin/streptomycin (Invitrogen) for 4 - 5 days. Subsequently, cells were used in the corresponding experiments. In order to induce glycogen depletion, myotubes were maintained in low glucose medium (DMEM 3 mM glucose, supplemented with 2 % iFCS), for 16 h. In order to activate AMPK, myotubes were maintained in high or low glucose for 16 hours as described above, and subsequently treated with oligomycin (5 μ M, Sigma) for 30 min.

Retroviral infections - In order to study protein-protein interactions in C2C12 myotubes, growing cells were (co-)infected with AMPK β 2-WT and/or R6 retroviruses. Briefly, retroviral systems and Phoenix helper-free retrovirus producer cell lines were used as published before (21-23). Amphotropic retroviral supernatants were produced as previously described (17). Briefly, 24 - 48 h after calcium phosphate/DNA transfection of producer cells, supernatants were harvested, filtered (0.45 micron filters; Corning, Germany) and used for infection of C2C12 cells in presence of 4 μ g/ml polybrene (Sigma). For infections, cells were incubated with virus particles for 6 - 8 h and then allowed to recover for 48 h on fresh medium, before selection pressure was applied. Stably infected cells were selected 2 days post-infection with 4 μ g/ml puromycin for 36 - 48 h, and 7 days post-infection with 300 μ g/ml Hygromycin B for 10 days preceding experiments.

Immunoprecipitation and Western blotting - Immunoprecipitation procedures were performed as previously described (17). Briefly, exogenous myc-AMPK α 1 was immunoprecipitated using anti-myc-tag antibody (9B11, Cell Signaling Technology, Beverly, MA), and exogenous R6 was immunoprecipitated using anti-FLAG-tag antibody (F3165, Sigma), followed by incubation with protein G-Sepharose beads (GE Healthcare). Western blot analysis was carried out using the following primary antibodies: myc-tag, tAMPK α , AMPK β 1, AMPK β 2, phospho-AMPK-T172 (all from Cell Signaling Technology, Beverly, MA), FLAG-tag (F3165, Sigma), R6 (AP13440a, Abgent), and phospho-AMPK β 2-T148 (17). R6 was detected using FLAG-tag (F3165, Sigma), unless otherwise stated. Detection was performed according to its primary antibody using anti-rabbit (Cell Signaling Technology) and anti-mouse (Dako) horse-radish peroxidase (HRP)-conjugated secondary antibodies, followed by chemiluminescence.

In order to investigate the role of glycogen, myc-AMPK α 1 was immunoprecipitated using the anti-myc-tag antibody after the addition of the glycogen-mimic β -cyclodextrin (5 mM, Sigma) for 1 h at 4 °C. Subsequently, immune complexes were electrophoresed by SDS-PAGE and analyzed by Western blot analysis, as described above.

Biochemical intracellular glycogen measurement - Intracellular glycogen content was measured, as previously described (17). Briefly, HEK293T cells (non-transfected or transfected) or stably-infected C2C12 myotubes were lysed in potassium hydroxide (30 %) and boiled at 70 °C for 30 min. Subsequently, samples were cooled to 25 °C before sodium sulfate (6 %, w/v) and EtOH (99.5 %, v/v) were added at a 1:1:3 ratio. After thorough mixing, samples were rotated top-over-top at 4 °C for 30 - 60 min. The precipitate was collected by centrifugation at 5000 rpm (Eppendorf Centrifuge 5415 R) for 5 min at 4 °C. To hydrolyze glycogen, pellets were dissolved in 1 M HCl and boiled at 100 °C for 2 h. Samples were cooled before

neutralization using 2 M NaOH. Hydrolysates were used for glucose determination using a glucose (GO) assay kit (Sigma), according to the manufacturer's instructions.

Yeast two-hybrid (Y2H) analyses - Yeast two-hybrid analysis was performed as previously described (15,19). Briefly, interaction analysis using yeast THY-AP4 strain (*MATa, ura3, leu2, lexA::lacZ::trp1, lexA::HIS3, lexA::ADE2*) co-transformed with the indicated combination of plasmids (see above). Transformants were grown in selective 4 % glucose synthetic complete medium lacking the corresponding supplements to maintain selection for plasmids. The strength of the interaction was determined by measuring β -galactosidase activity in permeabilized yeast cells and expressed in Miller units. In all Y2H analyses similar protein levels were obtained from the expression constructs, as verified in the crude extracts from the different yeast transformants.

Statistical analysis - All bar graph data are presented as mean $\pm$ S.E.M., unless otherwise stated. Statistical differences were evaluated using unpaired Student's *t* test and statistical analysis software Prism 4 (GraphPad Software, Inc.). P values equal to or less than 0.05 were considered statistically significant.

RESULTS

R6 preferentially interacts with AMPK β 2

We previously demonstrated that R6 (PPP1R3D), one of the glycogen-targeting subunits of the protein phosphatase 1 (PP1), physically interacts with AMPK β 1 in MIN6 pancreatic β cells (15). To verify AMPK β 1/R6 interaction in HEK293T cells, we overexpressed myc-tagged AMPK heterotrimers (myc- α 1, mCherry- β 1, γ 1), together with FLAG-tagged R6 (FLAG-R6) or empty vector control (EV). Physical interaction between AMPK β 1 and R6 was assessed by immunoprecipitation (IP) of AMPK from cells that were routinely cultured under glucose-rich conditions (25 mM glucose) (**Fig. 1A**). Pull-down of myc-AMPK α 1 resulted in co-immunoprecipitation of both mCherry-AMPK β 1 (65 kD) and FLAG-R6, indicating formation of the heterotrimeric AMPK complex and the interaction with R6 (**Fig. 1A**). As expected, FLAG-R6 signal was absent in cells transfected with EV. Next, we investigated the interaction between muscle-specific AMPK β 2 heterotrimers (myc- α 1, mCherry- β 2, γ 1) and R6 (FLAG-R6) using co-transfection and immunoprecipitation in HEK293T. Again both proteins co-immunoprecipitated, thus pointing to occurrence of a physical interaction between R6 and AMPK β 2 (**Fig. 1B**). Interestingly, co-immunoprecipitation appeared stronger in the presence of the AMPK β 2 compared to β 1. These data were further confirmed by yeast-two-hybrid (Y2H) analyses: in **Fig. 1C** it is shown that R6 binds to both AMPK β 1 and β 2, while the interaction between AMPK β 2 and R6 is significantly stronger than the one between AMPK β 1 and R6. Combined, these findings suggest that R6 more tightly interacts with AMPK β 2 if compared to AMPK β 1.

Figure 1. R6 preferentially interacts with AMPK β 2. (**A and B**) HEK293T cells transiently overexpressed FLAG-R6 and heterotrimers of AMPK β 1 (myc- α 1, γ 1, mCherry-AMPK β 1) (**A**) or AMPK β 2 (myc- α 1, γ 1, mCherry-AMPK β 2) (**B**). FLAG empty vector (EV) was used as control. Cells were continuously cultured under high glucose (25 mM) conditions and harvested 56 h post-transfection. Interaction between AMPK β 1/2 and R6 was assessed by immunoprecipitating the heterotrimeric AMPK complex from 800 μ g of lysate using the anti-myc-tag antibody. Western blots were assessed using the indicated antibodies. Representative Western blots have been shown. (**C**) Yeast THY-AP4 strain (see Materials and Methods) was transformed with the indicated combination of plasmids. Transformants were grown in high glucose (4 % glucose) containing medium and protein interactions were estimated by measuring the β -galactosidase activity. Empty vector pGADT7 (GAD- Φ) served as control. Values correspond to means from at least 6 different transformants (bars indicate S.D.). * $p < 0.001$ vs. LexA-AMPK β 1-WT + GAD-R6.

R6 interaction with AMPK β 2 requires its substrate-binding motif

Recently, a number of functionally distinct protein domains have been identified in the R6 glycogen-targeting subunit of PP1 (18). R6 is composed of domains that mediate binding to carbohydrates (via its CBM), binding to the PP1 catalytic subunit (PP1c; RVXF motif), and to the glycogen metabolism-related substrates of PP1 (via the highly conserved WDNND motif). To investigate whether and which of these motifs is involved in the interaction between the AMPK β subunit and R6, we performed studies in HEK293T cells co-expressing AMPK β 2 complex (myc- α 1, mCherry- β 2, γ 1) with FLAG-R6, the latter carrying various mutations corresponding to its protein motifs (18) (**Fig. 2A, B**). We again immunoprecipitated myc-AMPK α 1 using a myc-directed antibody. Of note, endogenous R6 was not detected either in lysate or in immunoprecipitated material, thereby substantiating the need for FLAG-R6 overexpression and interaction between AMPK β 2 and R6 (**Fig. 2B**: EV condition). R6 carried various domain-specific mutations: in both R6-RARA and R6-RAHA mutants, the hydrophobic valine and phenylalanine residues within putative R6-RVXF motifs were substituted for alanine, allowing us to probe the role of the PP1-binding motif in AMPK β 2/R6-binding (**Fig. 2B**). R6-RARA, a mutant known to have lost its capacity to bind to endogenous PP1c but not to PP1-substrates (18), presented a similar AMPK β 2-binding profile compared to wild-type, non-mutated R6 (R6-WT; **Fig. 2B**; right panel). The R6-RAHA mutant that carried mutations in a domain close to the substrate-binding motif, however, had completely lost its ability to interact with AMPK (**Fig. 2B**; right panel). This data was nicely reproduced in a reciprocal Y2H assay using AMPK β 2 as bait (**Fig. 2C**). Further, the substrate-binding motif (WDNND) was mutated to study its effect on AMPK β 2/R6 interaction. In one of the mutants, the two aspartate residues present within the WDNND motif were replaced by alanine (R6-WANNA mutant), whereas in the R6-WDNAD mutant, the second asparagine residue was replaced by alanine. Whereas the R6-WDNAD mutant was still capable of interacting with AMPK β 2, the WANNA mutation abolished AMPK β 2 binding (**Fig. 2B** right panel, **C**). In good agreement, Y2H analyses using AMPK β 1 or AMPK β 2 as bait confirmed that the binding of R6 to the AMPK β subunits depended on the R6 substrate-binding motif being intact (**Fig. 2C and 2D**). Thus, we show that the AMPK β /R6 interaction requires the R6 substrate-binding motif. In all subsequent experiments, we concentrated on AMPK β 2/R6 interaction.

Figure 2. R6 interaction with AMPKβ2 requires its substrate-binding motif. (A) Schematic model indicating the PP1- and substrate binding sites of R6, and its corresponding mutations. **(B)** HEK293T cells transiently co-expressing AMPKβ2 heterotrimers (myc-α1, γ1, mCherry-AMPKβ2) and FLAG-R6 wild-type (R6-WT) or mutant (R6-RARA, R6-RAHA, R6-WANNA, R6-WDNAD, respectively) were continuously cultured under high glucose (25 mM) conditions and harvested 48 h post-transfection. FLAG empty vector (EV) was used as control. Interaction between AMPKβ2 and R6 was assessed by immunoprecipitating the heterotrimeric AMPK complex from 450 μg of lysate using the anti-myc-tag antibody. Western blots were assessed using the indicated antibodies. Representative Western blots have been shown. **(C and D)** Yeast THY-AP4 strain (see Materials and Methods) was transformed with GAD-AMPKβ2 **(C)** or GAD-AMPKβ1 **(D)** plasmids and LexA-R6 plasmids as indicated. Transformants were grown in high glucose (4 % glucose) containing medium and protein interactions were estimated by measuring the β-galactosidase activity. Values correspond to means from at least 6 different transformants (bars indicate S.D.). * p<0.05 and ** p<0.001 vs. LexA-R6-WT.

AMPK β 2 Thr-148 mutant shows reduced interaction with R6

Previously, we showed that substitution of the glycine-147 residue for arginine (G147R) within the β 1-CBM domain abolished the interaction of AMPK with R6 (15), indicating that AMPK β 1 requires the CBM for interaction with R6. More recently, we demonstrated that autophosphorylation at the β -threonine-148 (T148) residue, centrally located in the CBM, prevents AMPK from binding to carbohydrates such as glycogen (17). As we show here that R6 also interacts with AMPK β 2, we next investigated whether Thr-148 is required for AMPK β 2/R6 interaction. To this end, a phospho-mimicking T148D AMPK β 2 mutant was generated. Transiently transfected HEK293T cells co-expressing FLAG-R6 and AMPK heterotrimers (myc- α 1 and γ 1 in combination with either mCherry- β 2-WT or mCherry- β 2-T148D), were cultured under high glucose conditions (25 mM) and used for co-immunoprecipitation analysis. Impaired interaction between AMPK β 2 and R6 was observed in cells overexpressing mutant AMPK β 2-T148D (**Fig. 3A**; right panel); relevantly, FLAG-R6 and mCherry- β 2 were expressed at comparable levels (**Fig. 3A**; left panel). Independent Y2H analyses corroborated these findings: T148D mutation decreased the interaction between AMPK β 2 and R6 (**Fig. 3B**). Taken together, our data indicate that Thr-148 mutation into Asp results in loss of AMPK β 2/R6 interaction, suggesting that an intact Thr-148 residue is essential for the formation of the AMPK β 2/R6 complex under glucose-rich culturing conditions.

Figure 3. AMPK β 2 Thr-148 mutant shows reduced interaction with R6. **(A)** HEK293T cells transiently transfected with myc- α 1, γ 1 and mCherry-AMPK β 2 wild-type (WT) or Thr-148 mutant (T148D) were co-transfected either with FLAG-R6 (+), or FLAG empty vector (-) as control. Cells were cultured under high glucose (25 mM) conditions, and harvested 56 h post-transfection. Interaction between AMPK β 2 and R6 was assessed by immunoprecipitating the heterotrimeric AMPK complex from 800 μ g of lysate using the anti-myc-tag antibody. Western blots were assessed using the indicated antibodies. Representative Western blots have been shown. **(B)** Yeast THY-AP4 strain was transformed with plasmids LexA-AMPK β 2-WT or LexA-AMPK β 2-T148D and GAD-R6 as indicated. Transformants were grown in high glucose (4 % glucose) containing medium and protein interactions were estimated by measuring the β -Galactosidase activity. Empty vector pGADT7 (GAD- Φ) served as control. Values correspond to means from at least 6 different transformants (bars indicate S.D.). * $p < 0.001$ vs. LexA-AMPK β 2-WT + GAD-R6.

Glycogen negatively regulates the interaction between AMPK β 2 and R6

To investigate whether the interaction between AMPK β 2 and R6 is responsive to variation in glycogen level, we aimed to deplete cellular glycogen content. Hence, HEK293T cells were either pharmacologically treated with forskolin, a compound inducing glycogen breakdown (24), or glucose-deprived, after which glycogen content was assessed. Because R6 is known to have glycogenic properties (19), we included non-transfected control cells and compared these with cells that were either transfected with FLAG-R6 alone, or co-transfected with all three subunits of AMPK (myc- α 1, mCherry- β 2-WT, γ 1).

As expected, cells overexpressing FLAG-R6 showed high glycogen content (both in the absence and presence of co-overexpression of AMPK β 2): basal glycogen levels were 30-40-fold increased compared to non-transfected control cells (**Fig. 4A**). Both forskolin-treatment and glucose-deprivation significantly lowered the intracellular glycogen levels in all cell lines, as compared to control (**Fig. 4A**). To assess the effect of cellular glycogen content on AMPK β 2/R6 interaction, HEK293T cells overexpressing both FLAG-R6 and AMPK β 2 heterotrimers were either treated with forskolin or glucose-deprived for 16 h. Reduction of cellular glycogen content, either by forskolin or glucose-deprivation, substantially enhanced the interaction between AMPK β 2 and R6 (**Fig. 4B**; right panel); as total levels of AMPK and R6 did not change within the experimental time frame (**Fig. 4B**; left panel), this ruled out any possible interference by altered expression. Collectively, our data suggest that the interaction between AMPK β 2 heterotrimers and R6 is inversely correlated to cellular glycogen level.

To independently examine a possible direct effect of glycogen on the AMPK/R6 binding, we next used β -cyclodextrin (β -CD), a model-sugar that mimics glycogen, in competitive binding-immunoprecipitation experiments, in HEK293T cells cultured under high glucose. Addition of β -CD completely disrupted the interaction between AMPK β 2 and R6 (**Fig. 4C**; right panel). Combined, this data suggests that under glucose-rich conditions glycogen disturbs AMPK β 2/R6 interaction, indicating that glycogen interferes with and therefore weakens the interaction between AMPK β 2 and R6.

Figure 4. Glycogen negatively regulates the interaction between AMPK β 2 and R6. (A and B) HEK293T cells either non-transfected, transiently transfected with FLAG-R6 alone, or co-transfected with FLAG-R6, myc- α 1, γ 1 and mCherry-AMPK β 2-WT were cultured under high glucose (25 mM) conditions. Prior to lysis, cells were treated with forskolin (100 μ M) or DMSO (control) under high glucose conditions, or cells were glucose-deprived (GD, 0 mM) for 16 h. (A) Intracellular glycogen level was determined as described in the section Materials and Methods. Levels were corrected for corresponding protein concentrations. * $p < 0.05$ vs. corresponding DMSO controls; $n = 2$. (B) Heterotrimeric AMPK was immunoprecipitated from 500 μ g of lysate using the anti-myc-tag antibody, followed by Western blot analysis using the indicated antibodies. (C) HEK293T cells were transiently co-transfected as indicated and harvested 56 h post-transfection. Cells were continuously grown under high glucose (25 mM) conditions. β -cyclodextrin (β -CD) was added prior to immunoprecipitation. Representative Western blots have been shown.

AMPK β 2-R6 interaction is enhanced in conjunction with increased AMPK β 2 Thr-148 phosphorylation

Next, we assessed the impact of Thr-148 phosphorylation on the glycogen-modulated AMPK/R6 interaction. To this end, we co-transfected HEK293T cells with AMPK heterotrimers (myc- α 1, γ 1, and either mCherry- β 2-WT or -T148D mutant) and FLAG-R6 and challenged the cells for 16 h with various concentrations of glucose. The expression of AMPK β 2-T148D mutant did not affect the glycogenic activity of FLAG-R6 in cells growing in high glucose conditions (Fig. 5A), indicating that glycogen production depends on the function of R6 and not on the interaction between R6 and AMPK β 2. Reduced availability of glucose (3 mM or 0 mM) correlated with significantly decreased intracellular levels of glycogen (Fig. 5A) in both AMPK β 2-WT and -T148D expressing cells (Fig. 5A). Interestingly, we observed increased level of Thr-148 phosphorylation in β 2-WT immunoprecipitates upon lowering glucose conditions, although we did not find changes in the levels of AMPK Thr-172 phosphorylation, neither in cellular lysates nor in the precipitates from β 2-WT or β 2-T148D expressing cells (Fig. 5B). Low glycogen content enhanced AMPK β 2/R6 interaction (Fig. 5B; cf. Fig. 4). Whereas AMPK β 2-WT/R6 binding was readily detectable, T148D mutation completely abolished AMPK β 2 interaction with R6 (Fig. 5B; cf. Fig. 3). To dissociate

AMPK from glycogen via Thr-148 phosphorylation and study the effect thereof on AMPK β 2/R6 interaction, we next exposed cells to the AMPK activator oligomycin in the context of high and low cellular glycogen. If glycogen content was low (*i.e.*, 3 mM glucose), oligomycin activated AMPK (Thr-172 phosphorylation; **Fig. 5C**), an effect that was overruled by high cellular glycogen content (*i.e.*, 25 mM glucose). Oligomycin-induced activation of AMPK and the consequential Thr-148 phosphorylation further enhanced the interaction of AMPK β 2-WT and R6 (**Fig. 5C**; right panel) under low glycogen conditions. This interaction was disrupted by high cellular glycogen or by T148D mutation (**Fig. 5C**). These results indicate that glycogen-depletion enhances both AMPK β 2/R6 interaction and phosphorylation of Thr-148. These results also indicate that physiological phosphorylation of AMPK β 2 at Thr-148 has a different outcome from AMPK β 2-T148D mutant: whereas the AMPK β 2-T148D mutant is not able to interact with R6, this interaction is improved under physiological conditions that enhance AMPK β 2 Thr-148 phosphorylation (**Fig. 5C**).

Figure 5. AMPK β 2/R6 interaction is enhanced in conjunction with increased AMPK β 2 Thr-148 phosphorylation. (A and B) HEK293T cells co-expressed AMPK β 2 heterotrimer (myc- α 1, γ 1, mCherry-AMPK β 2-WT or -T148D) and FLAG-R6. Prior to lysis, cells were cultured under various glucose conditions (25 mM, 3 mM, 0 mM) for 16 h. (A) Intracellular glycogen levels were biochemically determined and corrected for the corresponding protein concentrations. * $p < 0.01$ vs. WT 25 mM, # $p < 0.01$ vs. T148D 25 mM, ## $p < 0.001$ vs. T148D 25 mM; $n = 2$. (B) Heterotrimeric AMPK was immunoprecipitated from 350 μ g of lysate using the anti-myc-tag antibody, followed by Western blot analysis using the indicated antibodies. (C) Cells were serum-starved both under low (3 mM) and high (25 mM) glucose conditions for 16 h, prior to 1 h treatment with oligomycin (3 μ M) or DMSO (control). AMPK was immunoprecipitated from 500 μ g of lysate as described in panel B, followed by Western blot analysis using the indicated antibodies. Representative Western blots have been shown.

AMPK β 2 shows enhanced interaction with R6 upon glycogen depletion in C2C12 myotubes

Furthermore, we explored the significance of cellular glycogen with respect to AMPK β 2/R6 interaction in C2C12 myotubes, a more physiologically relevant model. Since endogenous R6 was below detection level in C2C12 cells (data not shown), we co-expressed AMPK β 2-WT and FLAG-R6 in C2C12 myoblasts (**Fig. 6A, B**). C2C12 cells were differentiated to myotubes and cultured under high (25 mM) or low (3 mM) glucose concentrations prior to oligomycin-treatment. Lowered glucose availability significantly decreased the myocellular glycogen content in cells expressing β 2-WT (**Fig. 6A**). As expected, R6 overexpression enhanced the levels of glycogen under basal and treated conditions (**Fig. 6A**). Oligomycin clearly induced AMPK activation in total cell lysates, under both high and low glucose conditions (**Fig. 6B**, left panel). However, as in HEK293T cells, oligomycin-mediated AMPK activation could only be detected in immunoprecipitates from cells that were cultured under low glycogen conditions (**Fig. 6B**; right panel). As expected, increases in interaction of AMPK β 2/R6 were detected after immunoprecipitating FLAG-R6 from cells treated with oligomycin under low glycogen conditions (**Fig. 6B**; right panel). Taken together, in good agreement with our observations in HEK293 cells, these data support the idea that cellular glycogen content controls AMPK β 2/R6 binding.

Figure 6. AMPK β 2 shows enhanced interaction with R6 upon glycogen depletion in C2C12 myotubes.

(A and B) C2C12 cells stably overexpressing both FLAG-R6 and wild-type AMPK β 2 (β 2-WT) were differentiated into myotubes for 4-5 days, and subsequently incubated with high (25 mM) or low (3 mM) glucose for 16 h, followed by 30 minutes treatment with oligomycin (5 μ M) or DMSO (control). Empty vector FLAG (EV) was used as control. **(A)** Myocellular glycogen levels were determined using biochemical glycogen assay, as described in Materials and Methods. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 3$. **(B)** Interaction between AMPK β 2 and R6 was assessed by immunoprecipitation using the anti-FLAG-tag antibody, followed by Western blot analysis using the indicated antibodies. Representative Western blots have been shown.

DISCUSSION

We recently showed that activation of AMPK precedes AMPK β Thr-148 autophosphorylation to preclude the AMPK complex from binding to glycogen (17). Further data suggested a possible role for Thr-148 phosphorylation in glycogen turnover. R6 is a known glycogen-binding protein and PP1-targeting subunit acting as a glycogenic driver (19). In the current study, we demonstrate the involvement of Thr-148 in the dynamic AMPK β 2/R6 interaction that is governed by glycogen content.

R6 recruits PP1 phosphatase to its substrates (*e.g.*, GS and GP) (18), thus playing a critical role in the regulation of glycogen metabolism. Most of the PP1-glycogen-targeting subunits exert their actions through binding to PP1 via a conserved N-terminal PP1-binding motif (RVXF), and interact with PP1-substrates via a conserved C-terminal substrate-binding (WXXGNYX(L/I)) motif (18). We have recently shown that R6 does utilize this conserved region to bind to its glycogenic substrates GS and GP (18). In line with these results, our work indicates that AMPK β /R6 interaction occurs also via the R6 substrate-binding motif (**Fig. 2**), as a mutation in this domain resulted in loss of interaction, a process that is independent of PP1 binding. Therefore, these results may suggest that R6 acts as a scaffold to bring AMPK β 2 in close proximity to the PP1 phosphatase, for instance, under conditions that require AMPK dephosphorylation and inactivation. However, we did not find evidence for R6/PP1-dependent AMPK dephosphorylation in our study under conditions where AMPK β 2/R6 interaction was enhanced. This may be attributed to the use of glycogen depletion and mitochondrial poisoning as triggers. Although we find these treatments as required to induce significant AMPK β 2/R6 interaction, their application necessarily goes along with elevated AMP levels and consequent protection from Thr-172 dephosphorylation (25).

In this work, we also aimed at better understanding the role of AMPK β 2 Thr-148 phosphorylation in glycogen metabolism. In accordance with earlier findings on AMPK β 1 (15), we show that AMPK β 2/R6 interaction is markedly increased under low glucose conditions, which correlated with decreased glycogen levels. Strong AMPK β 2 Thr-148 phosphorylation signals were only detected when glycogen levels were low. In this condition, oligomycin further enhanced the interaction between AMPK β 2 and R6, which augmented the phosphorylation of Thr-148. Therefore, Thr-148 phosphorylation is compatible with binding to R6 and even the interaction is enhanced in conjunction with this post-translational modification. In agreement with our earlier work, Thr-148 phosphorylation occurred if glycogen pools were diminishing. Thus, the autophosphorylation of Thr-148 by AMPK may be understood as a marker indicating detachment of AMPK from glycogen in conditions that challenge cellular glycogen level. Notably, in conjunction with lowered glycogen content, oligomycin-induced Thr-172 phosphorylation was higher. Therefore, consistent with our results obtained in HEK293T cells and C2C12 myotubes, glycogen may be interpreted as a storage site for inactive AMPK, which is in agreement with an

earlier study that suggested AMPK inhibition by binding to glycogen branch points (26).

Similar to the observations in yeast (27), the equivalent AMPK β 1-G147R mutation impaired the ability to interact with R6 (15). Since AMPK β Thr-148 is located adjacent to Gly-147, one would expect a similar loss of binding to R6 for Thr-148 mutant. In agreement, we report here that AMPK β 2/R6 interaction was completely lost in the AMPK β 2-T148D mutant. In our previous study, we found that T148D mutant did not bind glycogen (17). Nevertheless, the molecular mechanism of explaining the loss of binding to R6 as a result of T148D mutation remains unclear, since Thr-148 phosphorylation occurs in conjunction with R6 interaction. There are at least two possible explanations: (i) the T148D mutation causes a subtle modification in the conformation of the β 2 subunit resulting in the loss of interaction with R6, a process not shared by the physiological phosphorylation of this subunit; or (ii) the interaction of AMPK β 2-WT with R6 that is observed during glycogen degradation evolves from their initial binding at glycogen. While we formally cannot rule out the former, there are arguments in support of the latter notion. Firstly, both AMPK β 2 and R6 bind to glycogen via their respective CBM (9,28) and weakly interact under conditions of high glycogen content. This interaction might be indirect, *i.e.*, both proteins bind to glycogen independent of each other. It could be anticipated that localization of AMPK and R6 to glycogen would bring these proteins in closer proximity to each other and thus allow them to interact in a direct manner as a second step. This is indeed in accordance with our findings showing that depletion of intracellular glycogen resulted in enhanced AMPK β 2/R6 interaction. The treatment of AMPK β 2/R6 co-immunoprecipitations with β -CD accordingly showed that the AMPK β 2/R6 interaction was completely abolished under high intracellular glycogen content (**Fig. 4C**), whereas the interaction remained more stable if immunoprecipitations were performed from glucose-deprived cells (**Fig. 4B**). Similarly, in Y2H analyses, low glucose media augmented the AMPK β 1/R6 interaction (29). One step further, high glycogen content was found to decrease AMPK β 2/R6 interaction and thus glycogen may in fact rather perturb the direct binding between AMPK β 2 and R6. Although our experiments provide proof-of-principle for the inhibitory role of glycogen in AMPK β /R6 interaction, the exact details, *i.e.*, whether β -CD scavenges AMPK or R6 or both, are unknown. Yet, it might not be surprising if R6 prefers binding to glycogen over AMPK β 2 to further promote glycogenic activity, since the R6-substrate-binding motif partially overlaps with the CBM domain (18). Therefore, these data support the idea that both AMPK β 2 and R6, independently, are capable of binding to glycogen, thus facilitating the formation of multi-protein-glycogen complexes (30). The alternative direct binding mode is triggered by lowered glycogen content as further discussed below.

AMPK plays an essential role in various biological processes involved in restoring cellular energy homeostasis. Tuning of such processes requires adequate regulation, and involves AMPK compartmentalization and complex formation with

other proteins and substrates. For example, AMPK controls glycogen metabolism, as it is known to phosphorylate and inactivate GS *in vitro* and *in vivo* (31,32). In yeast, it was reported earlier that Gal83 (Snf1 β -subunit ortholog) is involved in binding to Reg1 (PP1 glycogenic subunit ortholog) and studies in mouse pancreatic β cells revealed that AMPK β 1 interacts with the PP1-glycogen targeting subunit R6 (15,33). In the present study, we show that AMPK β 2 interacts more strongly with R6 if compared to AMPK β 1, pointing to a possible role for this interaction in skeletal muscle. The CBM domain of AMPK β 2 possesses higher binding affinity for carbohydrates such as glycogen than β 1 (11). In a more detailed study the CBM domain of AMPK β 2 bound linear carbohydrates and single α 1,6-branched carbohydrates 4-30 fold tighter in comparison with β 1 (34). These data suggest that the AMPK β 2 isoform maybe more important for coordination of glycogen storage and breakdown. Furthermore, given that AMPK β 2, as well as R6, are highly expressed in skeletal muscle (3,35), a tissue active in controlling disposal of glucose into glycogen (36), it is likely that these proteins contribute to myocellular glycogen turnover. The C2C12 murine cell line is widely used as an *in vitro* model of skeletal muscle (37), and we used these cells upon differentiation into muscle-like myotubes to investigate the dynamic interaction between AMPK β 2 and R6. Consistent with the findings of Bujak *et al.* that prolonged fasting induces the breakdown of skeletal muscle glycogen content (38), we demonstrate the reduction in myocellular glycogen levels as a result of glucose deprivation (**Fig. 6A**). Although we are currently lacking a direct proof, this breakdown of glycogen may cause AMPK β 2 and R6 to leave glycogen, as was reported for GS (39,40). The possible mechanisms causing AMPK β 2 and R6 to leave the dwindling glycogen particle may include protein phosphorylation, such as Thr-148 phosphorylation, and the decreasing surface binding options. On a diminishing glycogen granule, we can indeed expect the concentration of potential interaction partners to increase dramatically, which may facilitate a direct binding between these proteins concomitant with their detachment from glycogen. In support of such notion, oligomycin-induced AMPK activation augments AMPK β 2/R6 interaction in skeletal muscle myotubes while further depleting glycogen and inducing Thr-148 phosphorylation (**Fig. 6A**), suggesting that these events are linked. In summary, we describe for the first time that R6 physically interacts with AMPK β 2 in C2C12 skeletal muscle myotubes, which is markedly enhanced if glycogen content is low. Vice versa, high availability of extracellular glucose and thus high intracellular glycogen are negative regulators of the direct AMPK β 2/R6 interaction. Given these findings, one would expect that dysregulation of AMPK β 2/R6 interaction may be linked to metabolic disorders such as type 2 diabetes or glycogen storage diseases, in which the glycogenic pathway is either impaired or hyper-activated, respectively.

Altogether, we envisage the following sequence of events: high glucose availability (**Fig. 7A**), mimicking an *in vivo* 'fed' state, maintains cellular glycogen stores. Glycogen-binding proteins such as AMPK β 2 heterotrimers and R6 associate with glycogen, thereby forming multi-protein-glycogen complexes. AMPK β 2-T148D

remains cytosolic, and is thus prevented from ‘indirectly’ interacting with R6. Upon nutrient deprivation (**Fig. 7B**), mimicking an *in vivo* ‘fasted’ state, the AMP:ATP ratio rises, and the cellular glycogen level decreases. In response, AMPK β 2 heterotrimers and R6 are increasingly concentrated at the surface of the glycogen particle until forced to release and simultaneously engage in a direct interaction. Under these conditions, AMPK is turned into an active state, and due to an AMP-mediated conformational change, it is further prevented from dephosphorylation by protein phosphatases such as PP1 (25). In addition, Thr-148 autophosphorylation prevents AMPK from returning to glycogen. Since the T148D mutant is not present at the glycogen particle during the departure of R6, these proteins are less likely to bind and form complexes. In response to glucose replenishment (**Fig. 7C**), mimicking an *in vivo* ‘re-fed’ state, ATP-binding makes AMPK a better substrate for PP1-R6 mediated dephosphorylation. Thus, increases in intracellular glycogen reinforce the formation of a multi-protein-glycogen complex. The model provides with a possible explanation for AMPK β 2/R6 interaction in situations of low energy, where AMPK requires additional surveillance by PP1 to switch-off once cellular ATP is restored. Despite the fact that this model is supported by the presented results and experimental data from various studies, we admit that further investigation is required to advance it into solid knowledge. For instance, the molecular interplay of AMPK and PP1-R6 as yet has not been addressed in cell free assays, which would allow for defined adenine nucleotide levels, and suitable genetic mouse models are lacking to further study AMPK functions in relation to PP1-R6 in tissues and the whole body context.

Figure 7. Myocellular glycogen content dynamically regulates AMPKβ2-R6 interaction. (A) Regulation by high glucose availability/glycogen content, mimicking an *in vivo* ‘fed’ state. **(B)** Regulation by low glucose availability/glycogen content, mimicking an *in vivo* ‘fasted’ state. **(C)** Regulation by glucose re-availability/glycogen replenishment, mimicking an *in vivo* ‘re-fed’ state. See main text for details.

REFERENCES

1. Jornayvaz, F. R., Samuel, V. T., and Shulman, G. I. (2010) The role of muscle insulin resistance in the pathogenesis of atherogenic dyslipidemia and nonalcoholic fatty liver disease associated with the metabolic syndrome. *Annu Rev Nutr* **30**, 273-290
2. Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J., and Winder, W. W. (1999) 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* **48**, 1667-1671
3. Thornton, C., Snowden, M. A., and Carling, D. (1998) Identification of a novel AMP-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle. *J Biol Chem* **273**, 12443-12450
4. Chen, Z., Heierhorst, J., Mann, R. J., Mitchelhill, K. I., Mitchell, B. J., Witters, L. A., Lynch, G. S., Kemp, B. E., and Stapleton, D. (1999) Expression of the AMP-activated protein kinase beta1 and beta2 subunits in skeletal muscle. *FEBS Lett* **460**, 343-348
5. McGee, S. L., Howlett, K. F., Starkie, R. L., Cameron-Smith, D., Kemp, B. E., and Hargreaves, M. (2003) Exercise increases nuclear AMPK alpha2 in human skeletal muscle. *Diabetes* **52**, 926-928
6. Kodiha, M., Rassi, J. G., Brown, C. M., and Stochaj, U. (2007) Localization of AMP kinase is regulated by stress, cell density, and signaling through the MEK-->ERK1/2 pathway. *Am J Physiol Cell Physiol* **293**, C1427-1436
7. Carling, D., Clarke, P. R., Zammit, V. A., and Hardie, D. G. (1989) Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. *Eur J Biochem* **186**, 129-136
8. Kazgan, N., Williams, T., Forsberg, L. J., and Brenman, J. E. (2010) Identification of a nuclear export signal in the catalytic subunit of AMP-activated protein kinase. *Mol Biol Cell* **21**, 3433-3442
9. Polekhina, G., Gupta, A., Mitchell, B. J., van Denderen, B., Murthy, S., Feil, S. C., Jennings, I. G., Campbell, D. J., Witters, L. A., Parker, M. W., Kemp, B. E., and Stapleton, D. (2003) AMPK β Subunit Targets Metabolic Stress Sensing to Glycogen. *Current Biology* **13**, 867-871
10. Hudson, E. R., Pan, D. A., James, J., Lucocq, J. M., Hawley, S. A., Green, K. A., Baba, O., Terashima, T., and Hardie, D. G. (2003) A Novel Domain in AMP-Activated Protein Kinase Causes Glycogen Storage Bodies Similar to Those Seen in Hereditary Cardiac Arrhythmias. *Current Biology* **13**, 861-866
11. Koay, A., Woodcroft, B., Petrie, E. J., Yue, H., Emanuelle, S., Bieri, M., Bailey, M. F., Hargreaves, M., Park, J. T., Park, K. H., Ralph, S., Neumann, D., Stapleton, D., and Gooley, P. R. (2010) AMPK beta subunits display isoform specific affinities for carbohydrates. *FEBS Lett* **584**, 3499-3503
12. Fong, N. M., Jensen, T. C., Shah, A. S., Parekh, N. N., Saltiel, A. R., and Brady, M. J. (2000) Identification of binding sites on protein targeting to glycogen for enzymes of glycogen metabolism. *J Biol Chem* **275**, 35034-35039
13. Heroes, E., Lesage, B., Gornemann, J., Beullens, M., Van Meervelt, L., and Bollen, M. (2013) The PP1 binding code: a molecular-lego strategy that governs specificity. *FEBS J* **280**, 584-595
14. Armstrong, C. G., Browne, G. J., Cohen, P., and Cohen, P. T. W. (1997) PPP1R6, a novel member of the family of glycogen-targeting subunits of protein phosphatase 1. *FEBS Letters* **418**, 210-214
15. Garcia-Haro, L., Garcia-Gimeno, M. A., Neumann, D., Beullens, M., Bollen, M., and Sanz, P. (2010) The PP1-R6 protein phosphatase holoenzyme is involved in the glucose-induced dephosphorylation and inactivation of AMP-activated protein kinase, a key regulator of insulin secretion, in MIN6 beta cells. *FASEB J* **24**, 5080-5091
16. Sanz, P., Alms, G. R., Haystead, T. A., and Carlson, M. (2000) Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. *Mol Cell Biol* **20**, 1321-1328
17. Oligschlaeger, Y., Miglianico, M., Chanda, D., Scholz, R., Thali, R. F., Tuerk, R., Stapleton, D. I., Gooley, P. R., and Neumann, D. (2015) The Recruitment of AMP-activated Protein Kinase to Glycogen Is Regulated by Autophosphorylation. *J Biol Chem* **290**, 11715-11728
18. Rubio-Villena, C., Sanz, P., and Garcia-Gimeno, M. A. (2015) Structure-Function Analysis of PPP1R3D, a Protein Phosphatase 1 Targeting Subunit, Reveals a Binding Motif for 14-3-3 Proteins which Regulates its Glycogenic Properties. *PLoS One* **10**, e0131476
19. Rubio-Villena, C., Garcia-Gimeno, M. A., and Sanz, P. (2013) Glycogenic activity of R6, a protein phosphatase 1 regulatory subunit, is modulated by the laforin-malin complex. *Int J Biochem Cell Biol* **45**, 1479-1488
20. Gimeno-Alcaniz, J. V., and Sanz, P. (2003) Glucose and type 2A protein phosphatase regulate the interaction between catalytic and regulatory subunits of AMP-activated protein kinase. *J Mol Biol* **333**, 201-209
21. Kinsella, T. M., and Nolan, G. P. (1996) Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum Gene Ther* **7**, 1405-1413
22. Morgenstern, J. P., and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* **18**, 3587-3596
23. Voncken, J. W., Niessen, H., Neufeld, B., Rennefahrt, U., Dahlmans, V., Kubben, N., Holzer, B., Ludwig, S., and Rapp, U. R. (2005) MAPKAP kinase 3pK phosphorylates and regulates chromatin association of the polycomb group protein Bmi1. *J Biol Chem* **280**, 5178-5187
24. Singh, P. K., Singh, S., and Ganesh, S. (2012) The laforin-malin complex negatively regulates glycogen synthesis by modulating cellular glucose uptake via glucose transporters. *Mol Cell Biol* **32**, 652-663
25. Suter, M., Riek, U., Tuerk, R., Schlattner, U., Wallimann, T., and Neumann, D. (2006) Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. *J Biol Chem* **281**, 32207-32216
26. McBride, A., Ghilagaber, S., Nikolaev, A., and Hardie, D. G. (2009) The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. *Cell Metab* **9**, 23-34
27. Momcilovic, M., Iram, S. H., Liu, Y., and Carlson, M. (2008) Roles of the glycogen-binding domain and Snf4 in glucose inhibition of SNF1 protein kinase. *J Biol Chem* **283**, 19521-19529

28. Machovic, M., and Janecek, S. (2006) Starch-binding domains in the post-genome era. *Cell Mol Life Sci* **63**, 2710-2724
29. Garcia-Haro, L., Garcia-Gimeno, M. A., Neumann, D., Beullens, M., Bollen, M., and Sanz, P. (2012) Glucose-dependent regulation of AMP-activated protein kinase in MIN6 beta cells is not affected by the protein kinase A pathway. *FEBS Lett* **586**, 4241-4247
30. Rybicka, K. K. (1996) Glycosomes the organelles of glycogen metabolism. *Tissue&Cell*
31. Carling, D., and Hardie, D. G. (1989) The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta* **1012**, 81-86
32. Jorgensen, S. B., Nielsen, J. N., Birk, J. B., Olsen, G. S., Viollet, B., Andreelli, F., Schjerling, P., Vaulont, S., Hardie, D. G., Hansen, B. F., Richter, E. A., and Wojtaszewski, J. F. (2004) The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* **53**, 3074-3081
33. Mangat, S., Chandrashekarappa, D., McCartney, R. R., Elbing, K., and Schmidt, M. C. (2010) Differential roles of the glycogen-binding domains of beta subunits in regulation of the Snf1 kinase complex. *Eukaryot Cell* **9**, 173-183
34. Mobbs, J. I., Koay, A., Di Paolo, A., Bieri, M., Petrie, E. J., Gorman, M. A., Doughty, L., Parker, M. W., Stapleton, D. I., Griffin, M. D., and Gooley, P. R. (2015) Determinants of oligosaccharide specificity of the carbohydrate-binding modules of AMP-activated protein kinase. *Biochem J* **468**, 245-257
35. Montori-Grau, M., Guitart, M., Garcia-Martinez, C., Orozco, A., and Gomez-Foix, A. M. (2011) Differential pattern of glycogen accumulation after protein phosphatase 1 glycogen-targeting subunit PPP1R6 overexpression, compared to PPP1R3C and PPP1R3A, in skeletal muscle cells. *BMC Biochem* **12**, 57
36. Ren, J. M., Marshall, B. A., Gulve, E. A., Gao, J., Johnson, D. W., Holloszy, J. O., and Mueckler, M. (1993) Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. *J Biol Chem* **268**, 16113-16115
37. Burattini, S., Ferri, P., Battistelli, M., Curci, R., Luchetti, F., and Falcieri, E. (2004) C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *Eur J Histochem* **48**, 223-233
38. Bujak, A. L., Crane, J. D., Lally, J. S., Ford, R. J., Kang, S. J., Rebalka, I. A., Green, A. E., Kemp, B. E., Hawke, T. J., Schertzer, J. D., and Steinberg, G. R. (2015) AMPK Activation of Muscle Autophagy Prevents Fasting-Induced Hypoglycemia and Myopathy during Aging. *Cell Metab* **21**, 883-890
39. Nielsen, J. N., Derave, W., Kristiansen, S., Ralston, E., Ploug, T., and Richter, E. A. (2001) Glycogen synthase localization and activity in rat skeletal muscle is strongly dependent on glycogen content. *J Physiol* **531**, 757-769
40. Prats, C., Cadefau, J. A., Cusso, R., Qvortrup, K., Nielsen, J. N., Wojtaszewski, J. F., Hardie, D. G., Stewart, G., Hansen, B. F., and Ploug, T. (2005) Phosphorylation-dependent translocation of glycogen synthase to a novel structure during glycogen resynthesis. *J Biol Chem* **280**, 23165-23172

Chapter 4

FRET-assisted reporting of intracellular AMPK activity based on Fluorescence Lifetime Imaging Microscopy

Yvonne Oligschläger*, Dimitris Kapsokalyvas*, Laura Schmidt, Dipanjan Chanda,
Marie Miglianico, Ricardo Rodriguez Calvo, Marc A.J.M van Zandvoort,
Dietbert Neumann

* Authors equally contributed

ABSTRACT

AMP-activated protein kinase (AMPK) is a metabolic enzyme that plays a key role in controlling cellular metabolism. AMPK is known to cycle between at least two different subcellular pools, namely cytoplasm and glycogen, in which the β -subunit carbohydrate-binding module (CBM) plays an important regulatory role. Previously, an AMPK-specific Förster Resonance Energy Transfer (FRET) biosensor, AMPKAR, was developed for measuring intracellular AMPK activity in living cells. Here, we present a novel, optimized AMPK-specific biosensor (ASS) that allows for monitoring of cytoplasmic AMPK activity in HL-1 cardiomyocytes, based on a sophisticated Fluorescence Lifetime Imaging Microscopy (FLIM) approach. A strong increase in basal FRET efficiency ($24.4 \pm 2.0 \%$) was observed in cells expressing wild-type ASS as a result of nutrient starvation, which impaired further monitoring of changes in average CFP lifetime induced by the AMPK activator oligomycin. Optimization of the experimental conditions revealed that glucose/serum-rich medium (non-starvation) minimized this effect ($4.9 \pm 2.9 \%$). Under this condition, FRET efficiency of wild-type ASS increased upon treatment with oligomycin (to $6.6 \pm 1.4 \%$), but this was mainly attributed to the DMSO vehicle. Interestingly, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an analogue of AMP capable of activating AMPK, enhanced FRET efficiency of wild-type ASS expressing cells to $9.9 \pm 1.1 \%$, which occurred in a time- but not dose-dependent manner. Altogether, our findings indicate that the wild-type ASS biosensor detects increased AMPK activity albeit dynamic range is limited. Hence, we provide evidence that FLIM/FRET can be used as an approach to accurately quantify fluctuations of intracellular AMPK activity. Because little is known about the activity of glycogen-localized AMPK, we also generated glycogen-targeted biosensors that in the near future could advance our understanding of the role and activity of glycogen-bound AMPK.

INTRODUCTION

Dynamic protein–protein interactions belong to a complex signal transduction network system that plays a key role in the regulation of cellular activities. Extracellular signal transduction to various subcellular localizations often involves protein kinases. In particular, the AMP-activated protein kinase (AMPK) is a master switch important for maintaining cellular and whole-body energy homeostasis by phosphorylating downstream targets to switch on catabolic energy-producing processes (*e.g.*, glycolysis and fatty acid oxidation), while inhibiting anabolic energy-requiring processes (*e.g.*, lipid and glycogen synthesis) (1). AMPK is a highly conserved sensor of increased levels of AMP and ADP originating from ATP depletion (2,3), which activates AMPK via allosteric regulation. This triggers upstream kinases, *e.g.*, liver kinase B1 (LKB1) (4), Ca²⁺/calmodulin-dependent protein kinase kinase β (CaMKK β) (5), and transforming growth factor- β -activated kinase 1 (TAK1) (6,7), to activate AMPK by phosphorylating the catalytic α -subunit at threonine-172 (Thr-172). Once the energy balance has been restored, AMPK undergoes dephosphorylation and inactivation by protein phosphatases (*e.g.*, PP1, PP2A, PP2C) (8-10).

Because phosphorylation events occur in a second-to-minute time frame (11,12), classical methods such as immunofluorescence microscopy or Western blotting do not provide sufficient *in situ* spatiotemporal resolution and quantitative real-time information, as these techniques are limited to the use of fixed or lysed cells, respectively. A biological breakthrough in reporting detailed information on dynamic changes in cellular signaling is the advent of fluorescent reporters, termed biosensors. A widely used technique enabling the visualization of molecular signaling events in living cells is Förster Resonance Energy Transfer (FRET) (13), representing the occurrence of non-radiative energy transfer between donor and acceptor fluorophore (14). FRET is a two-step process, *i.e.*, the donor needs to be excited from the ground state to the excited state (*i*) in order to non-radiatively transfer energy to the acceptor molecule through dipolar coupling between donor emission and acceptor excitation dipole moments (*ii*) (15). (For further details on the FRET principle, see section **Supporting Information** at the end of this chapter).

The use of FRET biosensors has become evident for gaining insight into spatiotemporal changes of signaling pathways in living cells (16,17). FRET biosensors are subdivided into two classes, intermolecular and intramolecular biosensors. The former is known as FRET pair (bimolecular), consisting of a donor fluorescent protein and an acceptor fluorescent protein. The latter (unimolecular) is most widely used (16,18), in which donor and acceptor fluorescent proteins are positioned in one single construct, allowing for high transfection efficiency, high signal-to-noise ratio, and relatively straightforward ratiometric image analysis (16,18). Recently, it was shown that FRET biosensors enable real-time monitoring of dynamic post-translational modifications, such as phosphorylation of lipids and proteins (19,20). An example of such intramolecular construct is Picchu (21), which monitors the *in vivo* tyrosine kinase activity of the CrkII adapter protein. This kind of backbones comprises a

substrate-specific sequence (sensor domain) that can be phosphorylated by the corresponding kinase (22). Subsequently, FRET occurs upon recognition of the sensor-phosphorylation by the ligand domain, bringing donor and acceptor fluorophores in close proximity, thereby enabling the visualization of subcellular kinase activity.

Because FRET efficiency of a specific intramolecular biosensor is predominantly affected by the distance and relative orientation of the two fluorophores (14), the development of optimized biosensors is growing rapidly. In order to render (available) biosensors distance-dependent, Komatsu *et al.* developed a backbone that consists of a long, flexible linker (Eevee backbone), as well as optimized fluorescent proteins (22), thereby improving the detection of FRET signals. Interestingly, Tsou *et al.* demonstrated that the AMPK biosensor AMPKAR displays enhanced FRET, predominantly in the cytoplasm, in response to AMPK-mediated phosphorylation of its target (23). Additionally, these researchers reported that, depending on its stimulus, phosphorylation of AMPKAR occurs in the cytoplasm and in the nucleus, suggesting that the biosensor serves as a molecular tool that enables the detection of spatiotemporal AMPK activity in living cells. Both studies (22,23) employed ratiometric biosensors (14), indicating that the level of FRET, *i.e.*, change in kinase activity, was calculated based on the ratio of FRET (fluorescence intensity measured in the YFP (yellow fluorescent protein) channel upon donor excitation) and CFP (fluorescence intensity of the CFP (cyan fluorescent protein) channel upon donor excitation), referred to as FRET/CFP ratio. Ratiometric FRET measurements are relevant for specific experimental configurations and cannot be compared with other setups (24-27). Although ratiometric FRET quantification enables live-cell detection of molecular interactions without the need for specialized equipment, the interpretation of intensity-based FRET measurements is limited by experimental conditions and potential artifacts, such as bleed-through, the concentration and labelling efficiency of the fluorochromes, photobleaching, and cross excitation, thus requiring careful and time-consuming and complicated correction measurements (24-27).

An alternative and more accurate method for the detection of FRET is Fluorescence Lifetime Imaging Microscopy (FLIM), as it overcomes the limitations associated with ratiometric FRET. FLIM/FRET is a technique based on the (average) donor fluorescence lifetime, which is a unique characteristic of a fluorophore in a certain environmental condition (28,29). Fluorescence lifetime is known as the average time that a molecule spends in the excited state before returning to its ground state. However, upon energy transfer to its acceptor, fluorescence lifetime of the donor decreases. The fluorescence lifetime measurements are insensitive to local concentration of the fluorophore or local excitation light intensity (28,30). Thus, FLIM is capable of precisely detecting the shorter donor (average) lifetime that results from FRET occurrence, thereby providing insight into, for instance, post-translational modifications in living cells (30).

In the present study, we modified a previously reported optimized FRET-biosensor backbone (22) to a novel optimized AMPK biosensor by exchanging the sensor domain with a previously reported AMPK-specific substrate (23), hereafter referred to as ASS biosensor. To our knowledge, this is the first time that an AMPK-specific biosensor, in combination with the FLIM/FRET technique, has been used to monitor and estimate cytoplasmic AMPK activity based on average donor lifetimes and derived FRET efficiencies.

EXPERIMENTAL PROCEDURES

Materials - Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). T₄ DNA Ligase and Polynucleotide Kinase (PNK) were acquired from Fermentas (Burlington, CA). All primers and oligonucleotides were obtained from Eurogentec (Seraing, BE). ECFP and EYFP control vectors were kindly provided by Dr. Aoki (Kyoto University, Kyoto). Antibodies directed against phospho-AMPK-Thr-172, phospho-ACC-Ser-79, GAP-DH and β -actin were purchased from Cell Signaling (Beverly, USA).

FRET Biosensor Construction - AMPK-specific substrate (ASS) FRET biosensors were engineered based on previous publications (31,32). Wild-type (WT) ASS motif sequence was derived from Tsou *et al.* (32), and a non-phosphorylatable mutant (MT) motif sequence was generated. ASS-WT and ASS-MT were subcloned, using oligonucleotide linkers, into the NotI and BspEI restriction sites of the cytoplasmic PKA activity construct, pAKAR3EV (kindly provided by Dr. Aoki) (22), thereby exchanging the sensor domain (*i.e.*, ASS substrate instead of PKA substrate), referred to as ASS-WT and ASS-MT constructs.

In order to monitor AMPK activity at glycogen, CBM β 2 sequence was fused with the ASS-constructs. The cDNA CBM β 2 (33) was amplified by PCR and initially subcloned into the EcoRI and MluI restriction sites of the PKC activity construct, pEevee-PKC (kindly provided by Dr. Aoki), thereby exchanging the optional domain (*i.e.*, CBM β 2 instead of C1 domain of PKC β), referred to as pEevee-CBM β 2. Subsequently, the ASS inserts (WT/MT) were subcloned into the EcoRI and XbaI restriction sites of the pEevee-CBM β 2, thereby exchanging the sensor domain (*i.e.*, ASS instead of PKC substrate), referred to as ASS-CBM β 2 (WT/MT) constructs. Primer sequences are available upon request. All of the constructs were verified by sequencing.

Cell culture - HL-1 cardiomyocyte cell line was kindly provided by Dr. W. Claycomb (Louisiana State University, New Orleans, LA), cultured on fibronectin (5 µg/ml; Sigma)/gelatin (0.01%; Merck)-coating in Claycomb medium (supplemented with 10% heat-inactivated fetal calf serum (iFCS, Bodinco BV, Alkmaar), 0.1 mmol/liter noradrenaline, 2 mmol/liter L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin) at 37 °C and 5% CO₂.

For transient transfections, HL-1 cells were seeded on coverslips (Ø 20 mm; Thermo Scientific) at a density of 90,000 cells/well in 12-well plates (Greiner Bio-One) 24 h before transfection. For fluorescence lifetime imaging microscopy, cells were transfected with 1 µg of plasmid DNA (AMPK activity reporter, see Results section) using Lipofectamine 2000 (Invitrogen) in antibiotic-free culture medium. Six to eight hours after transfection, transfection medium was replaced by normal growth medium. At 48 h after transfection, cells were used for FLIM-FRET studies.

For FLIM-FRET-based reporting of AMPK activity, two different protocols were used. For the starvation protocol, cells were cultured in low glucose, serum-starved medium (5.5 mM glucose), supplemented with 1% penicillin/streptomycin, 1% non-essential amino acids (Life Technologies) and 1% L-glutamine) at least 6 h prior to the experiment. To allow for microscopic monitoring, cells were switched to phenol-red free serum-starved, low glucose medium (DMEM 11880, 5.5 mM glucose), supplemented with HEPES (25 mM).

For the non-starvation protocol, cells were switched from normal growth medium to phenol-red free high glucose medium (25 mM), supplemented with 10% FBS and HEPES (25 mM) prior to the experiment. In order to visualize AMPK activity, cells were treated with or without oligomycin (Sigma, 5 µM) or AICAR (Sigma, 2 mM), and lifetime images were measured at different time points.

Western blotting - Cells were lysed in Ripa lysis buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitor mixtures (Roche Applied Science). Cell lysates were electrophoresed by SDS-PAGE and analyzed by Western blot.

Microscopical Imaging and Image Processing - Imaging was performed using a Leica TCS SP5 (Leica Microsystems GmbH, Wetzlar, Germany) two photon microscope. A Ti:Sapphire Chameleon Ultra II (Coherent, USA) mode locked at 800 nm was used as the excitation source. A Leica HCX APO L 20x/1.00 was used for excitation and epicollection. CFP and YFP fluorescence was detected in the 470-500 nm and 530-550 nm range, respectively. Emission spectra were acquired in the emission wavelength scanning mode. Sixteen images at 10 nm intervals from 450-600 nm were acquired. Images were analysed and reconstructed in MATLAB 7.1 (Mathworks Inc, Natick, USA) based on a custom routine. Spectra presented in figures are the normalized summed spectra over the entire cell surface.

The FLIM detection was performed using a Becker&Hickl TCSPC (Time Correlated Single Photon Counting) module (SPC 830, Becker&Hickl GmbH, Berlin, Germany) adapted on the Leica TCS SP5. Detection was performed with a photon counting PMT. CFP lifetime images were detected in the same range with the same PMT in the photon counting mode. Acquired images were visualized using the dedicated software SPCM 9.5 (Becker-Hickl GmbH, Berlin, Germany). Acquisition was performed on a 256 μm^2 region of interest with 256-pixel resolution and 256 ADC (Analog to Digital Converter) resolution. Exponential fits, de-convolution, and fluorescence decay analysis were performed with the dedicated software SPC-Image 3.5 (Becker-Hickl GmbH, Berlin, Germany) (34). The Instrument Response Function (IRF) was extracted from the rising time of the signal. A double exponential function, equation (1), was used to fit the fluorescence decay, where $F(t)$ is the time dependent fluorescence emission, F_0 the fluorescence at time zero, and t is time.

$$F(t) = F_0 \left(a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \right) \quad [1]$$

The fast (τ_1) and slow (τ_2) components of the CFP fluorescence with corresponding pre-exponential factors (α_1) and (α_2) were extracted from equation [1]. The mean lifetime was computed using the amplitude weighted mean lifetime formula $\tau_{\text{mean}} = \alpha_1 \cdot \tau_1 + \alpha_2 \cdot \tau_2$ [2], defined by $\alpha_1 + \alpha_2 = 1$ [3]. Lifetime images presented are colour coded in the range between 1000-2000 ps.

Photobleaching of YFP (acceptor) was performed using the 514 nm laser line. Selected regions of interested were continuously scanned with maximum laser intensity until the fluorescence intensity reduced to 90% of its initial value. Scanning was performed on 256×256 μm^2 regions with 512×512 pixel resolution at 100Hz line scan speed. In average, 100 scanned frames were required.

FRET efficiency (E_F) was calculated as the donor molecule (CFP) lifetime change according to the following formula: $E_F = 1 - (\tau_{\text{after}} / \tau_{\text{before}})$ [4], in which τ_{before} is the mean lifetime of CFP before biosensor activation, and τ_{after} is the mean lifetime after biosensor activation. The mean lifetime is calculated based on formula [2].

Autofluorescence levels of cells were investigated under normal cell density levels (section Cell Culture) and normal levels of laser power used for the ASS biosensor. The level of autofluorescence in the CFP channel was below detection levels.

RESULTS

Design of ASS biosensor for FLIM/FRET-based monitoring of intracellular AMPK activity

To generate a desirable FRET-based reporter of cytoplasmic AMPK activity, we replaced the sensor domain of the previously reported pAKAR3EV backbone (22) with the AMPK-specific substrate (ASS) (amino acid sequence: MRRVAT*LVDL) (23). This intramolecular biosensor carries four different functional domains that are expressed as a single polypeptide chain in mammalian cells, with an N-terminal acceptor YFP, and a C-terminal donor CFP. YFP is followed by an intrinsic FHA1 domain (22), serving as ligand domain, a long flexible EV-linker, and a Ser/Thr-kinase substrate sequence (ASS) as sensor domain (**Fig. 1A**). Upon AMPK-mediated phosphorylation, FHA1 recognizes the specific phosphorylation signal on ASS, thereby positioning CFP and YFP in close proximity and facilitating the transfer of energy (**Fig. 1B**; left panel: FRET). To exclude the possibility of measuring off-target effects due to the ASS sequence, we designed a non-phosphorylatable ASS mutant construct by replacing the threonine phosphorylation residue (T*) to alanine, referred to as ASS-MT. Therefore, ASS-MT serves as negative control due to its insensitivity for AMPK activity, thus preventing the occurrence of FRET (**Fig. 1B**; right panel: No FRET).

Figure 1. ASS biosensor. (A) Schematic representation of the biosensor including the different domains (adapted from Komastu *et al.* (22)). (B) Mechanism of action of ASS-WT *versus* ASS-MT. The ligand (FHA1) domain is attached to an acceptor fluorophore (YFP), while the sensor domain (ASS-WT) is linked to a donor fluorophore (CFP). Upon AMPK-mediated phosphorylation of ASS-WT, the flexible linker facilitates the binding of ASS-WT and FHA1 domain, which is capable of recognizing phosphorylation. Subsequently, CFP and YFP are in close proximity, and FRET occurs (left panel). The non-phosphorylatable ASS-MT prevents this from occurring, resulting in a constitutive 'No-FRET' situation (right panel).

Starvation significantly perturbs the average CFP lifetime of the ASS-WT biosensor

To monitor AMPK activity based on the ASS reporter, we employed the FLIM/FRET technique after transient transfection of ASS-WT in HL-1 cardiomyocytes. Both ASS-MT and ECFP, the latter which does not carry an acceptor molecule, served as negative control. Considering that serum-starvation, often in combination with glucose-deprivation, is a commonly used cell culture procedure to induce cell cycle synchronization and cellular senescence (35) or to enhance cellular responses to pharmacological agents (36), we speculated that under these conditions, oligomycin would activate AMPK, leading to changes in FRET signal. Therefore, HL-1 cardiomyocytes were cultured under low glucose (5.5 mM), serum-starved conditions for a period of 6 h prior to fluorescence-lifetime imaging, referred to as starvation, and subsequently treated with oligomycin (5 μ M) for 20 min (**Fig. 2**). At basal conditions, *i.e.* after a starvation period of 6 h, the ASS-WT construct provided a CFP donor average fluorescence lifetime of $1,291 \pm 16.81$ ps in HL-1 cells (**Fig. 2C**). The average CFP lifetime did not significantly decrease after treatment with oligomycin ($1,254 \pm 17$ ps) (**Fig. 2C**), an effect that was reflected by the corresponding color-coded lifetime images (**Fig. 2A**, middle panel), suggesting that the starvation condition already resulted in maximal FRET response. In order to assess whether oligomycin was capable of activating AMPK, we performed Western blot analysis to determine AMPK activity. As expected, oligomycin enhanced AMPK activation (pAMPK-Thr-172), both in ASS-transfected and non-transfected HL-1 cells (**Fig. 2D**). Hence, contrary to the expectations, AMPK activation by oligomycin in serum-starved cells did not yield decreased average CFP lifetimes.

Based on literature, CFP has a slightly higher average lifetime (2,200 ps (37)) as compared to the lifetimes we observed for the ASS-MT control (**Fig. 2C**). To test whether starvation, in cells expressing ASS-WT, was responsible for the initial drop in average CFP lifetime, we compared the lifetime before and after photobleaching of the YFP acceptor, as the transfer of energy from donor to acceptor is prevented in that case. After photobleaching of YFP in HL-1 cells expressing ASS-WT, we observed that average CFP lifetime increased to $1,766 \pm 40$ ps (**Fig. 2C**; reflected by **Fig. 2A**; right panel), which was not significantly different from the average CFP lifetime of cells overexpressing ASS-MT (**Fig. 2C**). Similarly, emission spectra of CFP did not significantly change before or after activation (**Fig. 2B**), whereas photobleaching of YFP resulted in an emission spectrum (peaks at 477 nm and 503 nm) that clearly corresponded to the ECFP control emission spectrum (38) (data not shown). Altogether, our data suggest that starvation causes a basal AMPK activation, which adversely induces FRET occurrence and limits the dynamic range of the biosensor.

Figure 2. Starvation significantly perturbs the average CFP lifetime of the ASS-WT biosensor. HL-1 cardiomyocytes overexpressing ASS-WT have been starved for 6 h prior to treatment with oligomycin (5 μ M). **(A)** Representative lifetime before activation and after activation with oligomycin, and after photobleaching. **(B)** Normalized corresponding spectra of the emission CFP, before activation (red), after activation (green) and after YFP photobleaching (blue). **(C)** Average CFP lifetimes of ASS-WT (sphere) and ASS-MT (square) biosensors before activation (black), after activation (dark grey), and subsequently after photobleaching (light grey) ASS-WT $n=10$, ASS-MT $n=6$; * $p < 0.01$; ** $p < 0.001$; n.s. non-significant **(D)** Representative Western blot showing that oligomycin activates AMPK both in non-transfected HL-1 cells and in HL-1 cells transfected with ASS-WT as compared to DMSO control.

The non-starvation protocol allows for detectable changes of FRET efficiency

To investigate whether starvation indeed involves basal AMPK activity, thereby affecting average CFP lifetime, we compared the behavior of ASS-WT with ASS-MT. ASS-MT is a mutant reporter that carries a non-phosphorylatable AMPK-specific motif sequence, which in turn should prevent the occurrence of FRET under conditions causing AMPK activation (*cf.* **Fig. 1B**; right panel: No FRET). After starvation, ASS-WT showed an average CFP lifetime of $1,291 \pm 17$ ps, whereas the average CFP lifetime of ASS-MT was significantly higher ($1,708 \pm 13$ ps) (**Fig. 3A**), and was found to be almost equivalent to the lifetime of cells expressing ECFP alone (data not shown). Thus, our results indicate that starvation causes significant basal AMPK activity, as shown by an increase in FRET signal detected by ASS-WT under starvation conditions compared to ASS-MT.

Next, we aimed to optimize FLIM/FRET-assisted reporting of intracellular AMPK activity. In order to dampen the effect of starvation on average CFP lifetime, we modified the experimental set-up and switched to measuring lifetime upon treatment with glucose-rich (25 mM), serum-containing medium (non-starvation). Interestingly, in comparison with starvation conditions, the average CFP lifetime of ASS-MT did not alter upon non-starvation conditions ($1,708 \pm 13$ ps vs. $1,725 \pm 28$ ps, respectively) (**Fig. 3A**). However, the average CFP lifetime of ASS-WT was significantly higher under non-starvation conditions compared to starvation conditions ($1,291 \pm 17$ ps vs. $1,641 \pm 15$ ps, respectively), and reached almost the average CFP lifetime of the ASS-MT control (**Fig. 3A**). To estimate the level of AMPK activity induced by starvation, we calculated the average difference in CFP lifetimes between ASS-WT (which allows FRET) and ASS-MT (which does not allow FRET) under starvation and non-starvation conditions, displayed as lifetime difference (%) (**Fig. 3B**). Starvation *per se* resulted in 24.4 ± 2.0 % increase in basal AMPK activity, whereas only 4.9 ± 2.9 % increase was observed in response to non-starvation conditions (**Fig. 3B**). Based on these results, we preceded our experiments with non-starvation conditions to limit basal AMPK activity, thereby increasing the dynamic range of ASS-WT.

Figure 3. Comparison of starvation and non-starvation conditions. HL-1 cardiomyocytes were transiently transfected with ASS-WT or ASS-MT. **(A)** Average CFP lifetimes of ASS-WT (sphere) and ASS-MT (square) biosensors after starvation (grey) or non-starvation (black) conditions. **(B)** Estimation of the percentage of basal AMPK activity upon starvation (grey) and non-starvation (black) conditions. Calculations were based on differences in average CFP lifetime of ASS-WT *versus* ASS-MT. ASS-WT starvation $n=10$; ASS-MT starvation $n=11$; ASS-WT non-starvation $n=16$; ASS-MT non-starvation $n=12$; * $p<0.01$; ** $p<0.001$.

Effect of oligomycin-induced AMPK activation on FRET efficiency

Oligomycin is known to induce AMPK activation by inhibiting mitochondrial ATP synthesis (39). To assess whether we could measure cytoplasmic AMPK activity using FLIM/FRET, we treated HL-1 cells with oligomycin and subsequently performed time-lapse imaging. After 10 minutes of incubation, oligomycin significantly enhanced FRET efficiency in cells expressing ASS-WT (6.6 ± 1.4 %), whereas it barely induced FRET in cells expressing ASS-MT (2.3 ± 0.5 %) (**Fig. 4A**). Furthermore, over a time period of 25 minutes, oligomycin gradually induced AMPK activity, which did not even reach maximal activation after 25 min (9.9 ± 1.6 %), as shown by the trend (**Fig. 4B**). Notably, the statistical sample number is small. Therefore values are not completely representative of the population as shown in **Figure 4A**. However, the trend is indicative of the actual situation, showing that AMPK activity does not reach plateau levels after 10 min. Although we did not follow-up the effect of oligomycin on AMPK activity over a longer period of time, these results indicate that oligomycin induced FRET in a time-dependent manner, suggesting that ASS-WT can be used for visualizing real-time AMPK activity based on a FLIM/FRET approach.

Figure 4. Effect of oligomycin-induced AMPK activation on FRET efficiency. HL-1 cardiomyocytes were transiently transfected with ASS-WT (sphere) or ASS-MT (square) and incubated with non-starvation medium. **(A)** FRET efficiency (%) of ASS-WT *versus* ASS-MT after 10 min incubation with oligomycin (5 μ M). ASS-WT $n=13$; ASS-MT $n=11$; * $p=0.01$. **(B)** Time-course of oligomycin to measure the change in average CFP lifetime in cells expressing ASS-WT (light grey) *versus* cells expressing ASS-MT (dark grey), presented as FRET efficiency (%); $n=3$.

Given that DMSO might have cellular and molecular side-effects (40), we assessed whether oligomycin induced FRET as a consequence of DMSO. Time-lapse imaging was performed and CFP average lifetimes of ASS-WT and ASS-MT in response to DMSO were measured in order to calculate FRET efficiencies (**Fig. 5**). Despite the fact that DMSO had minimal effect on ASS-MT (1.6 ± 0.6 %), a significant increase in FRET was observed in cells expressing ASS-WT (6.3 ± 1.4 %), suggesting that DMSO consequently induced basal AMPK activation. The induction in FRET signal was strongest after 15 min of incubation, after which it got stabilized, indicating that DMSO presumably interferes with the AMPK signaling cascade. Therefore, it is likely that the increase in AMPK activity caused by DMSO interferes with the measurements of FRET efficiency of the ASS-WT biosensor, thereby overestimating the effect resulting from oligomycin.

Figure 5. Effect of DMSO on FRET efficiency (%) of ASS-WT versus ASS-MT. HL-1 cardiomyocytes were transiently transfected with ASS-WT (spheres; light grey) or ASS-MT (squares; dark grey) and incubated with non-starvation medium. Time-course of DMSO to measure the change in average CFP lifetime in cells expressing ASS-WT versus cells expressing ASS-MT, presented as FRET efficiency (%); $n=3$.

ASS-WT biosensor supports the monitoring of AMPK activity in response to AICAR in a time-dependent manner

To circumvent the possible adverse effect caused by DMSO, we investigated whether 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an AMP analogue dissolved in phosphate-buffered saline (PBS), could have similar effects on FRET efficiency, *i.e.*, AMPK activity. Therefore, we performed time-lapse imaging and measured average CFP lifetimes from ASS-WT and ASS-MT (**Fig. 6C**). Representative images and spectra are shown in Figure 8A. Upon AICAR treatment, average CFP lifetime of ASS-WT decreased significantly after 10 min of incubation (1659 ± 18 ps vs. 1494 ± 20 ps, respectively), an effect that was reflected by the corresponding emission spectrum and FLIM image color change (**Fig. 6A**). Thus, AICAR induced a significant change in AMPK activity, represented by a FRET efficiency of 9.9 ± 1.1 % (**Fig. 6C**). As expected, we did not observe significant changes in average CFP lifetime

in cells expressing ASS-MT (1736 $\pm$ 11 ps vs. 1724 $\pm$ 17 ps, respectively) (**Fig. 6B**). This result was re-confirmed by measuring the FRET efficiency, providing a percentage that was close to non-detectable levels (0.6 $\pm$ 1.3 %) (**Fig. 6C**). Remarkably, in the following 20 min, FRET efficiency of ASS-WT slowly further increased to reach a level of 13.0 $\pm$ 1.0 % after 30 min of AICAR treatment, which did not even reach plateau phase, and this effect that was completely abolished by the ASS-MT biosensor. Taken together, these data suggest that AICAR allows for FLIM/FRET-assisted reporting of cytosolic AMPK activity in a time-dependent manner.

Figure 6. ASS-WT biosensor supports the monitoring of AMPK activity in response to AICAR in a time-dependent manner. Representative FLIM images and corresponding spectra before, and after 10 min of activation with AICAR (2 mM) from HL-1 cells overexpressing ASS-WT $n=11$ (**A**) or ASS-MT $n=11$ (**B**), * $p<0.001$. (**C**) FRET efficiencies of ASS-WT (spheres) versus ASS-MT (squares) in response to AICAR under non-starvation conditions. ASS-WT $n=10$; ASS-MT $n=8$; * $p<0.001$. (**D**) Time-course of AICAR to measure the change in average CFP lifetime in cells expressing ASS-WT (spheres; light grey) versus cells expressing ASS-MT (squares; dark grey), presented as FRET efficiency (%); $n=3$.

AICAR-induced AMPK activity is not regulated in a dose-dependent manner

To examine whether FLIM/FRET is an accurate technique for the detection of dynamic changes in AMPK activity, the amount of AICAR was based on a dose-response with concentrations of 0.5, 2 and 8 mM, which is either four times lower or higher, respectively, than the concentration of 2 mM frequently used for the stimulation of (cardio)myocytes (41-43). Consistent with our previous data (*cf.* **Fig. 6**), efficiency of FRET in cells overexpressing AMPK-WT increased in response to low (0.5 mM), medium (2 mM) and high (8 mM) doses of AICAR (11.7 ± 1.4 , 9.9 ± 1.1 and 12.0 ± 0.8 %, respectively), whereas the ASS-MT blunted these effects (6.2 ± 0.7 , 0.6 ± 1.3 and 3.4 ± 0.9 , respectively) (**Fig. 7A**). Consistently, Western blot analyses showed that AICAR induces AMPK activation (pAMPK-Thr-172 and its downstream target pACC-Ser-79, respectively) both in cells expressing ASS-WT and ASS-MT (**Fig. 7B**), but this activation was concentration-independent. Altogether, using the ASS biosensors, our results suggest that, in HL-1 cells, AICAR-induced activation of cytosolic AMPK is regulated in a dose-independent manner.

Figure 7. AICAR-induced AMPK activity is not regulated in a dose-dependent manner. (A) FRET efficiencies of ASS-WT (spheres) versus ASS-MT (squares) in response to medium (2 mM; ASS-WT $n=10$; ASS-MT $n=8$), low (0.5 mM; ASS-WT $n=16$; ASS-MT $n=19$) and high (8 mM; ASS-WT $n=26$; ASS-MT $n=15$) concentrations of AICAR under non-starvation conditions after 10 min. * $p<0.001$ (B) Representative and corresponding Western blot showing the level of AMPK activation in response to AICAR.

DISCUSSION

Fluorescent biosensors are used for reporting on various dynamic signaling events, such as post-translational modifications in living cells. Understanding these spatiotemporal regulations involves advanced microscopy techniques, such as FRET, and sophisticated image processing, which demands for specific correction measurements. To circumvent these kinds of corrections, we monitored cytoplasmic AMPK activity using a novel biosensor, ASS-WT, based on fluorescence-lifetime imaging (FLIM/FRET). Our results demonstrated that ASS-WT is sensitive to cytosolic AMPK activation. Optimization of experimental conditions revealed that starvation leads to high basal AMPK activity, thereby causing FRET before the addition of pharmacological AMPK activators, whereas a non-starvation condition prevented this basal FRET occurrence. Although both oligomycin and AICAR resulted in a FRET increase of 6.6 ± 1.4 % (**Fig. 4**) and 9.9 ± 1.1 % (**Fig. 6**), respectively, our results show that DMSO interferes with FLIM/FRET measurements of AMPK activity following oligomycin (**Fig. 5**). Altogether, we provide evidence that FLIM/FRET can be used as a technique for accurately monitoring cytoplasmic, or localized, AMPK activity, specifically using non-starvation and DMSO-free protocols.

Based on the fact that starvation is a standard procedure for increasing cellular responses to external stimuli, we hypothesized that this experimental condition would allow for maximal activation of AMPK in response to oligomycin, to be monitored on a FLIM/FRET-based approach. Surprisingly, starvation caused an unexpected induction in FRET signal in ASS-WT expressing cells. Given that the changes in AMPK activity were strong enough to prevent the detection of oligomycin-induced AMPK activation, these results suggest that ASS-WT is very sensitive to slight changes in cytoplasmic AMPK activation, and that the FLIM/FRET technique allows for the detection of such minor changes in AMPK activity. Therefore, we excluded the starvation protocol for monitoring cytoplasmic AMPK activity induced by pharmacological agents.

Optimization of the experimental conditions (starvation *versus* non-starvation) greatly improved the dynamic range of CFP regarding the ASS-WT biosensor, indicating that non-starvation has a negligible effect on basal AMPK activity. Based on these conditions, we showed that pharmacological AMPK activation resulted in enhanced FRET efficiency in HL-1 cardiomyocytes. Although oligomycin was shown to increase FRET efficiency to 9.9 ± 1.6 % after 25 min of incubation, we observed that these effects were DMSO-dependent. Strikingly, the percentage of DMSO was only 0.2 %, which is generally considered to be non-cytotoxic. Indeed, if we compared DMSO, often used as vehicle control (44,45), with oligomycin treatment in non-transfected or transfected HL-1 cells, we clearly observed oligomycin-induced AMPK activation determined by Western blot analysis. Yet, when monitoring oligomycin-induced AMPK activity based on FLIM/FRET microscopy, the increase in activity was largely due to DMSO. Given these findings, it can be speculated that FLIM/FRET offers encouraging prospect to visualize little AMPK activity changes,

which are below detection levels by conventional techniques, such as Western blot analyses or immunofluorescence studies.

An alternative pharmacological AMPK activator dissolved in PBS, AICAR, resulted in a $13.0 \pm 1.0\%$ change in FRET efficiency after 30 min of incubation, reconfirming that our FLIM/FRET-based approach is suitable for measuring cytoplasmic AMPK activity. Despite FLIM/FRET allowed for measurement of small changes in AMPK activity, we did not find significant difference in FRET efficiency when cells were treated with different concentrations (0.5, 2 or 8 mM) of AICAR, similar to what we found by Western blot analysis. To better understand the dynamic range of ASS-WT regarding the visualization of cytoplasmic AMPK activity, we tested very low concentrations of AICAR (50 μ M) (data not shown). Based on the fact that neither FLIM/FRET nor Western blot analysis allowed for detection of small changes in AMPK activity, we conclude that further optimization is required to advance our understandings of the physical properties of the ASS-WT biosensor, as the AMPK activity transition appears to occur between the range of 50–500 μ M of AICAR.

Although ECFP is widely used as a donor molecule in FRET studies, several studies report on the exchange of ECFP for alternative brighter cyan fluorescent protein variants, such as mTurquoise (46). As ECFP has bi-exponential fluorescence dynamics, *i.e.*, the existence of two different lifetimes (47), this complex fluorescent decay makes it rather complicated to acquire FLIM data that allow for accurate distinction between the fraction undergoing FRET (two lifetimes) and the fraction not undergoing FRET (two lifetimes) (48). Other technical difficulties to be considered are the experimental set-up, as it may possibly affect actual lifetime measurements. Based on the number of experimental conditions and repeats, the use of small coverslips was preferred over culturing dishes to perform lifetime measurements. However, as coverslips are prone to movement or floating, for instance after changing treatment condition, it occasionally caused a loss of individual or clusters of cells to be analyzed, thus influencing the accurate measurement of single-cell AMPK activity. An alternative approach overcoming these issues would be the use of an advanced microfluidic cell culture system, as described by Tsou *et al.* (23), enabling a controlled flow of medium, thereby facilitating the detection of small changes upon addition or removal of external stimuli.

A limitation in fluorescence microscopy could be the detection of autofluorescence, if it is not addressed adequately. Autofluorescence can originate from cellular components that have fluorescent properties, resulting in background fluorescence. Autofluorescence is dependent on cell confluence (49). However, if levels of transfection were moderate and cell confluence was controlled to normal cell density levels, autofluorescence did not influence the lifetime measurements. Additionally, spontaneous beating of the HL-1 cardiomyocytes might interfere with spatial resolution and accuracy; therefore, beating cells were excluded. As transient transfection is known to have a cell-to-cell variability, *e.g.*, regarding the concentration of the fluorescent biosensor observed as brightness, we performed measurements on

both high (bright) and moderate (less bright) expressing cells. Notably, we preferred the imaging of less bright cells over bright cells, as in certain cases cell-blebbing was observed in very bright cells, which could be attributed to phototoxicity and -damage. Furthermore, by selecting less bright cells, we also minimized the contribution to inter-construct FRET, which occurs in a concentration-dependent manner, only when donor and acceptor from two different constructs are in close proximity (50). Another limitation is that these lifetime measurements are time-consuming, taking into account (i) the measurement itself (40 sec per measurement), (ii) defining proper regions of interest (iii) following-up different regions of interest on the same coverslip in time.

To overcome the rather low transfection efficiency in HL-1 cells ($\pm 30\%$) as well as the variation in brightness, it might be an option to stably express the ASS FRET biosensors in these cells using retrovirus. However, high sequence homology between CFP and YFP might result in recombination during integration to the retroviral genome (18,51), which makes this strategy a challenge. Interestingly, various techniques have been developed to support the generation of stable cell lines carrying the FRET-based biosensor, such as the piggyBac gene transfer system (51).

Because AMPK is known to cycle between different cellular compartments, for instance between glycogen and the cytoplasm (52-54) (Oligschlaeger *et al.*, *submitted*), we hypothesized that FRET biosensors containing a muscle-specific glycogen-targeting domain, *i.e.* CBM β 2 (see Experimental Procedures), would enable the monitoring of glycogen-localized AMPK activity. Very recently, Miyamoto *et al.* (55) reported that osABKAR, an optimized and further developed AMPK biosensor, is capable of monitoring localized AMPK activity. Targeting of this biosensor to a wide variety of specific intracellular compartments, due to fusion with corresponding signal sequences for instance for plasma membrane, Golgi apparatus, ER, etc. provided insight into the cellular distribution of AMPK activity. However, these researchers did not report on the activity of glycogen-localized AMPK.

While optimization is required to draw solid conclusions on the regulation of AMPK activity, our results collectively suggest that ASS-WT is a valuable tool for visualizing AMPK activity at specific subcellular localizations by means of a FLIM/FRET approach. Future studies using the glycogen-targeting ASS-WT biosensor may enable assessment of the function and activity of glycogen-bound AMPK. These developments will create further opportunities to implement a cell-based screening that utilizes the glycogen-bound biosensor to study the role of small molecules on AMPK-glycogen binding and glycogen metabolism.

REFERENCES

- Winder, W. W., and Hardie, D. G. (1999) AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* **277**, E1-10
- Steinberg, G. R., and Kemp, B. E. (2009) AMPK in Health and Disease. *Physiol Rev* **89**, 1025-1078
- Mihaylova, M. M., and Shaw, R. J. (2011) The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* **13**, 1016-1023
- Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R., and Hardie, D. G. (2003) Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol* **2**, 28
- Hurley, R. L., Anderson, K. A., Franzoni, J. M., Kemp, B. E., Means, A. R., and Witters, L. A. (2005) The Ca²⁺/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *J Biol Chem* **280**, 29060-29066
- Momcilovic, M., Hong, S. P., and Carlson, M. (2006) Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. *J Biol Chem* **281**, 25336-25343
- Scholz, R., Sidler, C. F., Thali, R. F., Winssinger, N., Cheung, P. C., and Neumann, D. (2010) Autoactivation of transforming growth factor beta-activated kinase 1 is a sequential bimolecular process. *J Biol Chem* **285**, 25753-25766
- Garcia-Haro, L., Garcia-Gimeno, M. A., Neumann, D., Beullens, M., Bollen, M., and Sanz, P. (2012) Glucose-dependent regulation of AMP-activated protein kinase in MIN6 beta cells is not affected by the protein kinase A pathway. *FEBS Lett* **586**, 4241-4247
- Marley, A. E., Sullivan, J. E., Carling, D., Abbott, W. M., Smith, G. J., Taylor, I. W., Carey, F., and Beri, R. K. (1996) Biochemical characterization and deletion analysis of recombinant human protein phosphatase 2C alpha. *Biochem J* **320** (Pt 3), 801-806
- Gimeno-Alcaniz, J. V., and Sanz, P. (2003) Glucose and type 2A protein phosphatase regulate the interaction between catalytic and regulatory subunits of AMP-activated protein kinase. *J Mol Biol* **333**, 201-209
- Blazek, M., Santisteban, T. S., Zengerle, R., and Meier, M. (2015) Analysis of fast protein phosphorylation kinetics in single cells on a microfluidic chip. *Lab Chip* **15**, 726-734
- Homayouni, R., Byrne, J. H., and Eskin, A. (1995) Dynamics of protein phosphorylation in sensory neurons of Aplysia. *J Neurosci* **15**, 429-438
- Piston, D. W., and Kremers, G. J. (2007) Fluorescent protein FRET: the good, the bad and the ugly. *Trends Biochem Sci* **32**, 407-414
- Jares-Erijman, E. A., and Jovin, T. M. (2003) FRET imaging. *Nat Biotechnol* **21**, 1387-1395
- Sahoo, S. K., Bera, R. K., Kanungo, B. K., and Baral, M. (2012) Spectroscopic and pH-metric studies on the complexation of a novel tripodal amine-phenol ligand towards Al(III), Ga(III) and In(III). *Spectrochim Acta A Mol Biomol Spectrosc* **89**, 322-328
- Miyawaki, A. (2003) Visualization of the spatial and temporal dynamics of intracellular signaling. *Dev Cell* **4**, 295-305
- Allen, M. D., and Zhang, J. (2006) Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem Biophys Res Commun* **348**, 716-721
- Aoki, K., Kiyokawa, E., Nakamura, T., and Matsuda, M. (2008) Visualization of growth signal transduction cascades in living cells with genetically encoded probes based on Forster resonance energy transfer. *Philos Trans R Soc Lond B Biol Sci* **363**, 2143-2151
- Antal, C. E., and Newton, A. C. (2013) Spatiotemporal dynamics of phosphorylation in lipid second messenger signaling. *Mol Cell Proteomics* **12**, 3498-3508
- Zhang, J., and Allen, M. D. (2007) FRET-based biosensors for protein kinases: illuminating the kinome. *Mol Biosyst* **3**, 759-765
- Kurokawa, K., Mochizuki, N., Ohba, Y., Mizuno, H., Miyawaki, A., and Matsuda, M. (2001) A pair of fluorescent resonance energy transfer-based probes for tyrosine phosphorylation of the Crkl adaptor protein in vivo. *J Biol Chem* **276**, 31305-31310
- Komatsu, N., Aoki, K., Yamada, M., Yukinaga, H., Fujita, Y., Kamioka, Y., and Matsuda, M. (2011) Development of an optimized backbone of FRET biosensors for kinases and GTPases. *Mol Biol Cell* **22**, 4647-4656
- Tsou, P., Zheng, B., Hsu, C. H., Sasaki, A. T., and Cantley, L. C. (2011) A fluorescent reporter of AMPK activity and cellular energy stress. *Cell Metab* **13**, 476-486
- Chen, H., Puhl, H. L., 3rd, Koushik, S. V., Vogel, S. S., and Ikeda, S. R. (2006) Measurement of FRET efficiency and ratio of donor to acceptor concentration in living cells. *Biophys J* **91**, L39-41
- Zal, T., and Gascoigne, N. R. (2004) Photobleaching-corrected FRET efficiency imaging of live cells. *Biophys J* **86**, 3923-3939
- Elder, A. D., Domin, A., Kaminski Schierle, G. S., Lindon, C., Pines, J., Esposito, A., and Kaminski, C. F. (2009) A quantitative protocol for dynamic measurements of protein interactions by Forster resonance energy transfer-sensitized fluorescence emission. *Journal of The Royal Society Interface* **6**, S59-S81
- Tadross, M. R., Park, S. A., Veeramani, B., and Yue, D. T. (2009) Robust approaches to quantitative ratiometric FRET imaging of CFP/YFP fluorophores under confocal microscopy. *J Microsc* **233**, 192-204
- Ishikawa-Ankerhold, H. C., Ankerhold, R., and Drummen, G. P. (2012) Advanced fluorescence microscopy techniques--FRAP, FLIP, FLAP, FRET and FLIM. *Molecules* **17**, 4047-4132
- Buranachai, C., Kamiyama, D., Chiba, A., Williams, B. D., and Clegg, R. M. (2008) Rapid frequency-domain FLIM spinning disk confocal microscope: lifetime resolution, image improvement and wavelet analysis. *J Fluoresc* **18**, 929-942

30. Sun, Y., Day, R. N., and Periasamy, A. (2011) Investigating protein-protein interactions in living cells using fluorescence lifetime imaging microscopy. *Nat Protoc* **6**, 1324-1340
31. Komatsu, N., Aoki, K., Yamada, M., Yukinaga, H., Fujita, Y., Kamioka, Y., and Matsuda, M. Development of an optimized backbone of FRET biosensors for kinases and GTPases. *Mol Biol Cell* **22**, 4647-4656
32. Tsou, P., Zheng, B., Hsu, C. H., Sasaki, A. T., and Cantley, L. C. A fluorescent reporter of AMPK activity and cellular energy stress. *Cell Metab* **13**, 476-486
33. Oligschlaeger, Y., Miglianico, M., Chanda, D., Scholz, R., Thali, R. F., Tuerk, R., Stapleton, D. I., Gooley, P. R., and Neumann, D. (2015) The Recruitment of AMP-activated Protein Kinase to Glycogen Is Regulated by Autophosphorylation. *J Biol Chem* **290**, 11715-11728
34. Becker, W. (2008) *The bh TCSPC Handbook*, 3rd ed., Becker & Hickl GmbH, Berlin
35. Rosner, M., Schipany, K., and Hengstschlager, M. (2013) Merging high-quality biochemical fractionation with a refined flow cytometry approach to monitor nucleocytoplasmic protein expression throughout the unperturbed mammalian cell cycle. *Nat Protoc* **8**, 602-626
36. Ching, J. K., Rajguru, P., Marupudi, N., Banerjee, S., and Fisher, J. S. (2010) A role for AMPK in increased insulin action after serum starvation. *Am J Physiol Cell Physiol* **299**, C1171-1179
37. Becker, W., Bergmann, A., Hink, M. A., Konig, K., Benndorf, K., and Biskup, C. (2004) Fluorescence lifetime imaging by time-correlated single-photon counting. *Microsc Res Tech* **63**, 58-66
38. Hou, B. H., Takanaga, H., Grossmann, G., Chen, L. Q., Qu, X. Q., Jones, A. M., Lalonde, S., Schweissgut, O., Wiechert, W., and Frommer, W. B. (2011) Optical sensors for monitoring dynamic changes of intracellular metabolite levels in mammalian cells. *Nat Protoc* **6**, 1818-1833
39. Hawley, S. A., Gadalla, A. E., Olsen, G. S., and Hardie, D. G. (2002) The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* **51**, 2420-2425
40. Santos, N. C., Figueira-Coelho, J., Martins-Silva, J., and Saldanha, C. (2003) Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. *Biochem Pharmacol* **65**, 1035-1041
41. Segalen, C., Longnus, S. L., Baetz, D., Counillon, L., and Van Obberghen, E. (2008) 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside reduces glucose uptake via the inhibition of Na⁺/H⁺ exchanger 1 in isolated rat ventricular cardiomyocytes. *Endocrinology* **149**, 1490-1498
42. Schwenk, R. W., Luiken, J. J., and Eckel, J. (2007) FIP2 and Rip11 specify Rab11a-mediated cellular distribution of GLUT4 and FAT/CD36 in H9c2-hIR cells. *Biochem Biophys Res Commun* **363**, 119-125
43. Chabowski, A., Momken, I., Coort, S. L., Calles-Escandon, J., Tandon, N. N., Glatz, J. F., Luiken, J. J., and Bonen, A. (2006) Prolonged AMPK activation increases the expression of fatty acid transporters in cardiac myocytes and perfused hearts. *Mol Cell Biochem* **288**, 201-212
44. Kho, C., Lee, A., Jeong, D., Oh, J. G., Gorski, P. A., Fish, K., Sanchez, R., DeVita, R. J., Christensen, G., Dahl, R., and Hajjar, R. J. (2015) Small-molecule activation of SERCA2a SUMOylation for the treatment of heart failure. *Nat Commun* **6**, 7229
45. Aryal, B., Jeong, J., and Rao, V. A. (2014) Doxorubicin-induced carbonylation and degradation of cardiac myosin binding protein C promote cardiotoxicity. *Proc Natl Acad Sci U S A* **111**, 2011-2016
46. Goedhart, J., von Stetten, D., Noirclerc-Savoye, M., Lelimosin, M., Joosen, L., Hink, M. A., van Weeren, L., Gadella, T. W., Jr., and Royant, A. (2012) Structure-guided evolution of cyan fluorescent proteins towards a quantum yield of 93%. *Nat Commun* **3**, 751
47. Liptonok, S. P., Borst, J. W., Mullen, K. M., van Stokkum, I. H., Visser, A. J., and van Amerongen, H. (2010) Global analysis of Forster resonance energy transfer in live cells measured by fluorescence lifetime imaging microscopy exploiting the rise time of acceptor fluorescence. *Phys Chem Chem Phys* **12**, 7593-7602
48. Duncan, R. R. (2006) Fluorescence lifetime imaging microscopy (FLIM) to quantify protein-protein interactions inside cells. *Biochem Soc Trans* **34**, 679-682
49. Bird, D. K., Yan, L., Vrotsos, K. M., Eliceiri, K. W., Vaughan, E. M., Keely, P. J., White, J. G., and Ramanujam, N. (2005) Metabolic mapping of MCF10A human breast cells via multiphoton fluorescence lifetime imaging of the coenzyme NADH. *Cancer Res* **65**, 8766-8773
50. Grailhe, R., Merola, F., Ridard, J., Couvignou, S., Le Poupon, C., Changeux, J. P., and Laguitton-Pasquier, H. (2006) Monitoring protein interactions in the living cell through the fluorescence decays of the cyan fluorescent protein. *Chemphyschem* **7**, 1442-1454
51. Aoki, K., Komatsu, N., Hirata, E., Kamioka, Y., and Matsuda, M. (2012) Stable expression of FRET biosensors: a new light in cancer research. *Cancer Sci* **103**, 614-619
52. Salt, I., Celler, J. W., Hawley, S. A., Prescott, A., Woods, A., Carling, D., and Hardie, D. G. (1998) AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. *Biochem J* **334** (Pt 1), 177-187
53. Carling, D., and Hardie, D. G. (1989) The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta* **1012**, 81-86
54. McBride, A., Ghilagaber, S., Nikolaev, A., and Hardie, D. G. (2009) The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. *Cell Metab* **9**, 23-34
55. Miyamoto, T., Rho, E., Sample, V., Akano, H., Magari, M., Ueno, T., Gorshkov, K., Chen, M., Tokumitsu, H., Zhang, J., and Inoue, T. (2015) Compartmentalized AMPK Signaling Illuminated by Genetically Encoded Molecular Sensors and Actuators. *Cell Rep* **11**, 657-670

SUPPORTING INFORMATION

During FRET, upon illumination, the donor fluorescent molecule gets excited from the ground state to the excited state for a few nanoseconds (**Supp. Fig. 1A**). During this time, the donor molecule loses some of its energy and can relax to the ground state by either emission of photons (fluorescence), or by various other processes (mainly radiationless relaxation to the ground state) (**Supp. Fig. 1A**; left panel). In case another fluorescent molecule is in close proximity (1-10 nm), the donor transfers its energy to this acceptor molecule, which emits energy as photons (fluorescence) to return to the ground state. Thus, in the case where also FRET occurs, excitation of the donor fluorophore not only results in donor emission, but also in acceptor fluorescence (**Supp. Fig. 1A**; right panel).

The FRET phenomenon depends on a number of factors: (i) the distance between donor and acceptor (r ; maximally 1-10 nm; **Supp. Fig. 1A**); (ii) the spectral overlap between the donor emission and acceptor excitation (**Supp. Fig. 1B**), and (iii) the relative orientation of the fluorophores to allow dipole-dipole coupling and thus energy transfer (16,22).

Supplemental Figure 1. Basic principle of Förster Resonance Energy Transfer (FRET). (A) Jablonski diagram showing the principle of FRET. (B) Spectral overlap between donor emission and acceptor excitation (13).

Energy transfer efficiency is most sensitive to changes when the distance between donor and acceptor approaches the Förster distance (R_0) for the two molecules. R_0 can be calculated by means of aforementioned factors (i), (ii) and (iii). In other words, R_0 is the characteristic distance in which the FRET efficiency is 50%, indicating that half of the excitation energy of the donor is transferred to the acceptor. The Förster theory dictates that FRET efficiency (E_{FRET} ; **Supp. Fig. 2A**) varies with the sixth power of distance between the donor and acceptor (r) (13). Therefore, distances (r) smaller than R_0 have a FRET efficiency close to maximal, whereas distances larger than R_0 have an efficiency close to zero (13). The dynamic range of FRET biosensors represents the difference between zero and maximal FRET occurrence (22) (**Supp. Fig. 2B**). FRET efficiency is represented as the change in average CFP lifetime after activation normalized to the average CFP lifetime before activation.

Supplemental Figure 2. Schematic representations. (A) FRET efficiency *versus* distance (13). (B) Dynamic range of a FRET biosensor (adapted from Komatsu *et al.* (22)).

Chapter 5

Displacing AMPK from glycogen by pharmacological targeting of its carbohydrate-binding module

Marie Miglianico, **Yvonne Oligschlaeger**, Dipanjan Chanda, Kanin Wichapong, Will A Coumans, Armand Jaminon, Ricardo Rodriguez-Calvo, Özge D Özcete, Roy Schrijver, Xiaoqing Zhu, Ivo Bleylevens, Nynke MS van den Akker, Daniel GM Molin, Jan FC Glatz, Gerry AF Nicolaes, Dietbert Neumann

In preparation

ABSTRACT

As a central player of energy metabolism, AMP-activated protein kinase (AMPK) is a well-established pharmacological target. Several identified AMPK-activating small molecules have fueled further research into their mechanism of action and substantiated the beneficial effects of AMPK activation in multiple disease settings. Here, we employ a novel screening protocol focusing on the carbohydrate-binding module (CBM) of AMPK. The procedure combines virtual ligand screening, a carbohydrate-binding competition assay, and assessment of the CBM subcellular localization pattern. Among the identified hit molecules, a cluster of similar compounds scored well in all screening steps. Specifically, two of the identified compounds had micromolar affinities for displacement of AMPK–CBM in cells. Surface plasmon resonance confirmed the direct binding of these two compounds to the CBM of AMPK. Remarkably, cellular application of a high-affinity compound led to increased AMPK activity and phosphorylation of a downstream target. Furthermore, the compound stimulated both glucose and fatty acid uptake in cultured cardiomyocytes, which could be reversed with the AMPK inhibitor Compound C. In summary, blocking access of AMPK to glycogen by targeting the carbohydrate-binding pocket with small molecules could be a novel alternative approach to AMPK activity modulation.

INTRODUCTION

As a cellular hub that integrates many input signals (hormones, exercise, nutrient availability for instance) and balances energy expenditure and conservation, AMP-activated protein kinase (AMPK) is a recognized target for pharmaceutical modulation (1-5). Initially identified as a target of interest in type 2 diabetes (2), AMPK is now being investigated in a vast area of cardiovascular diseases as well as in cancer and neurological diseases (2,4,6).

Targeting AMPK with pharmacological agents is not a field in its beginning and no less than 3 different mechanisms have already been identified to pharmacologically activate AMPK (1,2,5). This heterotrimeric kinase, constituted of a catalytic subunit α and two regulatory subunits β and γ , is primarily activated by phosphorylation on the α -Thr172 and is stimulated by an elevation of AMP:ATP ratio sensed by binding domains on its regulatory γ -subunit (6). The first class of AMPK activators acts either indirectly by changing the adenine nucleotide ratio mainly via inhibition of the mitochondrial respiratory chain (*e.g.*, phenformin, oligomycin) or by mimicking the AMP molecule and directly binding to the γ -subunit (*e.g.*, 5-amino-4-imidazolecarboxamide ribonucleoside [AICAR]) (2,6). The activators of the second class (*e.g.*, A-769662) bind between the regulatory subunit β and the kinase domain of the catalytic subunit α (7), which induces a conformational change and allosterically activates AMPK (8) without necessarily changing its phosphorylation status (9,10), and protects the Thr172 from dephosphorylation (8). The compounds of the third class (*e.g.*, PT-1) act by relieving the auto-inhibition on the kinase domain within the α -subunit (11), although this mechanism of action has recently been doubted (12).

Over the years, AMPK regulation has emerged as a very fine and complex system, conveyed by post-translational modifications, subcellular localization and metabolite sensing (3). Specifically, the AMPK β -subunit contains a carbohydrate-binding module (CBM), which is able to bind carbohydrates without having any catalytic activity (13,14). In cells, the CBM is capable of tethering AMPK to glycogen particles, and this binding is prevented by β -T148 autophosphorylation (15). The physiological role of AMPK bound to glycogen was suggested to be the facilitation of the targeting of the kinase towards glycogen-bound partners (14). Indeed, AMPK phosphorylates several glycogen-related targets, such as glycogen synthase (16), and spatial proximity would allow for a quick modulation of their activities. However, it was recently reported that *in vitro* binding of AMPK to glycogen inhibited its kinase activity (17), suggesting the existence of a glycogen-bound reserve of inactive AMPK in the cell. Although seemingly in disagreement, these two roles of glycogen-localized AMPK may be dependent on the degree of glycogen branching and thus separated in time or space (17). The specific interplay of AMPK with glycogen, accordingly, remains an active area of research that furthers our understanding of AMPK's involvement in cellular glucose handling and glycogen metabolism.

Here we present a new screening protocol for identification of compounds that prevent AMPK from binding to glycogen, which combines a virtual ligand screening, a competition assay for binding of CBM to the carbohydrate β -cyclodextrin and a quantitative assessment of CBM subcellular localization. The most effective compounds resulting from this screening were further characterized for their binding affinity and cellular activity.

EXPERIMENTAL PROCEDURES

Protein target preparation - The X-ray structures of the isolated CBM β 1 protein domain in complex with β -cyclodextrin (PDB ID: 1Z0M, resolution: 1.91 Å, (18)) and of the isolated apoprotein domain CBM β 2 (PDB ID: 2F15, resolution: 2.00 Å) were retrieved from the PDB-Redo database (19). After removal of the co-crystallized β -cyclodextrin from the 1Z0M structure, the YASARA-WHAT IF twinset package (20) was used to add hydrogen and optimize both structures by energy minimization. The optimized structures were checked with the NIH structural analysis server which compiles 6 different programs (21-25) and were judged satisfactory after minor targeted residue optimizations. A short molecular dynamics simulation was run for 14 ns to sample possible conformations for both CBM proteins, with the md_run.mcr macro of the YASARA-WHAT IF Twinset package (with default parameters: AMBER03 as force field, 2.5 fs time step, and without constraint). Conformations of the snapshots taken every 25 ps were ranked according to volume of the carbohydrate-binding pocket. The 8 conformations with largest pockets that involved the loop formed by S100/T101 residues were selected for the virtual screening. For this step, icmPocketFinder (MolSoft) was used to quickly analyze all conformations. The selected conformers were then subjected to DoGSiteScorer (26) for further analysis of the targeted pocket. The sequence alignment was performed with ClustalX2 (27).

Ligand database preparation - A database of ~800,000 compounds composed of both the Express-Pick and the Combinatorial libraries of ChemBridge were retrieved (data from January 2012, hit2lead.com) and desalted with the Desalt script from Mobyle@RPBS webserver. FAF-Drugs2 (28) was used to select drug-like compounds according to their ADME/tox properties with Lipinski's rule of 5 (29) and the filter for undesirable moieties and pan-assay interference compounds (PAINS (30)). For rigid docking, the Omega2 software was used on the filtered library to generate multiple 3D conformations (~35 conformers/compound; OpenEye Scientific Software, version 2.4). For flexible docking, a single 3D conformation was generated with Omega2 for the selected compounds, since that step includes ligand flexibility.

Computer-based screen - The in silico screen of the compound library followed an established pipeline of successive rigid and flexible dockings (31-33). Briefly, the rigid-body docking software FRED (OpenEye Scientific Software, version 2.2.5) was first used to screen the complete collection of multiple compound conformers on 8 target protein conformations of CBM β 2 to rapidly remove unlikely ligands from the library. The remaining best 240,000 compounds based on an averaged consensus score of the 8 conformations were selected for flexible docking with Surflex (BioPharmics LLC, version 2.514). Next, compounds were prioritized based on their averaged Combo scores. The bound poses of the best-scoring 1,000 compounds were visually inspected and the remaining top-800 compounds were purchased from ChemBridge, diluted in DMSO and further tested in vitro. During the process and the following steps, the large amount of data was processed with Perl and Knime (34) using the CDK (35) and JChem Extension (ChemAxon, version 204) packages. Additionally, the webserver ChemMine was used to compare the compounds according to 2D similarity (36).

Re-docking and binding free energy calculations - Coordinates of the 3D structures of AMPK CBM β 1 in complex with β -cyclodextrin (PDB code 1Z0M) and apoprotein AMPK CBM β 2 (PDB code 2F15) were downloaded from the PDB-Redo database as mentioned above. Ligands were docked into the binding pocket of the AMPK CBM β -2 by application of the Autodock Vina module plugin for PyMol (37). The two isoforms were first superimposed and then coordinates of the β -cyclodextrin from the original X-ray structure of AMPK CBM β 1 (1Z0M.pdb) were selected as the center of the docking box for AMPK CBM β 2 (2F15.pdb). The dimension of the box was set to 26.5 x 26.5 x 26.5 Å to include all key residues at the binding pocket.

Binding free energy calculations such as MM/PB(GB)SA and recently developed QM/MM-GBSA were applied to first determine a likely binding mode of the potent compounds (6456019 and 6469172) and subsequently the best method was used to predict the binding affinity of these and other compounds as well. Generally, binding free energy ($\Delta G_{\text{binding}}$) is computed based on the equations:

$$\Delta G_{\text{binding}} = \Delta H_{\text{tot}} - T\Delta S \quad (1)$$

$$\Delta H_{\text{tot}} = E_{\text{MM or QM/MM}} + \Delta G_{\text{sol}} \quad (2)$$

in which the ΔH_{tot} term represents the binding enthalpy value and the $-T\Delta S$ term is the entropy change upon ligand binding. The enthalpy is derived by summation of the molecular mechanics (MM) or the hybrid quantum mechanics/molecular mechanics (QM/MM) interaction and the free energy of solvation (ΔG_{sol}). The QM method used in this study are AM1-DH+ and PM6-DH+ in which the dispersion and hydrogen bond correction are added (38). The generalized Born model 8 (GB8) is used to calculate the free energy of solvation as it has been shown recently that the GB8 model performs better than other GB models (39). The entropy change upon ligand binding

was calculated by normal analysis. The calculated binding free energy (ΔG_{cal}) either MM/PB(GB)SA or QM/MM-GBSA was computed by application of the -MMPBSA.py module (40) implemented in AMBER12 (41) using the protocols as described previously (42,43) except for those parameters mentioned above.

Plasmids - Constructs for mammalian transient overexpression of myc-tagged AMPK α 1, AMPK β 1 and AMPK γ 1 were kindly provided by Dr. D. Carling (Imperial College London, London, United Kingdom). The construct of GFP-tagged CBM β 1 and CBM β 2 for retroviral infection of mammalian cells are described elsewhere (15). The CBM of the β 2 subunit (amino acid 67-163) was amplified by PCR and inserted into the pScherry2 expression vector (Delphigenetics) via the EcoRI and Sall restriction sites, resulting in bacterial expression constructs for Scherry-tagged CBM β 2.

5

Cell-free in vitro screening - Scherry-tagged CBM β 2 was expressed in Rosetta2 (DE3) Escherichia coli cells (Novagen). Bacteria were grown in auto-induction medium as described before (15), with the exception that the culture was upscaled to a 4-liter fermentor and run at 20°C for 30 hours. The protein was purified using metal affinity chromatography as previously described (15), and was subsequently loaded onto a column of epoxy-activated sepharose 5B (GE Healthcare) coupled to β -cyclodextrin. The column saturation was monitored using the red color of the Scherry tag. After column wash, the protein-resin complex was resuspended in a 50% slurry with PBS supplemented with 0.5% v/v Tween 20.

The principle of the competition assay is to test the ability of selected compounds to dissociate the protein from the immobilized β -cyclodextrin. The slurry is aliquoted in a 96-well plate where the compounds, dissolved in DMSO, are added 4 at a time, then for the active mixes only, one compound at a time to a final concentration of 100 μ M (final concentration of DMSO: 4% for mixes, 1% for singles). After settlement of the resin, the protein concentration found in the supernatant was measured with the Pierce BCA Protein Assay Kit (Life Technologies). The direct effect of the compound on BCA was measured in absence of protein/resin and subtracted to the first measure. This normalized absorbance was compared to effect on protein dissociation of vehicle (4% for mixes, 1% DMSO for singles) or 100 μ M maltoheptaose (Sigma Aldrich). We arbitrarily defined a difference of absorbance with the vehicle of 0.01 or higher, as a hit, if the associated two-tailed Student t-test gave a p-value below 0.05 (significant) or below 0.1 (near-significant).

Cell-based screening - C2C12 cells stably expressing the GFP-tagged CBM β 1 or β 2 were plated in a black 96-well plate (Greiner Bio-One) with 10,000 cells/well in assay medium (DMEM with 4.5 g/L glucose, L-Glutamine, 25 mM Hepes, without phenol red [Life Technologies], supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin [Life Technologies] and with or without 5% heat-inactivated fetal calf

serum [iFCS; Bodinco BV, Alkmaar, The Netherlands]). The following day, cells were stained with Hoechst (1 $\mu\text{g}/\text{mL}$ final concentration, Life Technologies) for 30 minutes then treated for 8 hours at 37°C according to conditions described below, subsequently fixed with 2% paraformaldehyde for 10 minutes and finally kept in PBS supplemented with azide. Fixed cells were imaged for Hoechst and GFP with the high-content imaging system BD Pathway 855, with 16 pictures per well using a 40X magnification and autofocus for each view. Images were processed using the BD AttoVision software (version 1.7) to define cell circumferences using both nuclear staining and cell outline obtained with high gain imaging (typically >200) of the GFP signal. With low exposure images (typically 250 ms), the GFP speckles, presumably caused by the concentrated binding of the GFP-tagged CBM to intracellular glycogen particles, were delineated. Next, image data were imported in Kaluza software (version 1.2 Beckman Counter, Inc.) for image cytometric analyses, which gave the mean number of GFP speckle per cell for each well (typically 150-200 cells were imaged in every well). Alternatively, the percentage of cells showing the expected GFP pattern was retrieved for each well by applying gating for a minimal count of one GFP speckle per cell. During the first screen, cells were treated with 25 μM compound (0.25% DMSO) in plain DMEM without serum or antibiotics and supplemented with 10 $\mu\text{g}/\text{mL}$ digitonin (Sigma-Aldrich) to facilitate cellular entry of the compounds. We arbitrarily defined a reduction in speckle number of 35%, as compared with the control, as a hit. The dose response titrations were performed at varying compound concentrations (0-100 μM) with a constant 1% DMSO in DMEM supplemented with 5% iFCS but without digitonin. The negative and positive control of speckle levels were the vehicle and 100 μM forskolin (1% DMSO), respectively. The dose response curves were fit to a dose-response model using GraphPad Prism software (GraphPad Software, Inc., version 5.03) to obtain IC50 measurements.

Cell culture and treatment - C2C12 cells were cultured in high-glucose medium (DMEM with 4.5 g/L glucose, L-glutamine and pyruvate, supplemented with 10% iFCS, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin) and differentiated for 7 days into skeletal muscle myotubes with differentiation medium (high glucose DMEM, supplemented with 2% horse serum [Life Technologies], 100 units/ml penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin). Human embryonic kidney HEK293T and human hepatic HepG2 cells were cultured in the same high-glucose medium. Human fibroblast-derived near-haploid HAP1 cells, WT or knock-out for either AMPK- β 1 or - β 2, were cultured according to distributor guidelines (Horizon Genomics). All these cells were treated in their own DMEM but with only 5% iFCS, except for undifferentiated C2C12 which were treated in assay medium (described above) with or without 5% iFCS. Apart from screened compounds, treatment included oligomycin (Sigma-Aldrich), AICAR (Sigma-Aldrich), A-769662, and phenformin: the duration and concentrations are mentioned throughout the results. Following treatment, cells were quickly lysed and the lysates were prepared for Western blotting. Alternatively, cells

were fixed then stained with a glycogen antibody (courtesy of Dr. O. Baba, Tokyo Medical and Dental University, Tokyo, Japan) and imaged by confocal microscopy as described elsewhere (15).

Cytotoxicity assay - C2C12 cells were plated in assay medium at 10,000 cells/well of a 96-well plate. The following day, cells were treated with different doses of compound, vehicle (1% DMSO), 1% Triton-X or left untreated. After 2 hours of treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Life Technologies) was added to the cells according to the manufacturer's recommendations. Cells were incubated for 4 hours at 37°C then DMSO was added and mixed until complete dissolution of the crystals. Absorbance was measured at 545 nm and normalized to that of untreated cells.

5 Glucose and fatty acid uptake - Glucose and fatty acid uptake were performed as previously described (44). Briefly, cardiomyocyte HL-1 cells were cultured according to the guidelines of the establishing team (45), and were treated in high glucose DMEM without iFCS. After overnight serum starvation, cells were pre-treated with 10 μ M Compound C (Dorsomorphin, abcamBiochemicals) or with vehicle for an hour, then 25 μ M of compound or vehicle (0.5 % DMSO final concentration) was added for an extra hour. Alternatively, 100 nM of insulin was added for the last 20 minutes of treatment. After stimulation, a mixture of [3 H]deoxyglucose and [14 C]palmitate was added to the cells for 10 minutes then washed away. Glucose and fatty acid uptake was assessed by scintillation counting of the cell lysate (44). Part of the final lysate was used for Western Blot.

Surface plasmon resonance - SCherry-tagged CBM β 2 protein was expressed and purified as described above and then immobilized on a SPR Sensorchip HC 1500 m (Xantecbioanalytics GmbH) by amine coupling to a density of 24,000 RU. The direct binding of the compounds was assessed at different concentrations in the running buffer (PBS, 0.05% Tween 20, 5% DMSO (v/v), pH 7.4) by surface plasmon resonance (Biacore T200; GE Healthcare). Sensorgrams were corrected for binding to an empty reference flow cell and were solvent corrected to account for slight changes in DMSO content. Data were analyzed with the BIAevaluation software and next compiled with GraphPad to obtain equilibrium dissociation constants (K_D).

Immunoprecipitation and kinase activity assay - HEK293T cells were transfected with myc-tagged AMPK α 1, AMPK γ 1 and either AMPK β 1 or β 2 using Lipofectamine 2000. Forty eight hours after transfection, cells were lysed and AMPK was immunoprecipitated using a myc-tag antibody (Cell Signaling Technology). For studying interaction partners of AMPK, before immunoprecipitation, the cell lysate was incubated with 5 mM β -cyclodextrin (Sigma-Aldrich), 25 μ M tested compounds or vehicle (0.5 % DMSO). Following immunoprecipitation, the samples were washed 3

times with lysis buffer before preparation of the proteins for Western Blot. In the case of the kinase assay, the immunoprecipitate was washed with the lysis buffer supplemented with high salt (1 M NaCl) to remove interacting partners, then 3 additional washes were performed with Tris Buffer (80 mM Tris with protease and phosphatase inhibitors, pH 7.4). The kinase assay was conducted following an adapted protocol from Promega for AMPK activity measurement using the ADP-Glo kit. Briefly, after the last wash, the kinase assay reactants were placed on the resin used to immunoprecipitate AMPK in a 2-fold concentrated manner to account for the bead volume. Final assay concentrations were 40 mM Tris, 20 mM MgCl₂, 0.1 mg/mL BSA, 50 μM DTT, 100 μM AMP, 150 μM ATP, 100 μM SAMS, and either 100 μM compound or corresponding vehicle (5 % DMSO). The kinase assay was incubated at room temperature for 60 minutes with regular resuspension of the beads. Then 25 μL of supernatant was transferred to a white 96-well plate to follow the ADP-Glo procedure (Promega) and measure with a luminometer (Glomax 96 Microplate Luminometer, Promega). The amount of ATP consumed by the kinase under different conditions was extrapolated from the standard curves as described in the Promega protocol.

Statistical analysis - All statistical analyses as well as graphics were prepared with the GraphPad Prism software (GraphPad Software, Inc., version 5.03).

RESULTS

A screening coupling *in silico* and *in vitro* steps to identify glycogen-delocalizing molecules

Each of the three AMPK subunits exists as isoforms, which partly exhibit tissue-specific expression profiles and different functions. In case of the β -subunit, the $\beta 1$ isoform is rather ubiquitously expressed, whereas the $\beta 2$ is more selectively expressed in muscle and liver tissues in humans (3,46) and has a higher affinity towards carbohydrate compared to the $\beta 1$ isoform (47). Considering that muscle and liver are very relevant target tissues in metabolic diseases (6) and contain high levels of glycogen, we aimed at targeting the $\beta 2$ -subunit and focused on that isoform in the design of the screening. However, inspection of the X-ray structures and protein sequences of both CBM isoforms revealed a high sequence identity of 82% and sequence similarity of 93% (**Fig. 1A**). Amino acid sequence conservation is especially high in the carbohydrate-binding region. The main difference between the β -isoforms resides in the insertion of a threonine in position 101 (Thr101, according to numbering of the human $\beta 2$ sequence) enlarging the loop of the lower edge of the $\beta 2$ -isoform binding pocket (**Fig. 1B**) (47). In order to take advantage of this particularity, the selection of the protein conformations sampled with molecular dynamics was based on the inclusion of this extra space, as well as the more defined carbohydrate-binding region. Since the Thr101 points towards the outside of the pocket, its side chain is not part of the defined pocket. However, the targeted pocket was lined with the side chains of the residues Ser93, Ser95, Trp99, Lys102, Gln124, Lys216, Trp133, Ser144, Leu146, Thr148 and Asn150, therefore it harbors both hydrophobic features and polar residues, which may allow hydrogen bond formation. Among these protein conformations, the eight largest pockets occupied a volume between 254 and 305 Å³ according to icmPocketFinder (261 to 371 Å³ according to DoGSiteScorer). Despite their rather small volumes, the good chemical properties of the pocket allowed them to be judged fairly satisfactory druggable pockets by the DoGSiteScorer analysis, scoring 0.59 in average on a scale from 0, undruggable, to 1, druggable. Validated carbohydrate-binding pockets are known to score quite poorly in that kind of analysis, which means that a score around 0.6 is acceptable for such pocket (48). These eight protein conformations were thus selected for the virtual screening.

A commercially available database of small molecules was retrieved and screened first with the Lipinski rule of 5 to retrieve compounds which are more likely to possess drug-like properties: this resulted in exclusion of ~10% of the compounds (722,000 compounds kept of the original 800,000 compounds; **Fig. 1C**). Subsequently, two consecutive steps of the virtual ligand screening of eight different CBM $\beta 2$ conformers followed an established protocol of rigid docking to remove the geometrically most unlikely compounds from the database, and then of a more exhaustive flexible docking that allows ligand and target side chain flexibility (31-33). The docked poses of the 1,000 best scoring compounds were visually inspected and

the top-800 were ordered for further testing in a two-step *in vitro* screening, as described in the following section (**Fig. 1C**). At the end of the process, 10 compounds were found positive in both assays, leading to a hit-rate of 1.25%. Interestingly, six of the compounds clustered together when a cutoff of 0.5 was used in ChemMine Binning Clustering Tool, whereas each of the other four compounds was binned separately. Therefore, this cluster of compounds, which dominated the hits, is further described below.

Figure 1. A screening coupling *in silico* and *in vitro* steps to identify glycogen-delocalizing molecules. (A) 2D-alignment of both CBMs sequences (Homo sapiens, UniProt accession codes: Q9Y478.4 for β1 and O43741.1 for β2), with conservation represented in symbols (“*” full, “.” strong, and “-” weak conservation) and bar graph; numbering based on human β1-sequence.

Figure 1 continued. Black arrows represent the known carbohydrate-binding residues, the blue arrow highlights the Thr101 insertion (in position 102 due to the numbering based on human β 1-sequence). **(B)** 3D-representations of AMPK β 1- (left, PDB ID: 1Z0M) and β 2- (right, PDB ID: 2F15) CBM in grey ribbon with the loop of positions 100-101 represented in blue and the binding pocket volume highlighted in orange balls. **(C)** Scheme representing the screening steps with associated number of compounds. The *in silico* steps are represented in blue and the *in vitro* ones in green; in white are the filtering steps.

Identification of similar compounds with ability to dislocate the CBM

The initial *in vitro* screening was based on a cell-free, carbohydrate-binding competition assay (**Fig. 2A**). First, the recombinant AMPK CBM β 2 domain was allowed to bind to the glycogen α 1-6 turn analog, β -cyclodextrin, which was immobilized on a resin. The isolated CBM domain was chosen over the AMPK heterotrimer because its use increased the signal to noise ratio (**Supp. Fig. 1A**; 1.2 vs 3.8 using maltoheptaose for the heterotrimer and CBM, respectively). Secondly, the compounds were added to the protein-resin complex in solution: since active compounds compete for interaction with the CBM, they should displace the protein from the resin-bound carbohydrate to the supernatant. The protein amount in the supernatant was estimated by the difference in absorbance, named “normalized sample absorbance”, between signal measurements in the presence of a compound and of vehicle only (**Fig. 2A**). To alleviate a direct effect of compounds on the BCA protein determination, the BCA absorbance was measured with the compound alone, in the absence of protein, and subtracted with the absorbance of vehicle alone, to give the “normalized background absorbance”.

The 800 compounds were first tested as mixes of four compounds at a concentration of 100 μ M, and the mixes showing a significant difference between normalized background and sample absorbance were selected for individual testing. After initial screening of single compounds at a concentration of 100 μ M, 42 single compounds were judged active (*i.e.*, difference between background and sample absorbance of at least 0.01), with a significant difference in 30 cases and near-significant difference in 12. Interestingly, nine of these compounds showed a very similar structure with two benzene rings linked by a flexible chain of 6–7 atoms of carbon and oxygen (**Table 1**). The ring “A” shared the exact same features across all hit compounds, while the substitutions on ring “B” vary. Since the substitutions of the ring “A” correspond to those of the natural product isoeugenol, this group of nine compounds is referred to as isoeugenol derivatives in the rest of the text. Isoeugenol derivatives exhibited a lower background absorbance compared to the vehicle (negative normalized background absorbance) probably by either stabilizing the copper of the BCA assay in an oxidized state or influencing directly the absorbance measurement. Six of them showed a significant difference with the normalized sample absorbance, and the three others reached near-significance (**Fig. 2B**), and were therefore considered active in this assay and included in the subsequent screening step. Of the complete set of tested compounds, only one other compound shared the

isoeugenol feature of the common structure (7969338, **Table 1**), however this compound showed no activity in the carbohydrate-binding competition assay (**Fig. 2B**). The shorter linker and bulkier ring “B” found in this compound compared to the active isoeugenol derivatives point toward a structure–activity relationship.

Figure 2. Identification of similar compounds with ability to dislocate the CBM. (A) Schematic representation of the carbohydrate-binding competition assay. (B) Normalized absorbances, as described in the scheme (background in grey, sample in black, represented as means with SEM), of the identified isoeugenol derivatives in the carbohydrate-binding competition assay. As described in the text, isoeugenol derivatives presumably interfered with the BCA readout, explaining the consistently negative normalized absorbances. Each compound and maltoheptaose (MalHep) were used at 100 μ M. Statistical significance: Student's two-tailed *t*-test of sample vs. background absorbance, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, #: $p < 0.1$, $n = 4$. (C) Representative image of C2C12 cells stably overexpressing the GFP-tagged CBM $\beta 2$ (in green). Cells were fixed then stained with an anti-glycogen antibody that was detected by an Alexa647-labeled secondary antibody (in red) and with DAPI for nuclei (in blue), after which co-localization was assessed by confocal imaging. (D) Percentage of cells showing the GFP speckled pattern normalized to level of vehicle (0.25% DMSO). Forskolin control was used at 100 μ M while the compounds were tested at 25 μ M, in serum-free medium supplemented with 10 μ g/mL digitonin. Statistical significance: Student's *t*-test compound vs. DMSO, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, $n = 4$.

For the following screening step, we used C2C12 cells stably over-expressing the GFP-tagged CBM β 2, which is known to show a punctuate pattern of GFP-positive areas that co-localize with glycogen (**Fig. 2C**) (15). Cells were incubated in the absence or presence of compounds and were monitored for the (dis)appearance of this peculiar pattern upon treatment. As a proof-of-concept, use of forskolin, an agent that depletes glycogen through the cAMP pathway, led to an 80% reduction in speckle amount (**Fig. 2D**). For the initial screening process, a small amount of digitonin was added to the serum-free medium, which allowed a better crossing of the membrane (**Supp. Fig. 1B**) without showing any obvious cytotoxicity. In this experimental setting, three of the nine aforementioned compounds were unfortunately not sufficiently soluble for testing, probably due to the relatively high hydrophobicity of this class of compounds ($\log P \approx 5$). In contrast, all other six compounds were found to significantly reduce the incidence of speckled pattern of the GFP-tagged CBM β 2, as judged by the observed lowering in the percentage of speckled cells compared to vehicle (**Fig. 2D**). Since they all decreased the number of speckles by more than the arbitrary threshold of 35%, the six compounds were judged active in cells and therefore classified as hit molecules. In summary, our two-step screening process following the virtual ligand screening, allowed us to identify isoeugenol derivatives with effects on carbohydrate binding both in a cell-free *in vitro* assay and in a cellular assay on subcellular localization.

Table 1. Structures and activities of the identified isoeugenol derivatives

Backbone	Description					Activity		
	ID	R1	R2	R3	R4	BCD	Cell	IC50
6449841					Passed	Unsoluble	
	6456019			BB—CH ₃		Passed	Passed	Micro-molar
	6457231					Passed	Passed	Out of range
	6460160	BB—CH ₃			BB—CH ₃	Passed	Unsoluble	
	6478568			BB—CH ₃	BB—CH ₃	Passed	Passed	Failed
	6481670		BB—CH ₃		BB—CH ₃	Passed	Passed	Out of range
6455633		BB—CH ₃		BB—CH ₃	Passed	Passed	Out of range
	6469172					Passed	Passed	Micro-molar
	6475310	BB—CH ₃	BB—CH ₃			Passed	Unsoluble
7969338					Failed		

R1, R2, R3 and R4 correspond to the substitution on the benzene ring "B". "BB" stands for Backbone, the blank spaces mean either that the substitution is a hydrogen, or, for the last compound, no additional substitution. "BCD" stands for the β -cyclodextrin-based cell free assay.

Table 1 continued. “Cell” stands for the cellular assay of GFP speckle quantification; “IC50” refers to the IC50 determination in CBM β 2 expressing cells by quantification of GFP speckles.

Two of the identified compounds have a micromolar activity and directly bind the CBM

To follow up on the cellular activity of the 6 identified compounds, half maximal inhibitory concentrations (IC50) were measured in absence of digitonin and in presence of serum by testing different concentrations in the cell-based assay. The compounds 6456019 and 6469172 showed micromolar activity (1.3 [0.1-14] and 8.9 [4-19] μ M [95% confidence interval, CI95] respectively; **Fig. 3A**). The compound 6456019 only displaced up to 49% of the total amount of speckles, while addition of compound 6469172 maximally resulted in a 92% displacement. However, the compounds 6455633, 6457231 and 6481670 seem to have little activity at the described concentrations and their IC50 could not be determined. The compound 6478568 did not show a significant decrease in speckle amount at any of the concentrations. This lack of activity of the four latter compounds is inconsistent with the effects seen during the cellular screening, which might for example be explained by their inability to cross the membrane readily in the absence of digitonin.

For the two remaining hit molecules with low micromolar activity (6469172 and 6456019), surface plasmon resonance was performed on the immobilized CBM β 2 to assess direct interaction. The resulting dose-dependent response confirmed direct binding. Supporting a direct effect in the cellular assay, the estimated dissociation constants of both compounds were in the same range as their IC50, with overlapping 95% confidence intervals (5.4 [3.9-7.5] and 25 [19-33] μ M [CI95] for 6456019 and 6469172 respectively; **Fig. 3B**). Therefore, the compounds 6456019 and 6469172 show high activity towards speckle disappearance in cells even in the absence of digitonin, and can directly bind to the recombinant CBM β 2. To further confirm a potential role of the compounds in glycogen-related pathways and to test the effect on heterotrimeric AMPK, we investigated the effect of the compounds 6469172 and 6456019 on the interaction of AMPK with glycogen-bound downstream targets in a cell-free setting. The heterotrimer AMPK α 1 β 2 γ 1 was immunoprecipitated from co-transfected HEK293T cells (myc- α 1, β 2, γ 1). Under basal condition, glycogen synthase immunoprecipitated with AMPK (**Fig. 3C**). However, when the cell lysate was pre-incubated with the glycogen mimetic, β -cyclodextrin, glycogen synthase was no longer recovered in the immunoprecipitate of AMPK, suggesting that glycogen may act as a scaffold between the two proteins in the lysate and that β -cyclodextrin disrupts their glycogen binding. Both compounds 6469172 and 6456019 similarly decreased the interaction of AMPK with glycogen synthase, showing their ability to mimic β -cyclodextrin and therefore suggesting a common binding pocket and action on AMPK. To verify that this effect was not an artifact, the same experiment was carried out with a CBM-unrelated AMPK activator, oligomycin (**Supp. Fig. 2**).

As expected, oligomycin did not disrupt the co-immunoprecipitation of AMPK and glycogen synthase. Therefore, the effect of 6469172 and 6456019 on AMPK heterotrimer was specific and similar to that induced by β -cyclodextrin.

Figure 3. Two of the identified compounds have a micromolar activity and directly bind the CBM. (A, D) IC₅₀ determination based on the average number of speckles per cell following treatment with a range of concentration (0-100 μ M). Assay was carried out for a selected number of compounds with CBM β 2-expressing C2C12 cells (**A**) and for 6469172 with CBM β 1-expressing C2C12 cells (**D**). Max inhibition refers to the maximal percentage of speckles removed by the tested compound. CI95: 95% confidence interval, Stdev: standard deviation, $n=3$ repeats with >150 imaged cells each. (**B**) Determination of the equilibrium dissociation constant (K_D) by surface plasmon resonance using immobilized CBM β 2 and different concentrations of 6456019 and 6469172 (0-50 μ M for 6456019 and 0-100 μ M for 6469172); $n=4$. (**C**) Lysate of AMPK-overexpressing mammalian cells treated with either vehicle (0.5% DMSO), 25 μ M of compound 6469172 or 6456019, or 5 mM β -cyclodextrin (left panel). Immunoprecipitation of AMPK heterotrimer was performed with the myc antibody following the treatment (right panel). The used antibodies recognize total level of glycogen synthase (GS), total level of AMPK- β 2 subunit (AMPK- β 2), and phosphorylation status of AMPK at Thr172 (AMPK-pThr172); $n=2$

After confirming an effect of both compounds on the CBM β 2 and on the β 2-containing AMPK heterotrimer, we sought to check for isoform specificity. In view of possessing both micromolar IC₅₀ and high amplitude of action, compound 6469172 was selected for further testing on cells expressing the GFP-tagged CBM β 1 in the same setting as the described cellular screening. Expectedly, owing to the lack of pocket specificity, the determined IC₅₀ was similar for both protein isoforms (10.9 [2-50] μ M for CBM β 1 vs. previously mentioned 8.9 [4-19] μ M for CBM β 2 [CI95]; **Fig. 3A and 3D**), suggesting absence of β -isoform selectivity.

In summary, the compounds 6469172 and 6456019 are direct binders of the carbohydrate-binding module of AMPK and have an activity in the micromolar range to change the subcellular localization of AMPK away from glycogen.

Docking poses of the identified compounds suggest an explanation for the difference in affinity and absence of isoform-specificity of the identified compounds

The six compounds of the identified cluster tested for IC₅₀ (**Table 1**) were re-docked into the binding pocket of CBM β 2 structures with AutoDock Vina to obtain a more detailed insight into their structure–activity relationship. Molecular docking of the active compounds (6456019 and 6469172) yielded two different possible binding modes for each compound. All binding poses share a similar orientation of the common ring “A” toward the loop of Thr101 (numbering of the human sequence of AMPK- β 2), while the linker is positioned between Trp99 and the loop of Leu146 and the variable ring “B” is close to Gln124 (**Fig. 4A and 4B**, **Supp. Fig. 3A and 3B**). While in all conformations, the compounds occupy the carbohydrate-binding site (as seen in the right panel of **Fig. 4A** compared with pocket definition shown in the right panel of **Fig. 1B**), the orientation of the rings account for main differences between the two poses found for each compound.

Several binding free energy calculations (*e.g.*, MM/PB(GB)SA and QM/MM-GBSA), which were previously shown to perform well in predicting binding affinity (42,49) and binding mode (43,50,51), were applied to estimate the binding strength for the two compounds in these two different poses. For both compounds 6456019 and 6469172, the poses depicted in **Fig. 4A and 4B** (defined as mode 1) had consistently lower binding free energy across calculation methods, compared to those in **Supp. Fig. 3A and 3B** (defined as mode 2) (**Supp. Fig. 3C**). Therefore, the binding mode 1 is more favorable and represents a likely binding mode of these analogous compounds in the binding pocket of CBM β 2.

Interestingly, the position occupied by the compound in the proposed binding modes resides in an area that is identical between CBM β 1 and CBM β 2 (**Fig. 4C**). Although the ring “A” substitution points towards the loop formed by Thr101 in the β 2-isoform, the compound does not fully occupy the extra space and can therefore also be accommodated in the smaller pocket of the β 1-isoform. Besides, the Thr101 residue, which is not present in CBM β 1, is flipped towards the outside of the binding pocket and cannot form additional interactions. Thus, these data could explain the absence of isoform-specificity seen in the *in vitro* experiments (**Fig. 3D**). When looking at the poses of the two most active compounds, the linker of 6456019 seems more interesting than that of 6469172 since the extra oxygens can form hydrogen-bonds with Trp99 and Asn150 (**Fig. 4B**). Interestingly, substitutions to the ring B of both compounds interact with additional residues (**Fig. 4A and 4B**): the nitro group at the R1 of 6456019 induces an electrostatic interaction with Asn98, while in R1/R2 of 6469172, the pyridine ring forms a hydrogen bond with Trp99. In contrast, none of the four other compounds tested for IC₅₀ (6457231, 6478568, 641870 and 6455633) could form these stabilizing interactions since their substitutions on ring B are only hydrophobic groups (**Table 1**). Therefore, the extra hydrogen bonds with Trp99 and Asn98 formed by 6469172 and 6456019 could explain the difference seen in IC₅₀.

124

Among the different methods used to calculate binding free energy (**Supp. Fig. 3C**), the QM/MM-GBSA approach using PM6-DH+ method shows the best performance since the calculated binding free energy ($\Delta G_{\text{cal.}}$) of the 6456019 and 6469172 compound derived by this method is in close agreement with the available experimental data ($\Delta G_{\text{exp.}}$). The free energy differences of the 6456019 compound are $\Delta G_{\text{cal.}}$ -7.93 kcal/mol vs. $\Delta G_{\text{exp.}}$ -7.23 kcal/mol and for 6469172 $\Delta G_{\text{cal.}}$ -6.57 kcal/mol vs. $\Delta G_{\text{exp.}}$ -6.93 kcal/mol. Thus, this binding free energy calculation method was also applied to estimate binding affinity of the other 4 selected compounds. The inactive compounds (6457231, 6478568, 641870 and 6455633) gave positive or less negative binding free energy difference implying an unfavorable binding to CBM β 2, which is in agreement with the experimental data (**Table 2**).

Table 2. Experimental and predicted binding free energy of the compounds tested for IC50 determination.

Compound ID	K_D (μM)	$\Delta G_{\text{exp.}}$ (kcal/mol)	$\Delta G_{\text{cal.}}$ (QM/MM-GBSA, PM6-DH+)	$ \Delta G_{\text{exp.}} - \Delta G_{\text{cal.}} $
6481670			1.89	
6455633			4.98	
6456019	5.44	-7.23	-7.93	0.70
6457231			-1.19	
6478568			-1.67	
6469172	25.40	-6.31	-6.57	0.26

In summary, the re-docking data offer interesting explanations for the lack of isoform specificity and for differences in binding affinities between compounds. The agreement between virtual and experimental data support the suggested binding modes and thus suggests a direct binding in the carbohydrate-binding pocket of AMPK.

Compound 6469172 is a cellular but not an allosteric activator of AMPK

Compound 6469172, which showed the highest activity in the cell-based assay to measure subcellular localization (**Fig. 3A**), was next studied for its ability to influence the AMPK pathway in C2C12 cells. Dose-dependent activation of AMPK and phosphorylation of its downstream target acetyl-CoA carboxylase (ACC) were seen after compound treatment (**Fig. 5A**). At higher concentrations, the effect of compound 6469172 was similar to that of oligomycin, an indirect AMPK activator. This AMPK activating effect was not due to cytotoxicity of the compound, as appeared from the MTT assay results, where no difference in cell viability was seen at any concentration compared to vehicle (**Supp. Fig. 4**). Activation by 6469172 was also time-dependent with a quick onset of AMPK and ACC phosphorylation but reaching a maximal activation after a longer period of time (**Fig. 5B**). In contrast, the oligomycin effect hit a maximal action on phosphorylation quickly and then faded away, suggesting either a quicker absorption or a different mechanism of action for these two AMPK activators.

Based on the previous results (**Fig. 3 and 4**), we did not expect β -isoform specificity, which we sought to confirm using the near-haploid cell line HAP1. Analysis of HAP1 cell lysates showed clearly increased AMPK and ACC phosphorylation upon 6469172 treatment and a milder response for AICAR (**Fig. 5C**). Interestingly, A-769662 treatment led to an increase in ACC phosphorylation while AMPK phosphorylation remained low, suggesting that AMPK allosteric activation drives most of the effect in this cell line. In HAP1 cell lines, either of the two β -subunit isoforms were targeted using the CRISPR/Cas9 system resulting in clean knock-outs as can be seen from the β -subunit expression (**Fig. 5C**). As was expected, upon treatment with 6469172 both knockout cell lines showed strong Thr-172 phosphorylation signals comparable to the WT, confirming the absence of β -isoform specificity. Similarly, AICAR activated AMPK in all cell lines, although somewhat stronger Thr-172 phosphorylation signals were obtained for the β 1-knockout. A-769662 treatments were also comparably efficient in knockout cell lines and WT with regard to enhancement of ACC phosphorylation signal. In contrast to the WT and β 2-knockout cell lines, treatment with A-769662 increased AMPK Thr-172 phosphorylation in the β 1-knockout cell line, indicating that in absence of the β 1-isoform, the action of A-769662 on AMPK may follow another mechanism (*i.e.*, change in phosphorylation status rather than allosteric activation; see Discussion for details). Therefore, the 6469172 compound activates both β 1- and β 2- containing AMPK heterotrimers.

AMPK is mainly activated through three methods: allosteric activation, facilitation of Thr172 phosphorylation or protection from Thr172 dephosphorylation. To test whether 6469172 acted allosterically to upregulate AMPK activity, immunoprecipitated myc-tagged AMPK heterotrimers from triple-transfected HEK293T cells (myc- α 1, β 2, γ 1) were tested for kinase activity. The enzymatic activity assessed by this assay seemed truly specific to AMPK, since the addition of SAMS peptide (substrate for AMPK) led to a dramatic increase in ATP consumption (**Fig.**

5D). The presence of the known allosteric activator A-769662 increased basal AMPK activity of the $\alpha 1\beta 1\gamma 1$ but not $\alpha 1\beta 2\gamma 1$ complex. On the contrary, and as expected, the compound 6469172 failed to increase AMPK activity in both cases, suggesting that 6469172-induced AMPK activation in cells may rather depend on increased Thr172 phosphorylation than on a direct allosteric effect. Therefore, we conclude that 6469172 is a novel AMPK activator without β -isoform specificity, whose mechanism of action does not rely on allosteric effect.

Figure 5. Compound 6469172 is a cellular but not an allosteric activator of AMPK. (A) Dose-dependent response of 6469172 treatment on AMPK and ACC phosphorylation. Assay carried out in C2C12 cells after 1.5 hour-treatment, doses in μM with a constant amount of vehicle (1% DMSO). Oligomycin was used at 5 μM for 1.5 hour. (B) Time-dependent response of AMPK and ACC phosphorylation in C2C12 cells after 15, 30 or 60-minute treatment with 25 μM of 6469172 compound or 5 μM oligomycin with a constant amount of vehicle (1% DMSO, 60 minute-treatment). (C) Treatment of the haploid cell line HAP1, in its WT form, or with a knock-out of either the AMPK- $\beta 1$ ($\beta 1$ -KO) or - $\beta 2$ subunit ($\beta 2$ -KO). 2-hour treatment of vehicle (0.25% DMSO) or 50 μM 6469172, or 1-hour treatment of 1.5 mM AICAR or 100 μM A769662. (A–C) The used antibodies recognize total level of ACC protein (ACC), phosphorylation status of ACC at Ser79 (ACC-pSer79), total level of AMPK- α subunit (AMPK- $\alpha 1/2$), total level of AMPK- β subunit (AMPK- $\beta 1/2$), phosphorylation status of AMPK at Thr172 (AMPK-pThr172) and total level of β -actin (B-actin) which was used as loading control. (D) Kinase activity assay on myc-tagged AMPK heterotrimers $\alpha 1\beta 1\gamma 1$ (left panel) or $\alpha 1\beta 2\gamma 1$ (right) immunoprecipitated from mammalian cells. Assays were done in the absence (white bars) or presence (grey bars) of 100 μM SAMS peptide, an AMPK substrate, and with 100 μM 6469172 or 50 μM A769662 in constant vehicle content (5% DMSO).

The identified compound affects metabolism-related cell lines and pathways

Muscle and liver are important organs for glycogen storage and therefore are of particular interest for our study. To assess the ability of 6469172 to activate AMPK in more physiologically relevant cell types, C2C12 cells were differentiated into myotubes. The treatment with 6469172 induced a strong AMPK and ACC phosphorylation comparable to that of other known AMPK activators (**Fig. 6A**). Secondly, the human hepatoma cell line HepG2 was also sensitive to 6469172 and other AMPK activator treatments, showing a strong AMPK and ACC phosphorylation (**Fig. 6B**). Therefore, the compound 6469172, which was shown to have the most interesting effect on CBM displacement from glycogen, also activates the AMPK pathway in relevant cell lines.

Figure 6. The identified compound affects metabolism-related cell lines and pathway. (A) Treatment of C2C12 cells differentiated in myotubes: 2-hour treatment with vehicle (1% DMSO) or 75 μ M 6469172, or 1-hour treatment with 1.5 mM AICAR, 100 μ M A769662 or 1.5 mM phenformin. (B) Treatment of the hepatic cell line HepG2: 2-hour treatment with vehicle (0.75% DMSO) or 75 μ M 6469172, or 1-hour treatment with 1.5 mM AICAR or 100 μ M A769662. (C) Glucose uptake measurement following treatment with vehicle (0.5% DMSO), 100 nM insulin or 25 μ M 6469172, with (grey bars) or without (white bars) pre-treatment with 10 μ M Compound C. Statistical significance: Student's *t*-test of vehicle without Compound C vs other treatment, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, $n = 4$. (D) Western Blot resulting from the previously described glucose uptake experiment. CC = Compound C pre-treatment. (A–B, D) The used antibodies recognize total level of ACC protein (ACC), phosphorylation status of ACC at Ser79 (ACC-pSer79), total level of AMPK- α subunit (AMPK- α 1/2), total level of AMPK- β subunit (AMPK- β 1/2), phosphorylation status of AMPK at Thr172 (AMPK-pThr172), phosphorylation status of AS160 at Thr642 (AS160-pThr642), total level of Akt protein (Akt), phosphorylation status of Akt at Ser473 (Akt-pSer473) and total level of β -actin (β -actin) which was used as loading control.

A well-documented role of AMPK is the increase of glucose and fatty acid uptake by triggering transporter translocation to the membrane (6). In HL-1 cardiomyocytes, treatment with 6469172 resulted in an increase in glucose uptake similar to that induced by insulin (**Fig. 6C**, left panel). Surprisingly, pre-treatment with the AMPK inhibitor Compound C raised the unstimulated levels of glucose uptake. A trend toward a further increase was seen upon insulin co-treatment, whereas the compound 6469172 in conjunction with Compound C did not lead to any further increase. Regarding fatty acid entry into HL-1 cardiomyocytes, the same increase in unstimulated uptake was seen with Compound C (**Fig. 6C**, right panel). With or without Compound C, insulin failed to trigger an increase in fatty acid uptake in that cell line. On the contrary, the compound 6469172 led to a 1.6-fold increase compared to basal, which was blunted after Compound C treatment. These results were mirrored by the Western Blot results (**Fig. 6D**): insulin strongly upregulated Akt and AS160 phosphorylation and slightly decreased AMPK and ACC phosphorylation, whereas 6469172 had the exact opposite effect on both pathways. Compound C reduced the AMPK and ACC phosphorylation even in presence of 6469172 and triggered the insulin pathway, as seen with the increased Akt and AS160 phosphorylation in each treatment, which could explain the increased nutrient uptake in Compound C treated samples. These results point to a potential beneficial effect of treatment with 6469172, since showing increased glucose and fatty acid uptake in an insulin-independent manner.

DISCUSSION

5 In this study, we first established a new protocol to target the CBM of AMPK by means of virtual ligand screening followed by a carbohydrate binding competition assay and monitoring of CBM subcellular localization. Using this workflow, we identified a set of structurally related compounds, the isoeugenol derivatives, with satisfactory scores in all screening steps. Two of these were found to bind directly to the CBM by surface plasmon resonance and mimic the β -cyclodextrin induced dissociation of the AMPK heterotrimer from another glycogen-bound protein, *i.e.*, glycogen synthase. The differences in activity seen in the cluster of isoeugenol derivatives (**Table 1**) could be explained by the presence of additional hydrogen-bonds in the most active ones that are predicted to stabilize the interaction between the compound and the CBM. The compound 6469172 additionally potently activated AMPK in several relevant cell lines, but did not allosterically activate AMPK as determined by cell-free kinase assays. Furthermore, 6469172 triggered increased nutrient uptake in HL-1 cardiomyocytes that was correlated to AMPK activation and blunted by treatment with the AMPK inhibitor Compound C.

The method of screening we established here is meant to identify agents that disrupt the glycogen–AMPK interaction. The *in silico* screen coupled with a two-step *in vitro* assessment led to a hit rate of 1.25%, which is in the reported range for a typical virtual screening (1–5 %) and better than that of classical high-throughput screenings (≤ 0.01 % success) (52). Higher success rates are generally achieved in virtual screenings when the amount of data on receptor structure and known ligands is high (52). In our case, only carbohydrates were known to bind to the CBM at the start of the project and they cannot be used for training in a classical virtual screening since requiring specific docking methods (53). The identification of a first set of active and inactive compounds, and the production of direct-binding experimental data, is therefore a great opportunity for further *in silico* screening attempts. In particular in the phase of compound development and optimization, computer-based techniques help to prioritize the possible compounds to be tested (54). In this study, the binding free energy calculations support the proposed binding modes since the results of the QM/MM-GBSA approach on these poses are in conformity with the experimentally determined values. However, in other studies, this method has also been successfully applied prior to compound ordering to further discriminate potential active and inactive compounds (43). Given the promising results obtained in this application, we expect such calculations to be useful for further lead generation of the isoeugenol derivatives as well.

The absence of β -isoform specificity for the identified compounds could be foreseen by the high global sequence similarity between the two isoforms and especially within the binding pocket (**Fig. 1A**). However, the predicted binding poses found by re-docking of the active compounds, which are supported by binding free energy calculations, show that the identified compounds do not fully take advantage of the larger pocket induced by the Thr101 insertion in the β 2-isoform (**Fig. 4A**).

Hence, it identifies a strategy for further hit-to-lead optimization to possibly reach isoform specificity. Nevertheless, isoform preference rather than specificity may be a more appropriate goal in the AMPK field, considering the close resemblance between AMPK holoenzymes and their complex regulation. For instance, the well-characterized activator A-769662 was often reported as being β 1-isoform specific: in recombinant protein activity assays, only β 1-containing complexes are activated independently of the α - and γ -isoforms (55), while in cells and tissues expressing mainly β 2 (56) as well as in β 1 knockouts (57), A-769662 fails to activate AMPK and AMPK-dependent pathways. In agreement with this view, in the kinase activity assay of this study, the immunoprecipitated β 1-containing complex, but not the β 2 heterotrimer, was activated with 50 μ M A-769662 (**Fig. 5D**). However, in the haploid cell lines, AMPK was activated upon 100 μ M A-769662 treatment independently of the expressed β -isoform (**Fig. 5C**). This result falls in line with other reports that showed that β 2-complexes can bind the A-769662 (58) with a dissociation constant higher than that of β 1 complexes but still in the low micromolar range (7) and that β 2-expressing tissues, like heart (59) and skeletal muscle (10,60,61), were sensitive to the compound. Moreover, A-769662 can provide a certain degree of protection against dephosphorylation to β 2-containing heterotrimers *in vitro* (57): this may be the mechanism at play in the β 1-deficient HAP1 cells as the phosphorylation degree of AMPK was increased upon treatment in this cell line compared to the WT and β 2-knockout cells (**Fig. 5C**). Therefore, even in the case of the well-established A-769662 compound, isoform preference remains a delicate puzzle. As described above, in the case of compound 6469172, a possible optimization strategy remains to be exploited based on our first results, which may lead to preference for β 2-containing complex, and therefore give a profile reciprocal to that of A-769662.

The identification of a cluster of six isoeugenol derivatives among the hit molecules provides with a head start for future studies on the structure–activity relationship. Isoeugenol is a natural compound found in essential oil of allspice (*Pimenta dioica*) or in Petunia flowers, for instance (36). In industry, this compound is widely found in fragrances and flavors, and is also used as precursor for the synthesis of vanillin (62,63). Although isoeugenol is classified as one of the most important fragrance allergens (62), the known applications of isoeugenol testify of a rather benign compound, which bodes well for the development of isoeugenol derivatives for possible use as drugs. Moreover, the risk profile may well be altered by the second ring found in all active derivatives of isoeugenol connected to the first by a carbon-oxygen linker of 6–7 atoms (**Fig. 4, Table 1**). As preliminary toxicological data, we did not find effects on cell viability (**Supp. Fig. 4**). To refine the definition of necessary features for both safety and potency, more derivatives of the identified compounds will need to be screened. In this future attempt, our protocol of *in vitro* screenings should come of use to quickly identify more active compounds.

Importantly, our screening is based exclusively on the CBM of AMPK and not on the heterotrimer, and thus relies on the assumption that AMPK–glycogen binding depends mainly on the CBM and little to no cooperative effect of the heterotrimer is needed. While we cannot formally exclude any influence on the CBM from the surrounding parts of the AMPK heterotrimer, several lines of evidence indicate the dominant contribution of CBM to glycogen binding. Firstly, for each of the β -subunit isoforms the reported dissociation constants for carbohydrates were similar for the isolated CBM (47) and the heterotrimer (64), *i.e.*, only one to three-fold difference with better binding for the isolated CBM. Secondly, the CBM was recently reported to directly interact with the kinase domain of the α -subunit and notably form the binding pocket needed to accommodate A-769662 (7,46). However, β -cyclodextrin binding to the CBM prevented interaction with the kinase domain indicating that the carbohydrate-binding ability of AMPK does not rely on interaction with the α -subunit (65). Thirdly, hydrogen/deuterium exchange mass spectroscopy showed that the β -cyclodextrin binding did not affect the α - and γ -subunits but only the CBM (58). Finally, since our identified compound mimicked the effect of β -cyclodextrin binding on the immunoprecipitated AMPK heterotrimer, we remain confident that focusing on the CBM during the screening was a sensible approach to target the heterotrimer.

The intended goal of the screening was to find a compound inducing the AMPK subcellular localization change from glycogen-bound to cytosolic free. Unexpectedly, our screening led to the identification of a new cellular activator of AMPK. The 6469172-stimulated AMPK activation was correlated with an increase in glucose and fatty acid uptake in a cardiomyocyte cell line. Similarly, also AICAR treatment and contraction cause an insulin-independent upregulation of glucose (66-68) and fatty acid (69) uptake in cardiac and skeletal muscle. Interestingly, these AMPK-dependent effects, as well as others including glycogen synthase phosphorylation, depend on glycogen levels (70,71). Despite some discrepancy, several papers reported an interdependence between AMPK activity and glycogen (3). McBride *et al.* tried to reconcile the different views by suggesting that although AMPK can bind to both branching points of glycogen, only the α 1-6 branches are inhibitory and thus different glycogen levels may lead to different effects on AMPK (17). This hypothesis was thoroughly tested in cell-free experiments but could not be tested in cells. As mentioned above, another study reported disruption of the CBM and kinase domain interaction upon carbohydrate binding which may explain the inhibitory effect of glycogen on AMPK (65). However, the precise mechanism through which glycogen branching points inhibits AMPK is largely unknown at this stage. In contrast to the branching points, 6469172 activates AMPK, but although we provide evidence that the compound does not act in an allosteric manner in cell-free kinase assays, the exact mechanism triggering this activation requires further clarification.

In summary, we established a new screening procedure for identification of compounds capable of preventing AMPK from binding to glycogen. The implementation of the screening uncovered an interesting cluster of isoeugenol derivatives able to compete the binding of the CBM domain of AMPK to the carbohydrate β -cyclodextrin and change the subcellular localization of the AMPK molecule. The compound 6469172 directly binds the recombinant CBM, activates cellular AMPK and triggers glucose and fatty acid uptake. Although many questions remain open, the duality of action on AMPK glycogen-binding and activity points to possible uses in models of metabolic disease.

REFERENCES

1. Yun, H., and Ha, J. (2011) AMP-activated protein kinase modulators: a patent review (2006 - 2010). *Expert Opin Ther Pat* **21**, 983-1005
2. Rana, S., Blowers, E. C., and Natarajan, A. (2015) Small molecule adenosine 5'-monophosphate activated protein kinase (AMPK) modulators and human diseases. *J Med Chem* **58**, 2-29
3. Viollet, B., Horman, S., Leclerc, J., Lantier, L., Foretz, M., Billaud, M., Giri, S., and Andreelli, F. (2010) AMPK inhibition in health and disease. *Crit Rev Biochem Mol Biol* **45**, 276-295
4. Wang, S., Song, P., and Zou, M. H. (2012) AMP-activated protein kinase, stress responses and cardiovascular diseases. *Clin Sci (Lond)* **122**, 555-573
5. Hardie, D. G. (2014) AMPK--sensing energy while talking to other signaling pathways. *Cell Metab* **20**, 939-952
6. Fogarty, S., and Hardie, D. G. (2010) Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. *Biochim Biophys Acta* **1804**, 581-591
7. Xiao, B., Sanders, M. J., Carmena, D., Bright, N. J., Haire, L. F., Underwood, E., Patel, B. R., Heath, R. B., Walker, P. A., Hallen, S., Giordanetto, F., Martin, S. R., Carling, D., and Gamblin, S. J. (2013) Structural basis of AMPK regulation by small molecule activators. *Nat Commun* **4**, 3017
8. Sanders, M. J., Ali, Z. S., Hegarty, B. D., Heath, R., Snowden, M. A., and Carling, D. (2007) Defining the mechanism of activation of AMP-activated protein kinase by the small molecule A-769662, a member of the thienopyridone family. *J Biol Chem* **282**, 32539-32548
9. Scott, J. W., Ling, N., Issa, S. M., Dite, T. A., O'Brien, M. T., Chen, Z. P., Galic, S., Langendorf, C. G., Steinberg, G. R., Kemp, B. E., and Oakhill, J. S. (2014) Small molecule drug A-769662 and AMP synergistically activate naive AMPK independent of upstream kinase signaling. *Chem Biol* **21**, 619-627
10. Ducommun, S., Ford, R. J., Bultot, L., Deak, M., Bertrand, L., Kemp, B. E., Steinberg, G. R., and Sakamoto, K. (2014) Enhanced activation of cellular AMPK by dual-small molecule treatment: AICAR and A769662. *Am J Physiol Endocrinol Metab* **306**, E688-696
11. Pang, T., Zhang, Z. S., Gu, M., Qiu, B. Y., Yu, L. F., Cao, P. R., Shao, W., Su, M. B., Li, J. Y., Nan, F. J., and Li, J. (2008) Small molecule antagonizes autoinhibition and activates AMP-activated protein kinase in cells. *J Biol Chem* **283**, 16051-16060
12. Jensen, T. E., Ross, F. A., Kleinert, M., Sylow, L., Knudsen, J. R., Gowans, G. J., Hardie, D. G., and Richter, E. A. (2015) PT-1 selectively activates AMPK-gamma1 complexes in mouse skeletal muscle, but activates all three gamma subunit complexes in cultured human cells by inhibiting the respiratory chain. *Biochem J* **467**, 461-472
13. Hudson, E. R., Pan, D. A., James, J., Lucocq, J. M., Hawley, S. A., Green, K. A., Baba, O., Terashima, T., and Hardie, D. G. (2003) A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. *Curr Biol* **13**, 861-866
14. Polekhina, G., Gupta, A., Michell, B. J., van Denderen, B., Murthy, S., Feil, S. C., Jennings, I. G., Campbell, D. J., Witters, L. A., Parker, M. W., Kemp, B. E., and Stapleton, D. (2003) AMPK beta subunit targets metabolic stress sensing to glycogen. *Curr Biol* **13**, 867-871
15. Oligschlaeger, Y., Miglianico, M., Chanda, D., Scholz, R., Thali, R. F., Tuerk, R., Stapleton, D. I., Gooley, P. R., and Neumann, D. (2015) The Recruitment of AMP-activated Protein Kinase to Glycogen Is Regulated by Autophosphorylation. *J Biol Chem* **290**, 11715-11728
16. Jorgensen, S. B., Nielsen, J. N., Birk, J. B., Olsen, G. S., Viollet, B., Andreelli, F., Schjerling, P., Vaulont, S., Hardie, D. G., Hansen, B. F., Richter, E. A., and Wojtaszewski, J. F. (2004) The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* **53**, 3074-3081
17. McBride, A., Ghilagaber, S., Nikolaev, A., and Hardie, D. G. (2009) The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. *Cell Metab* **9**, 23-34
18. Polekhina, G., Gupta, A., van Denderen, B. J., Feil, S. C., Kemp, B. E., Stapleton, D., and Parker, M. W. (2005) Structural basis for glycogen recognition by AMP-activated protein kinase. *Structure* **13**, 1453-1462
19. Joosten, R. P., Joosten, K., Cohen, S. X., Vriend, G., and Perrakis, A. (2011) Automatic rebuilding and optimization of crystallographic structures in the Protein Data Bank. *Bioinformatics* **27**, 3392-3398
20. Krieger, E., Koraimann, G., and Vriend, G. (2002) Increasing the precision of comparative models with YASARA NOVA--a self-parameterizing force field. *Proteins* **47**, 393-402
21. Laskowski, R. A., Moss, D. S., and Thornton, J. M. (1993) Main-chain bond lengths and bond angles in protein structures. *J Mol Biol* **231**, 1049-1067
22. Vriend, G. (1990) WHAT IF: a molecular modeling and drug design program. *J Mol Graph* **8**, 52-56, 29
23. Pontius, J., Richelle, J., and Wodak, S. J. (1996) Deviations from standard atomic volumes as a quality measure for protein crystal structures. *J Mol Biol* **264**, 121-136
24. Eisenberg, D., Luthy, R., and Bowie, J. U. (1997) VERIFY3D: assessment of protein models with three-dimensional profiles. *Methods Enzymol* **277**, 396-404
25. Colovos, C., and Yeates, T. O. (1993) Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Sci* **2**, 1511-1519
26. Volkamer, A., Kuhn, D., Grombacher, T., Rippmann, F., and Rarey, M. (2012) Combining global and local measures for structure-based druggability predictions. *J Chem Inf Model* **52**, 360-372
27. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-2948
28. Lagorce, D., Sperandio, O., Galons, H., Miteva, M. A., and Villoutreix, B. O. (2008) FAF-Drugs2: free ADME/tox filtering tool to assist drug discovery and chemical biology projects. *BMC Bioinformatics* **9**, 396
29. Pollastri, M. P. (2010) Overview on the Rule of Five. *Curr Protoc Pharmacol* **Chapter 9**, Unit 9 12

30. Baell, J. B., and Holloway, G. A. (2010) New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J Med Chem* **53**, 2719-2740
31. Du, J., Bleyeleven, I. W., Bitorina, A. V., Wichapong, K., and Nicolaes, G. A. (2014) Optimization of compound ranking for structure-based virtual ligand screening using an established FRED-Surflex consensus approach. *Chem Biol Drug Des* **83**, 37-51
32. Zarzycka, B., Seijkens, T., Nabuurs, S. B., Ritschel, T., Grommes, J., Soehnlein, O., Schrijver, R., van Tiel, C. M., Hackeng, T. M., Weber, C., Giehl, F., Kieser, A., Lutgens, E., Vriend, G., and Nicolaes, G. A. (2015) Discovery of small molecule CD40-TRAF6 inhibitors. *J Chem Inf Model* **55**, 294-307
33. Miteva, M. A., Lee, W. H., Montes, M. O., and Villoutreix, B. O. (2005) Fast structure-based virtual ligand screening combining FRED, DOCK, and Surflex. *J Med Chem* **48**, 6012-6022
34. Berthold, M., Cebon, N., Dill, F., Gabriel, T., Kötter, T., Meinel, T., Ohl, P., Sieb, C., Thiel, K., and Wiswedel, B. (2008) KNIME: The Konstanz Information Miner. in *Data Analysis, Machine Learning and Applications* (Preisach, C., Burkhardt, H., Schmidt-Thieme, L., and Decker, R. eds.), Springer Berlin Heidelberg, pp 319-326
35. Beisken, S., Meinel, T., Wiswedel, B., de Figueiredo, L. F., Berthold, M., and Steinbeck, C. (2013) KNIME-CDK: Workflow-driven cheminformatics. *BMC Bioinformatics* **14**, 257
36. Backman, T. W., Cao, Y., and Girke, T. (2011) ChemMine tools: an online service for analyzing and clustering small molecules. *Nucleic Acids Res* **39**, W486-491
37. Seeliger, D., and de Groot, B. L. (2010) Ligand docking and binding site analysis with PyMOL and Autodock/Vina. *J Comput Aided Mol Des* **24**, 417-422
38. Korth, M. (2010) Third-Generation Hydrogen-Bonding Corrections for Semiempirical QM Methods and Force Fields. *Journal of Chemical Theory and Computation* **6**, 3808-3816
39. Karaman, B., and Sippl, W. (2015) Docking and binding free energy calculations of sirtuin inhibitors. *Eur J Med Chem* **93**, 584-598
40. Miller, B. R., McGee, T. D., Swails, J. M., Homeyer, N., Gohlke, H., and Roitberg, A. E. (2012) MMPBSA.py: An Efficient Program for End-State Free Energy Calculations. *Journal of Chemical Theory and Computation* **8**, 3314-3321
41. Case DA, Darden TA, Cheatham TE, I., Simmerling CL, Wang J, Duke RE, Luo R, Walker RC, Zhang W, Merz KM, Roberts B, Hayik S, Roitberg A, Seabra G, Swails J, Götz AW, Kolossváry I, Wong KF, Pae-sani F, Vanicek J, Wolf RM, Liu J, Wu X, Brozell SR, Steinbrecher T, Gohlke H, Cai Q, Ye X, Wang J, Hsieh MJ, Cui G, Roe DR, Mathews DH, Seetin MG, Salomon-Ferrer R, Sagui C, Babin V, Luchko T, Gusarov S, Ko-valenko A, and PA, K. (2012) Amber 12. University of California, San Francisco; San Francisco, CA
42. Wichapong, K., Lawson, M., Pianwanit, S., Kokpol, S., and Sippl, W. (2010) Postprocessing of protein-ligand docking poses using linear response MM-PB/SA: application to Wee1 kinase inhibitors. *J Chem Inf Model* **50**, 1574-1588
43. Wichapong, K., Rohe, A., Platzer, C., Slynko, I., Erdmann, F., Schmidt, M., and Sippl, W. (2014) Application of docking and QM/MM-GBSA rescoring to screen for novel Myt1 kinase inhibitors. *J Chem Inf Model* **54**, 881-893
44. Dirx, E., Schwenk, R. W., Coumans, W. A., Hoebers, N., Angin, Y., Viollet, B., Bonen, A., van Eys, G. J., Glatz, J. F., and Luiken, J. J. (2012) Protein kinase D1 is essential for contraction-induced glucose uptake but is not involved in fatty acid uptake into cardiomyocytes. *J Biol Chem* **287**, 5871-5881
45. Claycomb, W. C., Lanson, N. A., Jr., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Bahinski, A., and Izzo, N. J., Jr. (1998) HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A* **95**, 2979-2984
46. Calabrese, M. F., Rajamohan, F., Harris, M. S., Caspers, N. L., Magyar, R., Withka, J. M., Wang, H., Borzilleri, K. A., Sahasrabudhe, P. V., Hoth, L. R., Geoghegan, K. F., Han, S., Brown, J., Subashi, T. A., Reyes, A. R., Frisbie, R. K., Ward, J., Miller, R. A., Landro, J. A., Londregan, A. T., Carpino, P. A., Cabral, S., Smith, A. C., Conn, E. L., Cameron, K. O., Qiu, X., and Kurumbail, R. G. (2014) Structural basis for AMPK activation: natural and synthetic ligands regulate kinase activity from opposite poles by different molecular mechanisms. *Structure* **22**, 1161-1172
47. Koay, A., Woodcroft, B., Petrie, E. J., Yue, H., Emanuelle, S., Bieri, M., Bailey, M. F., Hargreaves, M., Park, J. T., Park, K. H., Ralph, S., Neumann, D., Stapleton, D., and Gooley, P. R. (2010) AMPK beta subunits display isoform specific affinities for carbohydrates. *FEBS Lett* **584**, 3499-3503
48. Aretz, J., Wamhoff, E. C., Hanske, J., Heymann, D., and Rademacher, C. (2014) Computational and experimental prediction of human C-type lectin receptor druggability. *Front Immunol* **5**, 323
49. Uciechowska, U., Schemies, J., Scharfe, M., Lawson, M., Wichapong, K., Jung, M., and Sippl, W. (2012) Binding free energy calculations and biological testing of novel thioibarbiturates as inhibitors of the human NAD⁺ dependent histone deacetylase Sirt2. *MedChemComm* **3**, 167-173
50. Wang, J., Morin, P., Wang, W., and Kollman, P. A. (2001) Use of MM-PBSA in reproducing the binding free energies to HIV-1 RT of T1BO derivatives and predicting the binding mode to HIV-1 RT of efavirenz by docking and MM-PBSA. *J Am Chem Soc* **123**, 5221-5230
51. Gleeson, M. P., and Gleeson, D. (2009) QM/MM as a tool in fragment based drug discovery. A cross-docking, rescoring study of kinase inhibitors. *J Chem Inf Model* **49**, 1437-1448
52. Segers, K., Sperandio, O., Sack, M., Fischer, R., Miteva, M. A., Rosing, J., Nicolaes, G. A., and Villoutreix, B. O. (2007) Design of protein membrane interaction inhibitors by virtual ligand screening, proof of concept with the C2 domain of factor V. *Proc Natl Acad Sci U S A* **104**, 12697-12702
53. Prats, C., Cadefau, J. A., Cusso, R., Qvortrup, K., Nielsen, J. N., Wojtaszewski, J. F., Hardie, D. G., Stewart, G., Hansen, B. F., and Ploug, T. (2005) Phosphorylation-dependent translocation of glycogen synthase to a novel structure during glycogen resynthesis. *J Biol Chem* **280**, 23165-23172
54. Phatak, S. S., Stephan, C. C., and Cavasotto, C. N. (2009) High-throughput and in silico screenings in drug discovery. *Expert Opin Drug Discov* **4**, 947-959

55. Scott, J. W., van Denderen, B. J., Jorgensen, S. B., Honeyman, J. E., Steinberg, G. R., Oakhill, J. S., Iseli, T. J., Koay, A., Gooley, P. R., Stapleton, D., and Kemp, B. E. (2008) Thienopyridone drugs are selective activators of AMP-activated protein kinase beta1-containing complexes. *Chem Biol* **15**, 1220-1230
56. Treebak, J. T., Birk, J. B., Hansen, B. F., Olsen, G. S., and Wojtaszewski, J. F. (2009) A-769662 activates AMPK beta1-containing complexes but induces glucose uptake through a PI3-kinase-dependent pathway in mouse skeletal muscle. *Am J Physiol Cell Physiol* **297**, C1041-1052
57. Hawley, S. A., Fullerton, M. D., Ross, F. A., Schertzer, J. D., Chevtzoff, C., Walker, K. J., Pegg, M. W., Zibrova, D., Green, K. A., Mustard, K. J., Kemp, B. E., Sakamoto, K., Steinberg, G. R., and Hardie, D. G. (2012) The ancient drug salicylate directly activates AMP-activated protein kinase. *Science* **336**, 918-922
58. Landgraf, R. R., Goswami, D., Rajamohan, F., Harris, M. S., Calabrese, M. F., Hoth, L. R., Magyar, R., Pascal, B. D., Chalmers, M. J., Busby, S. A., Kurumbail, R. G., and Griffin, P. R. (2013) Activation of AMP-activated protein kinase revealed by hydrogen/deuterium exchange mass spectrometry. *Structure* **21**, 1942-1953
59. Kim, A. S., Miller, E. J., Wright, T. M., Li, J., Qi, D., Atsina, K., Zaha, V., Sakamoto, K., and Young, L. H. (2011) A small molecule AMPK activator protects the heart against ischemia-reperfusion injury. *J Mol Cell Cardiol* **51**, 24-32
60. Guigas, B., Sakamoto, K., Taleux, N., Reyna, S. M., Musi, N., Viollet, B., and Hue, L. (2009) Beyond AICA riboside: in search of new specific AMP-activated protein kinase activators. *IUBMB Life* **61**, 18-26
61. Goransson, O., McBride, A., Hawley, S. A., Ross, F. A., Shpiro, N., Foretz, M., Viollet, B., Hardie, D. G., and Sakamoto, K. (2007) Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. *J Biol Chem* **282**, 32549-32560
62. Dela, F., Prats, C., and Helge, J. W. (2014) Exercise interventions to prevent and manage type 2 diabetes: physiological mechanisms. *Med Sport Sci* **60**, 36-47
63. Kerzmann, A., Fuhrmann, J., Kohlbacher, O., and Neumann, D. (2008) BALLDock/SLICK: a new method for protein-carbohydrate docking. *J Chem Inf Model* **48**, 1616-1625
64. Bieri, M., Mobbs, J. I., Koay, A., Louey, G., Mok, Y. F., Hatters, D. M., Park, J. T., Park, K. H., Neumann, D., Stapleton, D., and Gooley, P. R. (2012) AMP-activated protein kinase beta-subunit requires internal motion for optimal carbohydrate binding. *Biophys J* **102**, 305-314
65. Li, X., Wang, L., Zhou, X. E., Ke, J., de Waal, P. W., Gu, X., Tan, M. H., Wang, D., Wu, D., Xu, H. E., and Melcher, K. (2015) Structural basis of AMPK regulation by adenine nucleotides and glycogen. *Cell Res* **25**, 50-66
66. Hunter, R. W., Treebak, J. T., Wojtaszewski, J. F., and Sakamoto, K. (2011) Molecular mechanism by which AMP-activated protein kinase activation promotes glycogen accumulation in muscle. *Diabetes* **60**, 766-774
67. Miyamoto, L., Toyoda, T., Hayashi, T., Yonemitsu, S., Nakano, M., Tanaka, S., Ebihara, K., Masuzaki, H., Hosoda, K., Ogawa, Y., Inoue, G., Fushiki, T., and Nakao, K. (2007) Effect of acute activation of 5'-AMP-activated protein kinase on glycogen regulation in isolated rat skeletal muscle. *J Appl Physiol* (1985) **102**, 1007-1013
68. Russell, R. R., 3rd, Bergeron, R., Shulman, G. I., and Young, L. H. (1999) Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol* **277**, H643-649
69. Luiken, J. J., Coort, S. L., Willems, J., Coumans, W. A., Bonen, A., van der Vusse, G. J., and Glatz, J. F. (2003) Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. *Diabetes* **52**, 1627-1634
70. Wojtaszewski, J. F., Jorgensen, S. B., Hellsten, Y., Hardie, D. G., and Richter, E. A. (2002) Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle. *Diabetes* **51**, 284-292
71. Nielsen, J. N., Derave, W., Kristiansen, S., Ralston, E., Ploug, T., and Richter, E. A. (2001) Glycogen synthase localization and activity in rat skeletal muscle is strongly dependent on glycogen content. *J Physiol* **531**, 757-769

SUPPLEMENTAL FIGURE LEGENDS

Supp. Fig. 1. (A) Normalized absorbance (sample in black and background in grey) of 100 µM maltoheptaose in the carbohydrate-binding competition assay, using either the $\alpha 1\beta 2\gamma 1$ AMPK heterotrimer or the isolated CBM- $\beta 2$; $n=3$. (B) Dose-dependent effect of digitonin on permeabilization. C2C12 were treated with a constant concentration of the non-permeable substance NTA-Atto 647N (Sigma-Aldrich) and with increasing doses of digitonin (0 to 25 µg/mL). After treatment, cells were washed once in PBS and imaged with a fluorescent microscope.

Supp. Fig. 2. Lysate of AMPK-overexpressing mammalian cells untreated or treated with either vehicle (0.5 % DMSO), 5 µM of oligomycin, or 5 mM β -cyclodextrin (Left panel). Immunoprecipitation of AMPK heterotrimer was performed with the myc antibody following the treatment (right panel). The used antibodies recognize total level of myc tagged-protein (myc), total level of glycogen synthase (GS), total level of AMPK- α subunit (AMPK- $\alpha 1/2$), and phosphorylation status of AMPK at Thr172 (AMPK-pThr172); $n=2$.

Supp. Fig. 3. (A–B) Alternative predicted binding mode of 6469172 (A) and 6456019 (B) in CBM β 2 (defined as mode 2). (C) Experimental and predicted binding free energy calculations for both binding modes of 6469172 and 6456019 with different methods.

Supp. Fig. 4. Toxicity measurement by MTT method with increasing amount of 6469172 (doses in μ M with constant level of vehicle, *i.e.*, 1 % DMSO). Absorbances were normalized to untreated cell absorbance, which gave 100% cell viability. Positive control was 1 % triton-X (TX).

Chapter 6

General Discussion – Tour d'AMPK: *Arrivée Finale*

AMPK is a key metabolic energy sensor for maintaining cellular and whole-body energy homeostasis (1), and is involved in regulating various signaling pathways that contribute to the dysregulation of metabolic homeostasis as observed during metabolic syndromes (MetS). One of the key features of MetS is a perturbation of glucose metabolism which, ultimately, leads to aberrant glycogen metabolism. To better understand this pathophysiological condition, in this thesis, the central focus is the interplay between the intracellular glycogen level and the localization of AMPK. Our research has made a valuable contribution to understand the critical crosstalk between AMPK's action and glycogen availability, thus unravelling possible novel links between AMPK and glycogen metabolism. Based on current advances in the field, this final section of the thesis allows for recapitulation of previous and novel findings and overall discussion. Furthermore, some additional data (that have been collected during the thesis work but are not part of the previous chapters) will be presented, and future experimental approaches will be suggested.

6 Principally, the research described in this thesis has provided (I) insight into the mutually exclusive molecular signaling event involved in regulating the recruitment of AMPK to glycogen (**Chapter 2**). Additionally, we have reported on (II) the dynamic interplay between AMPK and R6 with respect to intracellular glycogen content (**Chapter 3**). Having this research focus in mind, we attempted to visualize (III) localized AMPK activity using FLIM-FRET applications (**Chapter 4**). Given the role of AMPK in glycogen metabolism, and its function in metabolic diseases, our insights served as a basis for (IV) the design of a drug-targeting strategy that allows for the identification of CBM-specific compounds capable of shifting AMPK away from glycogen (**Chapter 5**). In this final section, the first three topics will be discussed in further detail.

AMPK activation has metabolic impact on a wide variety of tissues, and this effect may differ among tissues most probably due to the tissue-specific expression of individual AMPK subunits. From extensive studies (2-5), it has become evident that the presence of a CBM in the N-terminal part of the β -subunit allows for AMPK localization at glycogen. Thereby, the CBM can bridge AMPK to downstream targets such as glycogen synthase (GS) or glycogen phosphorylase (GP) for regulation of glycogen metabolism (6). However, AMPK localizes at many different cellular compartments. Similar to its upstream kinase liver kinase B1 (LKB1) (7,8), AMPK shuttles between the nucleus (9-11) and the cytoplasm (12,13) (**Figure 1**), where it phosphorylates and inactivates cytoplasmic acetyl-CoA carboxylase (ACC), or regulates nuclear targets such as peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1 α) and peroxisome proliferator-activated receptors (PPARs) to control transcriptional processes (14,15), respectively. The fact that exposure to metabolic stress, for instance, promotes nuclear AMPK localization (16), implies the existence of specific regulatory mechanisms that tightly control the nuclear–cytoplasmic and glycogen–cytoplasmic cycling of AMPK.

Figure 1. Subcellular localizations of AMPK. AMPK cycles between the nucleus (9,10) and cytoplasm (12,13), and between glycogen (2,4,5) and the cytoplasm.

Insight into the binding of AMPK to glycogen has come from studies in which AMPK was overexpressed in cellular systems, showing that the presence of an intact β -CBM allows for AMPK-glycogen binding in cultured cells (2,4,17), and that AMPK physically interacts with (2,4) and phosphorylates GS and GP (18). AMPK-induced phosphorylation at Ser-7 supports Ser-10 phosphorylation by casein kinase-1 (CK-1), and together, these phosphorylations result in inactivation of GS (19). Treatment of cultured human muscle cells with the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) resulted in increased glucose uptake and was

inversely correlated with GS activity (20), results that were further confirmed *in vivo* (21). Furthermore, they also showed that AMPK inactivates GS by virtue of Ser-7 phosphorylation (22), providing evidence that AMPK regulates GS in skeletal muscle. However, in contrast to its inhibitory role on glycogen synthesis, long-term AMPK activation caused accumulation of glycogen in skeletal muscle (23). Furthermore, it was shown that AICAR treatment promotes glycogen accumulation in skeletal muscle via increasing glucose uptake (24). A possible explanation for these contradictory observations is that AICAR-induced AMPK activation increases glucose uptake and subsequently elevates the intracellular content of G6P, which allosterically activates GS, thereby overriding the inhibitory phosphorylation by AMPK (25). These findings suggest that AMPK may adapt its mechanism of action presumably depending on the immediate metabolic state of the cell. Alternatively, AMPK's response to various metabolic situations depends on its subcellular localization. Although mutational studies on AMPK β -CBM contributed to a better understanding of AMPK-glycogen binding (2,3,5), the regulatory mechanism by which AMPK glycogen-cytoplasmic cycling is achieved had yet to be determined.

6

Glycogen-cytoplasmic cycling of AMPK: another way of 'exercise' to combat type 2 diabetes?

One of our main focuses of study was to shed light on the regulatory mechanisms involved in the release of AMPK from glycogen. As described in **Chapter 2** and summarized in **Figure 2**, we present AMPK β -Thr-148 autophosphorylation following AMPK activation as a mechanism to regulate AMPK-glycogen binding (17). Under normal physiological conditions, AMPK is quiescent and binds to glycogen via its CBM (**Fig. 2**; clockwise). In response to metabolic stress, this glycogen-bound pool of AMPK may be activated by upstream kinases (AMPKK) such as LKB1. Subsequently, AMPK may phosphorylate and inactivate the glycogen-binding protein GS in order to inhibit glycogen synthesis. If AMPK activation occurs in the cytoplasm, cytoplasmic AMPK α -Thr-172 phosphorylation causes rapid β -Thr-148 autophosphorylation in order to inhibit AMPK-glycogen localization, presumably ceasing the inhibitory effect of AMPK on glycogen synthesis (**Fig. 2**; counterclockwise). Hence, dephosphorylation of cytoplasmic AMPK by protein phosphatases allows for AMPK 're-cycling' towards glycogen. However, once bound to glycogen, the Thr-148 autophosphorylation event will not occur, because the Thr-148 residue, which is centrally situated in the binding-pocket, is occupied. Thus, as depicted in the figure below, our findings suggest that AMPK cycles between glycogen and cytoplasm, a mechanism that is properly controlled by autophosphorylation of Thr-148.

Figure 2. Tour d'AMPK: glycogen-cytoplasmic cycling of AMPK. Inactive AMPK localizes to glycogen (clockwise). There, it can be activated by upstream kinases (AMPK-K), presumably leading to phosphorylation and inactivation of GS. Alternatively, cytosolic AMPK can be activated by upstream kinases (counterclockwise), resulting in autophosphorylation of the β -Thr-148 residue, which is situated in the center of the β -CBM. Importantly, once phosphorylated at Thr-148, AMPK is prevented from binding to glycogen. This is a mutually exclusive situation, indicating that glycogen-bound AMPK does not undergo autophosphorylation at Thr-148, as it is occupied due to glycogen binding. Hence, activation of cytosolic AMPK might not only result in increased glucose uptake and increased glycolysis, thus improving blood glucose levels, but might also cease the AMPK-mediated inhibition of glycogen synthesis (Chapter 2).

The fact that $\beta 1$ and $\beta 2$ isoforms each show a distinct tissue distribution pattern (26,27) indicates a tissue-specific functional role of AMPK. Skeletal muscle is the main organ for insulin-dependent glucose storage into glycogen (28) and it has been shown that muscle-specific AMPK $\beta 2$ has a higher binding affinity for glycogen as compared to $\beta 1$. In **chapter 2**, we investigated the molecular mechanism causing AMPK to refrain from glycogen and found that autophosphorylation of Thr-148 prevents AMPK from binding to glycogen. Because we expected that 'free' cytosolic AMPK might have beneficial impact on skeletal muscle glucose and/or glycogen metabolism, as displayed in **Figure 2**, *i.e.* increased glucose uptake in conjunction with increased glycogen storage, we attempted to investigate the potential role(s) of localized AMPK in mouse skeletal muscle myotubes under physiological conditions. Hence, we performed studies using C2C12 cells that were stably infected with wild-type AMPK $\beta 2$ (glycogen-bound AMPK) and the phospho-mimicking $\beta 2$ -T148D mutant (cytosolic AMPK). In agreement with Halse *et al.* (20,29), preliminary data (unpublished) showed that 16 h treatment of C2C12 myotubes with media, in which glucose concentrations are lowered in a stepwise manner, resulted concomitantly in a decrease in intracellular glycogen content (**Fig. 3A**), and an increase in glucose uptake (**Fig. 3B**), presumably as a result of AMPK activation. These data support the findings

that AMPK mediates glucose import when cellular energy stores are depleted (30). Remarkably, our data also revealed that cytoplasmic AMPK ($\beta 2$ -T148D) further induced glucose uptake both under high and low intracellular glycogen content, when compared to glycogen-bound AMPK ($\beta 2$ -WT), and this effect was correlated with increased basal levels of glycogen. Accordingly, this data (**Fig. 3**) implies a novel role for glycogen-cytoplasmic cycling of AMPK in skeletal muscle glycogen metabolism, as cytoplasmic AMPK localization ($\beta 2$ -T148D) may promote glucose import, presumably stored in the form of glycogen if cellular energy levels are sufficient and used for glycolysis in case of energy deficit. Previously, Prats *et al.* proposed that the intracellular compartmentalization of glycogen itself plays a role in the regulation of metabolic pathways (31). It should also be noted that the dynamic binding and dissociation of glycogen-binding proteins to glycogen particles is essential for adequate coordination of glycogen synthesis and breakdown. Besides the fact that the T148D mutation prevented AMPK from binding to glycogen (**Chapter 2**), we found a negative correlation regarding AMPK-R6 interaction (**Chapter 3**): cytoplasmic AMPK ($\beta 2$ -T148D) was precluded from binding to R6. Hence, it could be speculated that R6 may have enhanced glycogenic properties if AMPK is not in close proximity, which implies that AMPK may function as gatekeeper for initiating R6-mediated glycogen synthesis.

Figure 3. Physiological impact of shifting AMPK away from glycogen on glycogen metabolism. C2C12 cells stably infected with AMPK $\beta 2$ -WT or AMPK $\beta 2$ -T148D were differentiated to myotubes. Briefly, at 85 % confluence, cells were cultured in glucose-rich containing medium (25 mM) supplemented with 2 % horse-serum for 5 days. Subsequently, myotubes were treated with high (25 mM), low (3 mM) or no glucose (0 mM) medium for 16 h. **(A)** Intracellular glycogen levels were determined as described in the Experimental Procedures of Chapter 3; $n=2$. \$ $p<0.05$, # $p<0.05$ vs. T148D 25 mM, * $P<0.01$ vs. WT 25 mM **(B)** Glucose uptake protocol was adapted from Schwenk *et al.* (32). Following treatment, cells were incubated with glucose uptake buffer containing ^3H -labeled deoxyglucose and 10 μM deoxyglucose for 10 min. Unbound substrate was removed by washing the cells with ice-cold uptake buffer and cells were lysed in 0.1 M NaOH. Incorporated glucose was assessed by scintillation counting of ^3H ; $n=3$. \$ $p<0.01$ compared to WT. Student's test was performed to assess statistical significance.

The data displayed above provide a basis for better understanding the role of glycogen-localized AMPK in metabolic diseases, suggesting that activation of cytoplasmic AMPK might be beneficial in type 2 diabetes. Under normal physiological conditions, insulin activates PP1 to dephosphorylate and activate GS, inhibits GSK-3-mediated GS phosphorylation and inactivates GP, all pointing towards increased glycogen synthesis (33). Insulin resistance is associated with reduced GS activity and thus impaired glycogen synthesis (34) as a consequence of impaired insulin sensitivity, indicating that a defect in insulin action not only impairs the ability to maintain normal blood glucose levels but also alters skeletal muscle glycogen metabolism, a common feature in people with type 2 diabetes (34). According to Højlund *et al.* (35), obese subjects, both with and without type 2 diabetes, showed a lack of dephosphorylation of GS Ser-7 and Ser-10. Previously, both in lean and type 2 diabetic subjects, AICAR treatment has been shown to increase GLUT4 translocation and subsequent glucose import in skeletal muscle (6). These data suggest that AMPK is indeed able to stimulate non-insulin dependent glucose uptake in insulin-resistant skeletal muscle, but what proportion of AMPK is localized to either glycogen or the cytoplasm remains unknown and possible roles of R6-PP1 are undefined as yet. Given the concept that insulin resistance reduces the rates of glycogen synthesis in type 2 diabetic muscle (36), which may be due to reduced glucose import, it is rational that muscle glycogen content is found significantly lowered in type 2 diabetes (34,37).

To further explore the role of cytoplasmic AMPK in the context of type 2 diabetes, we used an established approach to make cells insulin resistant by subjecting them to high palmitate treatment (38), similar to insulin resistance in patients being characterized by elevated plasma levels of both insulin and fatty acids (39). First, we observed in such insulin resistant cells that localization of AMPK to the cytosol (β 2-T148D) increased AMPK-Thr-172 phosphorylation levels when compared to cells expressing β 2-wild-type AMPK (**Fig. 4A**), suggesting that AMPK activity was higher upon dissociation from glycogen. These findings support the concept that glycogen mildly inhibits the catalytic activity of AMPK activation (5). With respect to insulin signaling, we observed that high palmitate treatment impaired insulin-stimulated Akt phosphorylation in cells containing wild-type (glycogen-bound) AMPK (β 2-WT) (**Fig. 4A**), suggesting that glycogen-bound AMPK does not help to overcome lipid-induced insulin resistance. In contrast, overexpression of cytosolic (β 2-T148D) AMPK partially prevented the onset of insulin resistance upon lipid oversupply, as can be deduced from the partially preserved insulin-stimulated Akt and AS160 phosphorylation (**Fig. 4A**). Concomitantly, palmitate-treatment impaired insulin-induced glucose uptake when AMPK was glycogen-bound (β 2-WT). Cytosolic (β 2-T148D) AMPK markedly increased glucose uptake (2-fold) both under basal and palmitate-treated conditions (**Fig. 4B**). Accordingly, our data indicate that shifting AMPK away from glycogen positively affects AMPK activity, thereby enhancing glucose uptake under insulin resistant conditions. Although we did not see an improvement of insulin-stimulated glucose uptake, the marked enhancement in

AMPK-mediated glucose uptake could have beneficial effects on lowering of blood glucose levels *in vivo*, in type 2 diabetic animal models. Complementary to AMPK's beneficial effects on glucose uptake, Vitzel *et al.* showed that chronic AICAR treatment increases glycogen content in glycolytic muscle of insulin resistant rats (40). These findings might be related to an increased availability of G6P to override AMPK-mediated GS phosphorylation (25). On the other hand, it might be that cytosolic AMPK promotes glucose uptake without inhibiting glycogen synthesis. Our results clearly indicate significant differences in the signaling pattern and in glucose uptake between glycogen-bound ($\beta 2$ -WT) AMPK and cytosolic ($\beta 2$ -T148D) AMPK. Notably, the endogenous expression of wild-type AMPK $\beta 2$ may still weaken the effects that we have observed in $\beta 2$ -T148D cells. Future *in vivo* studies will be required to determine the physiological impact of altering subcellular AMPK localization on glycogen metabolism, a topic that is further discussed in the paragraph Future perspectives.

Figure 4. Effect of disrupting AMPK-glycogen binding in the context of insulin resistance. C2C12 cells stably infected with AMPK $\beta 2$ -WT or AMPK $\beta 2$ -T148D were differentiated to myotubes. Briefly, at 85 % confluence, cells were cultured in glucose-rich containing medium (25 mM) supplemented with 2 % horse-serum for 5 days. Briefly, C2C12 myotubes were incubated in the presence or absence of the palmitate (HP; 16 h, 750 μ M) before stimulation with insulin (ins, 100 nM, 15-20 min) (41). Prior to lysis in 0.1 M NaOH, cells were incubated with glucose uptake buffer containing 3 H-labeled deoxyglucose and 10 μ M deoxyglucose for 10 min. **(A)** Lysates were subjected to Western blot and assessed using the indicated antibodies. Representative Western Blot is shown. **(B)** Incorporated glucose was assessed by scintillation counting of 3 H as described (32); $n=3$ with internal triplo measurements. * $p<0.001$ compared to $\beta 2$ -WT Basal-; ** $p<0.001$ compared to $\beta 2$ -WT Basal+; # $p<0.01$ compared to $\beta 2$ -WT. Student's test was performed to assess statistical significance.

It remains unclear whether the increase in myocellular glycogen level is beneficial. Several studies have shown that slight increases in the absolute concentration of muscle glycogen were correlated with an increase in insulin resistance in human muscle cells (42,43). Also, glycogen depletion as a result of physical activity has been shown to improve insulin resistance (44). In contrast, with respect to exercise training, it appears beneficial to maintain a high intramuscular

glycogen content at the onset of training (45). Thus, exploring the *in vivo* effects of AMPK dissociation from glycogen will provide novel insights into the role of glycogen-localized AMPK. Furthermore, it is important to know what proportion of AMPK is bound to glycogen; however, to our knowledge, this aspect has never been unraveled, presumably due to technical limitations. Yet, this information would be relevant to better understand to what extent AMPK–glycogen localization contributes to the various physiological settings.

The interplay of AMPK with interaction partners: glycogen-localized effect on cellular physiology?

It is well-known that the CBM domain of the muscle-specific $\beta 2$ isoform of AMPK has higher binding affinity for carbohydrates than $\beta 1$ (26). Very recently, Mobbs *et al.* showed that $\beta 2$ -CBM binds to linear carbohydrates and single $\alpha 1,6$ -branched carbohydrates with 4–30 fold greater affinity than $\beta 1$. From studies with knockout mouse models, it has been concluded that each of the two β -subunits exerts specific functions in metabolism. For instance, it has been reported that loss of $\beta 1$ reduces hepatic AMPK activity and appetite, and protects from high fat diet-induced adverse effects (46). In contrast, AMPK $\beta 2$ null mice have reduced AMPK activity in skeletal muscle, are insensitive to AICAR treatment and have reduced exercise tolerance in spite of overcompensation of $\beta 1$ protein expression (47). Thus, the $\beta 2$ subunit is a critical player in glucose and glycogen metabolism, especially in skeletal and cardiac muscle, which are tissues active in coordinating intracellular glucose disposal into glycogen. Other important regulatory proteins involved in glycogen metabolism are the glycogen-targeting subunits of protein phosphatase PP1, which bind to glycogen themselves and target PP1 to downstream substrates such as GS. Knowing that cellular glycogen is occupied with glycogen-binding proteins, and that AMPK $\beta 1$ -R6 physical interaction is disrupted by a single amino acid mutation in the $\beta 1$ -CBM, we addressed the question of AMPK-binding to the glycogenic protein R6 (**Chapter 3**), which shows the highest level of expression in skeletal and cardiac muscle (48). From our work, we concluded that AMPK $\beta 2$ /R6 interaction has an inverse relationship with intracellular glycogen: AMPK $\beta 2$ /R6 interaction was weak with high intracellular glycogen content, whereas glycogen depletion strongly increased AMPK interaction with R6. Collectively, this data describes a possible model for glycogen–cytoplasmic cycling of AMPK under different metabolic conditions (Fig. 5). Under basal conditions, both glycogen-binding proteins, and possibly other glycogen-binding proteins such as GS, independent of each other, predominantly localize to glycogen to form a protein–glycogen complex in order to facilitate glycogen-bound signaling (**Fig. 5A; (i)**). In skeletal muscle, glycogen synthesis under normal conditions is mostly controlled by the inhibition of GS (through phosphorylation by various kinases including AMPK). However, upon glycogen depletion muscle GS is also capable of translocating to the

nucleus (49) or cytoplasm (50). Therefore, we presume that glycogen-binding proteins are forced to leave the glycogen granule upon degradation in energy stress conditions, which supports the cytoplasmic physical (direct) interaction between AMPK β 2 and R6, but may also induce the binding and phosphorylation of GS (**Fig. 5A; (ii)**). As R6 forms a PP1-R6 holoenzyme, one could expect that glycogenolysis initiates the formation of a cytosolic multi-protein complex, consisting of AMPK, R6, PP1, and GS amongst others, in which PP1 dephosphorylates its substrates, depending on the energy status of the cell (**Fig. 5A; (iii)**). In addition to increased AMPK β 2/R6 interaction, we observed that glycogen breakdown increases Thr-148 phosphorylation (**Fig. 5A; (ii)**), an effect that is even further induced by the AMPK activator oligomycin (**Chapter 2 and 3**). Under these conditions, we assume that AMPK is part of a multi-protein complex. Altogether, the depletion of glycogen stores with consequent cytosolic shift of AMPK, which involves Thr-148 phosphorylation to block the return of AMPK to glycogen, as well as the maintained high catalytic activity of AMPK that changes its conformation to protect it from PP1-mediated dephosphorylation, facilitate sustained insulin-independent glucose import.

The question remains open under which conditions AMPK would phosphorylate GS. In the previous paragraph it was suggested that GS could be leaving the glycogen particle together with AMPK and R6, which may facilitate its AMPK-dependent phosphorylation (**Fig. 5A; (ii)**). Further evidence for this notion is found below (Figure 6). From an evolutionary point of view, we propose that prompt glycogen replenishment prepares the cell for new “fight or flight” events. Indeed, previously it was shown that during exercise, glycogen mainly functions as energy substrate (51), and that the rate of glucose import and glycogen synthesis increased after a bout of exercise in order to replenish glycogen stores (45). However, it is important to keep in mind that decreasing glycogen concentrations may increase GS activity via coordinated dephosphorylation of GS (50,52), whereas under these conditions, increased AMPK activity may simultaneously decrease GS activity by phosphorylating it. According to our data (**Chapter 3**), under low glucose conditions AMPK would leave glycogen as a result of breakdown. Hence, it may be speculated that during glycogen replenishment conditions the predominately cytoplasmic AMPK would be unable to affect GS activity, as AMPK would not be in close proximity to glycogen and its downstream target GS. However, our data points towards the idea that AMPK if localized to glycogen is rather inactive, whereas active AMPK predominantly localizes to the cytoplasm as a consequence of glycogen breakdown (**Chapter 3**). This would support the idea that cytosolic, active AMPK acts as a surveillant to properly control glycogen synthesis. Thus, a possible initial and transient AMPK-dependent inhibition of GS that occurs in the cytosol after glycogen depletion could serve the purpose of using glucose as an energy source to replenish cellular ATP before approving glycogen resynthesis as an energy costly process. In contrast, in absence of an additional energetic challenge the net effect of a cytosolic shift of AMPK (by means of β 2-T148D overexpression) will rather be the glycogen

synthesis. Accordingly, the $\beta 2$ -T148D mutant is a valid tool for studying the physiological effects of cytosolic AMPK on glycogen metabolism, as this mutation precludes AMPK from binding to glycogen (17), enhances glucose uptake, and presumably prevents the inhibitory effect of AMPK on GS (**Fig. 5B**). Consequently, carbohydrates can be increasingly stored as muscle glycogen, thus allowing the cell to prepare for the next energy demand. Therefore, dissociation of AMPK from glycogen will likely have beneficial impact on myocellular glucose uptake and glycogen storage in the context of type 2 diabetes.

Figure 5. Glycogen-cytoplasmic cycling of AMPK under different metabolic conditions. The possible impact of wild-type AMPK (**A**) vs. T148D mutant AMPK (**B**). (i) Glycogen-rich condition; (ii) glycogen-depleted condition; (iii) glycogen-replenishment following increased glucose uptake. (adapted and extended from Chapter 3).

Our data revealed that AMPK/R6 interaction is decreased by culturing of cells in high glucose-containing media, which was leading to high glycogen content (**Chapter 3**). Vice versa, enhanced interaction between AMPK and R6 was observed under a glucose-deprived, low glycogen situation and occurs in conjunction with Thr-148 phosphorylation (**Chapter 3**). Given that high glucose may induce PP1-R6-mediated dephosphorylation of AMPK, we tested the effect of glucose recovery on AMPK β 2/R6 interaction. As depicted in **Figure 6**, glucose recovery resulted in reduced AMPK activity (as shown by Thr-148 phosphorylation) and reduced the interaction between AMPK β 2 and R6, and a similar pattern was found for GS. However, Thr-172 phosphorylation only transiently decreased around 5 minutes after glucose re-availability, but increased thereafter to levels similar to control (25 mM glucose continuous culturing). Due to technical limitations with available antibodies we could not show physical interaction with PP1. Nevertheless, these results are suggesting a sequential mechanism involving PP1-R6. We speculate that re-availability of glucose (glucose recovery) is sufficient to meet the immediate intracellular energy demand. Accordingly, AMPK will change its conformation due to ATP bound, thus allowing for PP1-mediated dephosphorylation of AMPK at α -pT172. R6-PP1 may then return to glycogen to support for resynthesis of glucose stores. With some delay, dephosphorylation of β 2-Thr-148 will allow AMPK re-localization to glycogen (*c.f.*, **Fig. 5A (iii)**). As yet, however, we do not fully understand the transient Thr-172 dephosphorylation and relatively high level of AMPK activation also in control conditions (25 mM continuous culturing). Nevertheless, these unknown mechanisms are apparently unrelated to AMPK β 2/R6 interaction, since the complex has dissociated completely 30 minutes after recovery.

Figure 6. Effect of glucose re-availability on AMPK-R6 interaction HEK293T cells transiently co-transfected with AMPK β 2-WT (myc- α 1, mCherry- β 2 and γ 1) and R6 (FLAG-R6). Briefly, cells were cultured under serum-starved low glucose conditions (3 mM) for 16 h. Subsequently, cells were recovered with serum-starved high glucose medium (25 mM) in a time-dependent fashion, as indicated. Exogenous myc-AMPK α 1 was immunoprecipitated using a myc-tag antibody, followed by incubation with protein G-Sepharose beads, as described in Chapter 3. Western blot analysis was carried out using the indicated antibodies.

Interestingly, Mobbs *et al.* (53) provide with a different hypothesis that the Thr-101 insertion in AMPK β 2, as compared to β 1, further expands the pocket to make it more accessible for branched carbohydrates such as partially degraded glycogen. These findings would argue that the degradation of glycogen, which presumably occurs gradually per tier, allows for increased glycogen-binding of AMPK β 2, suggesting that AMPK β 2 might remain bound to the inner tiers of the glycogen particle, thereby enhancing the partnering of AMPK with glycogen-bound R6 and GS (Fig. 5A (ii)). Although this possibility is not ruled out, our data clearly indicate that glycogen breakdown results in increased phosphorylation of Thr-148. Since this post-translational modification occupies the carbohydrate-binding pocket, it prevents the recruitment of AMPK to glycogen. Conversely, if AMPK would remain bound to glycogen, the increase in Thr-148 phosphorylation would not have taken place, since in the bound situation Thr-148 is not accessible for phosphorylation. Therefore, it is more likely that AMPK cycles away from glycogen, at the same time finds its interaction partners, and regulates glycogen replenishment from a cytosolic localization. Yet, whether the increase in AMPK/R6 interaction is a consequence of glycogen degradation *per se* or is enhanced by Thr-148 phosphorylation remains unclear at this moment. Future experiments employing the use of glycogen breakdown inhibitors (*i.e.*, CP-91149), in addition to oligomycin for activating AMPK, could possibly clarify the role of Thr-148 phosphorylation on AMPK β 2–R6 interaction.

AMPK signaling: a possible role for other interaction partners

AMPK signaling may also occur at specific cytosolic domains through its interaction with scaffold proteins, which promote the interaction of various signaling pathways. For instance, AMPK interacts with the TAK-1-binding protein-1 (TAB1), a scaffold protein mediating TAK-1 activation and p38 mitogen activated protein kinase autophosphorylation (54–56). Based on our results, we assume that AMPK, or R6, may also act as scaffold proteins for other interacting partners.

Interestingly, a potential direct protein–protein interaction between Fyn and R6 was identified using the peptide arrays target screening (PATS) method (57). However, the physical interaction has not been further verified as yet. Increases in muscle energy demands activate AMPK and induce sarcolemmal recruitment of fatty acid translocase CD36, which plays a role in regulating cellular lipid utilization. CD36 facilitates long-chain fatty acid uptake (58) and AMPK is known to increase fatty acid β -oxidation by inhibiting acetyl-CoA carboxylase (ACC) through its phosphorylation (59). It has been shown that CD36 physically interacts with the Src family kinase Fyn (60), and regulates AMPK activity by decreasing Thr-172 phosphorylation in order to activate ACC and decrease fatty acid oxidation. Recently, Samovski *et al.* reported that AMPK regulation by CD36 is linked to fatty acid supply and multi-protein complex formation of CD36/Fyn/LKB1/AMPK (61). These researchers provided evidence that low exogenous concentrations of fatty acid exposure keeps AMPK in a quiescent state

by promoting Fyn-dependent LKB1 phosphorylation, which drives nuclear localization of LKB1. Upon fatty acid binding to CD36, Fyn dissociates from the complex, by which nuclear translocation of LKB1 is prevented. This allows LKB1 to remain cytosolic and activate AMPK. Consequently, LKB1 induces cytosolic activation of AMPK to promote fatty acid oxidation. Therefore, a direct protein-protein interaction between Fyn and R6 could provide with an interesting link. Given that AMPK activation status is regulated by phosphorylation/dephosphorylation, the findings that Fyn interacts with CD36/LKB1 to inhibit LKB1-mediated AMPK activation under quiescent conditions, suggest that Fyn could recruit PP1-R6 to the CD36/LKB1 complex to initiate dephosphorylation of AMPK. Together, this would suggest that AMPK, R6 and Fyn could play a very significant role, *i.e.* linking glycogen and fatty acid metabolism.

Another group of researchers has shown that AXIN, a central regulator of Wnt signaling, recruits LKB1 for phosphorylation of AMPK. Rises in intracellular AMP concentration increases the affinity of AMPK for AXIN, which at the same time, recruits LKB1, thereby promoting the activation of AMPK by Thr-172 phosphorylation. This model suggests for the vicinity of phosphatases to allow for immediate adaptation to metabolic changes. Indeed, Luo *et al.* reported that PP1 interacts with AXIN. This interaction was investigated in the context of Wnt signaling, showing that PP1 controls the AXIN/GSK-3 complex by means of coordinated phosphorylation and dephosphorylation of AXIN. GSK-3 has been shown to play a role in insulin-stimulated glucose transport and GS activation. Therefore, PP1/AXIN/GSK-3 may take part in the multi-protein complex in response to AMPK dissociation from glycogen, suggesting that those AMPK interacting proteins may be involved in glycogen metabolism.

Excluding AMPK from access to glycogen in order to apply the brakes on glycogen synthesis?

Whereas in type 2 diabetes the disposal of plasma glucose into muscular glycogen is diminished, studies have also demonstrated that chronic activation of AMPK results in glycogen accumulation in both skeletal and cardiac muscles, thus adding a layer of complexity. However, whether the subcellular localization of AMPK has a major impact in the dysregulation of glycogen deposition, to our knowledge, has never been addressed.

The γ -subunit is an important subunit for regulation of AMPK as it senses the intracellular AMP:ATP ratio. Traditionally, binding of AMP allosterically activates the enzyme and protects it from dephosphorylation at Thr-172. The naturally-occurring R302Q mutation in the AMPK γ 2 subunit is associated with the Wolff-Parkinson-White (WPW) syndrome (62,63). WPW is characterized by excessive intramyocellular glycogen deposition and cardiac hypertrophy that is associated with ventricular pre-

excitation and consequent impairment of electric conduction (64). Although a number of γ 2-mutations have been associated with increased AMPK activity (63), previous studies described discrepancies in AMPK activity as a consequence of the γ 2-R302Q mutation. Several reports suggested that the observed increases in glycogen storage were due to the inability of AMPK to inhibit GS as a result of impaired AMP-induced AMPK activation (65,66), implying that R302Q is a dominant negative mutation. Paradoxically, others have reported that R302Q was associated with an increased AMPK activity and resulted in increased glucose transport and glycogen synthesis (67), suggesting that R302Q might be a gain-of-function mutation that constitutively activates the holoenzyme and overrides the normal inhibition of glycogen synthesis. Alternatively, our model supports the concept that β -Thr-148 autophosphorylation, which makes AMPK to remain cytosolically localized, might occur as a consequence of constitutive AMPK activation thereby ceasing the inhibition of GS activity (68). Does this mean that the subcellular localization of AMPK is the underlying cause, or in other words, that relocalization of AMPK to glycogen is capable of reversing the glycogen accumulation and consequent disease symptoms seen in WPW?

To address this kind of questions, we developed biological tools to investigate localization-dependent AMPK function and/or activation (**Chapter 3**; Experimental Procedures). These tools consist of the smallest available tag (FLAsH-tag; optimized tag 12 AA) for protein-labeling in living cells, based on the affinity of the green FLAsH or red ReAsH reagents. Thus, in order to optimize co-localization studies on muscle-specific AMPK β 2, we decided to exchange the large mCherry tag (27 kD) with the FLAsH tag, and generate stable cell lines. Alternatively, we generated both adenoviral and retroviral constructs encompassing the γ 2R302Q mutation in order to investigate the role of β -Thr-148 in the context of WPW (not shown). Currently, all tools are available for studying, for instance, the physiological implications of β -Thr-148 phosphorylation on AMPK localization in a variety of cellular models mimicking these pathophysiological conditions. We hypothesize that interference with cytosolic AMPK localization, thus driving AMPK towards glycogen, may inhibit glycogen synthesis, an implication that is expected to be beneficial for WPW patients.

Another crucial link between AMPK and glycogen metabolism, either glycogen accumulation (WPW) or glycogen deficit (type 2 diabetes), may be the glycophagic process. This process is distinct from normal macrophagy, as it selectively targets glycogen for lysosomal degradation via autophagic recruitment, for instance in hepatocytes from newborns (69). More recently, it has been reported that the glycophagic pathway also exists in the adult heart (70,71), which is responsive to insulin and exposure to extracellular high glucose. Besides the prominent role in classical autophagic pathways, AMPK is also involved in glycophagy (70). Activation of glycophagy might have potential beneficial effects on glycogen-storage diseases, such as WPW, Pompe's disease (72), Danon disease (73), and Lafora disease (74), each of which are characterized by aberrant accumulation of glycogen. Furthermore, glycophagy may be an alternative mechanism to control glucose homeostasis under

conditions of sugar demand, such as acute metabolic stress. Based on the fact that AMPK is involved in glycophagy, it might be speculated that AMPK subcellular localization may have great impact on this process, thereby regulating glycogen metabolism. Thus, further investigations on the role of AMPK in glycophagy, particularly in relation to glycogen–cytoplasmic cycling of AMPK, will be informative.

Visualization of glycogen-localized AMPK

The precise coordination of basic cellular and molecular processes is essential for the existence of living cells/organisms. Fluorescent protein-based biosensors coupled with live-cell imaging allow for the visualization of such events (75) and, for instance, have advanced our current understanding of certain signaling pathways on a subcellular level. Förster resonance energy transfer (FRET)-based reporters provide information about dynamic posttranslational modifications, such as phosphorylation in living cells (76), which cannot be detected using traditional biochemical techniques (75). For instance, a high-throughput method, using ratiometric FRET-based fluorescent sensors, has been applied for the reading of dynamic kinase activities (77). As mentioned before, the particular role of AMPK at different subcellular localizations remains unclear. Tsou *et al.* reported on the visualization of cytoplasmic *versus* nuclear endogenous AMPK activity in living cells using an AMPK-specific biosensor (78). Recently, Miyamoto *et al.* provide insight into the compartmentalized regulation of AMPK using various AMPK biosensors, for detection of cytosolic, nuclear, membrane, Golgi, ER, mitochondrial and lysosomal fractions, respectively, which rely on high spatiotemporal precision (79). Consistent with our findings (**Chapter 4**), these researchers demonstrate that the cytosolic FRET efficiency, determining the AMPK activity in the cytoplasm, increases in response to glucose-deprivation (79). For the first time, they provided evidence that genetically encoded compartment-specific fluorescent biosensors, which can be targeted to specific subcellular localizations, represent a powerful real-time approach for understanding the intracellular and spatiotemporal behavior of AMPK. In this respect, it is of particular interest to study the dynamic glycogen-localized AMPK activity, thus addressing the dynamic interplay of AMPK and glycogen, an area of research that, to our knowledge, has not been studied yet. To achieve this, we have been exploring novel AMPK biosensors (**Chapter 4**), which by means of the CBM β 2 sequence are targeted to glycogen, and thus should allow for the monitoring of glycogen-localized AMPK activity (**Fig. 7**) using a FLIM/FRET approach. While modest in scope, our study provides a compelling stimulus for further work to develop a more complete understanding of the dynamic regulation of localized (glycogen *versus* cytoplasmic) AMPK activity. Given the rapidly evolving drug discovery field, the application of genetically encoded fluorescent biosensors might further advance our understandings of the mechanism of action of potential novel drug candidates, such as presented in **Chapter 5**.

Figure 7. Principle of FRET-based reporting of glycogen-localized AMPK activity using the $\beta 2$ -CBM (adapted from Chapter 4).

Future perspectives

The research described in this thesis has highlighted several important facets of AMPK regulation. Although the finishing line of the Tour d'AMPK has not been reached yet, the current status of research provides both advanced insight and ample novel opportunities for furthering our understanding with regard to exact regulation and physiological outcome of the glycogen–cytoplasmic cycling of AMPK. Translating our knowledge into more complex *ex vivo/in vivo* model systems, *e.g.*, using transgenic rodents or established disease models, will further clarify how interference with the AMPK–glycogen dynamics could have beneficial effects in the context of metabolic diseases such as type 2 diabetes (decreased cellular glycogen content) and WPW (increased cellular glycogen content). Several options for future research on the interplay between AMPK and glycogen metabolism and the role of modulators involved in this process are sketched below.

In vivo dynamic regulation of AMPK

Translation of the *in vitro* cell culture data into experimental animal models would allow for further studying the role of Thr-148 phosphorylation and yield physiologic data, and drawing conclusions on interaction between organs. To directly assess the physiological impact of Thr-148 phosphorylation on glucose and glycogen metabolism, we could make use of transgenic mice overexpressing muscle-specific (MLCK or MCK-driven) $\beta 2$ -WT and $\beta 2$ -T148D. These mice would offer the unique perspective of determining the whole body metabolic effects of changing the subcellular localization of AMPK. Full characterization of the mouse will include determination of muscle glycogen, as well as measurement of insulin-stimulated

glucose uptake. High-fat diet induced insulin resistance or exercise regimes may be required to further investigate the metabolic stability of these transgenics. On the molecular level, it might be interesting to check for the AMPK activity status and AMPK-dependent signaling (*i.e.*, ACC-Ser-79 and GS-Ser-7 phosphorylation). Tissue sections may show structural differences between the muscles of the mutants and may be further investigated using immunological techniques also to show AMPK localization and to visualize glycogen.

An alternative, interesting approach to study the physiological role of tissue-specific AMPK β 2-Thr-148 phosphorylation is the *in vivo* transfection of plasmids in fully differentiated myotubes of rodents (80). To achieve this, it would be of interest to express fluorescently-tagged genetically modified forms of AMPK (*i.e.*, transfection of mCherry-tagged β 2-T148D into skeletal muscle of one leg, while the contralateral muscle receives the mCherry-tagged β 2-WT control plasmid) in order to assess its functional relevance in more detail, *e.g.*, with respect to the endogenous distribution of AMPK and the interaction of AMPK with binding partners and with glycogen. Fluorescent microscopy and 2-photon laser scanning microscopy would allow for identification of the overall expression, whereas the latter would also aim for high-resolution imaging deep into muscle tissue of living rodents (*e.g.*, C57BL/6 mice), gaining detailed insight into the intracellular localization. To overcome the physiological consequence of interference by endogenous protein, one could apply this technique in whole-body AMPK β 2 knockout mice (47) or muscle-specific AMPK β 1 β 2 double knockouts (81).

Another *in vivo* experimental approach to determine the potential direct effect of glycogen content on Thr-148 phosphorylation or AMPK/R6 interaction is to deplete glycogen by altering the pre-exercise glycogen levels in one single leg of a rodent by means of single-legged (treadmill) exercise prior to performing two-legged exercise (82). The advantage of single-legged exercise is to have a second leg serving as an internal control. Parameters to be considered may be the phosphorylation status of AMPK (*e.g.*, p-Thr-172, p-Thr-148) and AMPK-dependent signaling (*e.g.*, p-ACC-Ser-79, pGS-Ser-7), rates of glucose uptake, fatty acid oxidation and muscle glycogen content, synthesis, and enzyme activity and protein levels (*e.g.*, GS and GP), measurements of blood glucose levels, and AMPK-dependent signaling pathways on transcriptional and translational level.

In addition, to evaluate, in an *in vivo* setting, whether AMPK-R6 interaction is largely attributed to changes in intracellular glycogen content, rodents (*e.g.*, C57BL/6 mice) could undergo prolonged fasting followed by refeeding. We anticipate that starvation followed by refeeding will induce an excessive glycogen accumulation, providing information on the dynamic regulation of AMPK-R6 interaction by glycogen, *e.g.*, by assessing protein-protein interactions, skeletal muscle glycogen storage, glycogen fractionation and signaling pathways on transcriptional and translational level. Alternatively, the effect of glycogen on AMPK-R6 interaction and/or Thr-148 phosphorylation can be assessed by comparing a group of mice that

only performed acute treadmill exercise with a second group that is exposed to a carbohydrate-rich diet following acute treadmill exercise. A third group may serve as sedentary controls. Parameters to be measured may be similar to the ones described above. Based on our results, we believe that AMPK-R6 interaction is under control of intracellular glycogen, showing that this interaction is required for proper coordination of glycogen metabolism.

Ultimately, gathered knowledge could be translated into established disease models (*e.g.*, diabetic (*ob/ob*) mice). Such established disease models would enable for assessing the occurrence and/or significance of Thr-148 phosphorylation or, alternatively, whether changes in AMPK subcellular localization can considerably improve obesity and/or type 2 diabetic outcomes, *i.e.* by investigating the concept of inhibiting the binding of AMPK to glycogen in insulin resistance. Muscle-specific effects could be addressed in such diseased models by means of recombinant adenoviral vectors for gene delivery to the target tissue (83). We anticipate that shifting AMPK away from glycogen (AMPK β 2-T148D) improves glycogen metabolism in a type 2 diabetic situation by increasing glucose uptake and ceasing the inhibition of glycogen synthesis, thus increasing glycogen storage.

It might also be interesting to translate our knowledge to established WPW disease models (84), which have been characterized by excessive glycogen accumulation and consequent cardiac defects. Such models would allow for drawing solid conclusions on the significance of glycogen-bound vs. free cytosolic AMPK, as we anticipate that driving AMPK towards glycogen may inhibit glycogen synthesis, an effect that is expected to be beneficial for WPW patients.

Finally, as autophosphorylation of Thr-148 causes loss of glycogen-binding capacity to glycogen, the CBM can be viewed as a new target for therapeutic intervention in the development of drugs to shift AMPK away from its original destination at glycogen. Therefore, the administration of AMPK- β -CBM-targeted drugs (**Chapter 5**) to the *in vivo* models as described above might help in the search for novel therapeutic drugs to combat metabolic diseases such as type 2 diabetes.

Concluding Remarks

The interplay between AMPK and glycogen metabolism and the role of interacting proteins, especially PP1-glycogen-targeting subunit R6, involved in this process has been elaborated with focus on normal physiological conditions. Further studies are required to clarify the metabolic consequences for the heart under pathophysiological conditions, but also for skeletal muscle and liver in particular, because these latter tissues are quantitatively the major sites of glycogen storage.

REFERENCES

1. Hardie, D. G. (2008) AMPK: a key regulator of energy balance in the single cell and the whole organism. *Int J Obes (Lond)* **32 Suppl 4**, S7-12
2. Polekhina, G., Gupta, A., Michell, B. J., van Denderen, B., Murthy, S., Feil, S. C., Jennings, I. G., Campbell, D. J., Witters, L. A., Parker, M. W., Kemp, B. E., and Stapleton, D. (2003) AMPK β Subunit Targets Metabolic Stress Sensing to Glycogen. *Current Biology* **13**, 867-871
3. Polekhina, G., Gupta, A., van Denderen, B. J., Feil, S. C., Kemp, B. E., Stapleton, D., and Parker, M. W. (2005) Structural basis for glycogen recognition by AMP-activated protein kinase. *Structure* **13**, 1453-1462
4. Hudson, E. R., Pan, D. A., James, J., Lucocq, J. M., Hawley, S. A., Green, K. A., Baba, O., Terashima, T., and Hardie, D. G. (2003) A Novel Domain in AMP-Activated Protein Kinase Causes Glycogen Storage Bodies Similar to Those Seen in Hereditary Cardiac Arrhythmias. *Current Biology* **13**, 861-866
5. McBride, A., Ghilagaber, S., Nikolaev, A., and Hardie, D. G. (2009) The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. *Cell Metab* **9**, 23-34
6. Viollet, B., Lantier, L., Devin-Leclerc, J., Hebrard, S., Amouyal, C., Mounier, R., Foretz, M., and Andreelli, F. (2009) Targeting the AMPK pathway for the treatment of Type 2 diabetes. *Front Biosci (Landmark Ed)* **14**, 3380-3400
7. Dorfman, J., and Macara, I. G. (2008) STRADalpha regulates LKB1 localization by blocking access to importin-alpha, and by association with Crm1 and exportin-7. *Mol Biol Cell* **19**, 1614-1626
8. Boudeau, J., Baas, A. F., Deak, M., Morrice, N. A., Kieloch, A., Schutkowski, M., Prescott, A. R., Clevers, H. C., and Alessi, D. R. (2003) MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. *EMBO J* **22**, 5102-5114
9. McGee, S. L., Howlett, K. F., Starkie, R. L., Cameron-Smith, D., Kemp, B. E., and Hargreaves, M. (2003) Exercise increases nuclear AMPK alpha2 in human skeletal muscle. *Diabetes* **52**, 926-928
10. Kodiha, M., Ho-Wo-Cheong, D., and Stochaj, U. (2011) Pharmacological AMP-kinase activators have compartment-specific effects on cell physiology. *Am J Physiol Cell Physiol* **301**, C1307-1315
11. Lamia, K. A., Sachdeva, U. M., DiTacchio, L., Williams, E. C., Alvarez, J. G., Egan, D. F., Vasquez, D. S., Juguilon, H., Panda, S., Shaw, R. J., Thompson, C. B., and Evans, R. M. (2009) AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science* **326**, 437-440
12. Salt, I., Celler, J. W., Hawley, S. A., Prescott, A., Woods, A., Carling, D., and Hardie, D. G. (1998) AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. *Biochem J* **334 (Pt 1)**, 177-187
13. Kazgan, N., Williams, T., Forsberg, L. J., and Brenman, J. E. (2010) Identification of a nuclear export signal in the catalytic subunit of AMP-activated protein kinase. *Mol Biol Cell* **21**, 3433-3442
14. Hong, Y. H., Varanasi, U. S., Yang, W., and Leff, T. (2003) AMP-activated protein kinase regulates HNF4alpha transcriptional activity by inhibiting dimer formation and decreasing protein stability. *J Biol Chem* **278**, 27495-27501
15. Yang, W., Hong, Y. H., Shen, X. Q., Frankowski, C., Camp, H. S., and Leff, T. (2001) Regulation of transcription by AMP-activated protein kinase: phosphorylation of p300 blocks its interaction with nuclear receptors. *J Biol Chem* **276**, 38341-38344
16. Kodiha, M., Rassi, J. G., Brown, C. M., and Stochaj, U. (2007) Localization of AMP kinase is regulated by stress, cell density, and signaling through the MEK-->ERK1/2 pathway. *Am J Physiol Cell Physiol* **293**, C1427-1436
17. Oligschlaeger, Y., Miglianico, M., Chanda, D., Scholz, R., Thal, R. F., Tuerk, R., Stapleton, D. I., Gooley, P. R., and Neumann, D. (2015) The Recruitment of AMP-activated Protein Kinase to Glycogen Is Regulated by Autophosphorylation. *J Biol Chem* **290**, 11715-11728
18. Carling, D., and Hardie, D. G. (1989) The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta* **1012**, 81-86
19. Skurat, A. V., Wang, Y., and Roach, P. J. (1994) Rabbit skeletal muscle glycogen synthase expressed in COS cells. Identification of regulatory phosphorylation sites. *J Biol Chem* **269**, 25534-25542
20. Halse, R., Fryer, L. G., McCormack, J. G., Carling, D., and Yeaman, S. J. (2003) Regulation of glycogen synthase by glucose and glycogen: a possible role for AMP-activated protein kinase. *Diabetes* **52**, 9-15
21. Wojtaszewski, J. F., Jorgensen, S. B., Hellsten, Y., Hardie, D. G., and Richter, E. A. (2002) Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle. *Diabetes* **51**, 284-292
22. Jorgensen, S. B., Nielsen, J. N., Birk, J. B., Olsen, G. S., Viollet, B., Andreelli, F., Schjerling, P., Vaulont, S., Hardie, D. G., Hansen, B. F., Richter, E. A., and Wojtaszewski, J. F. (2004) The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* **53**, 3074-3081
23. Holmes, B. F., Kurth-Kraczek, E. J., and Winder, W. W. (1999) Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. *J Appl Physiol (1985)* **87**, 1990-1995
24. Aschenbach, W. G., Hirshman, M. F., Fujii, N., Sakamoto, K., Howlett, K. F., and Goodyear, L. J. (2002) Effect of AICAR treatment on glycogen metabolism in skeletal muscle. *Diabetes* **51**, 567-573
25. Hunter, R. W., Treebak, J. T., Wojtaszewski, J. F., and Sakamoto, K. (2011) Molecular mechanism by which AMP-activated protein kinase activation promotes glycogen accumulation in muscle. *Diabetes* **60**, 766-774
26. Koay, A., Woodcroft, B., Petrie, E. J., Yue, H., Emanuelle, S., Bieri, M., Bailey, M. F., Hargreaves, M., Park, J. T., Park, K. H., Ralph, S., Neumann, D., Stapleton, D., and Gooley, P. R. (2010) AMPK beta subunits display isoform specific affinities for carbohydrates. *FEBS Lett* **584**, 3499-3503
27. Bieri, M., Mobbs, J. I., Koay, A., Louey, G., Mok, Y. F., Hatters, D. M., Park, J. T., Park, K. H., Neumann, D., Stapleton, D., and Gooley, P. R. (2012) AMP-activated protein kinase beta-subunit requires internal motion for optimal carbohydrate binding. *Biophys J* **102**, 305-314

28. DeFronzo, R. A., Gunnarsson, R., Bjorkman, O., Olsson, M., and Wahren, J. (1985) Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J Clin Invest* **76**, 149-155
29. Halse, R., Bonavaud, S. M., Armstrong, J. L., McCormack, J. G., and Yeaman, S. J. (2001) Control of glycogen synthesis by glucose, glycogen, and insulin in cultured human muscle cells. *Diabetes* **50**, 720-726
30. Mu, J., Brozinick, J. T., Jr., Valladares, O., Bucan, M., and Birnbaum, M. J. (2001) A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* **7**, 1085-1094
31. Prats, C., Gomez-Cabello, A., and Hansen, A. V. (2011) Intracellular compartmentalization of skeletal muscle glycogen metabolism and insulin signalling. *Exp Physiol* **96**, 385-390
32. Schwenk, R. W., Dirckx, E., Coumans, W. A., Bonen, A., Klip, A., Glatz, J. F., and Luiken, J. J. (2010) Requirement for distinct vesicle-associated membrane proteins in insulin- and AMP-activated protein kinase (AMPK)-induced translocation of GLUT4 and CD36 in cultured cardiomyocytes. *Diabetologia* **53**, 2209-2219
33. Groop, L., and Orho-Melander, M. (2008) New insights into impaired muscle glycogen synthesis. *PLoS Med* **5**, e25
34. Shulman, G. I., Rothman, D. L., Jue, T., Stein, P., DeFronzo, R. A., and Shulman, R. G. (1990) Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by ¹³C nuclear magnetic resonance spectroscopy. *N Engl J Med* **322**, 223-228
35. Hojlund, K., Birk, J. B., Klein, D. K., Levin, K., Rose, A. J., Hansen, B. F., Nielsen, J. N., Beck-Nielsen, H., and Wojtaszewski, J. F. (2009) Dysregulation of glycogen synthase COOH- and NH₂-terminal phosphorylation by insulin in obesity and type 2 diabetes mellitus. *J Clin Endocrinol Metab* **94**, 4547-4556
36. DeFronzo, R. A., and Tripathy, D. (2009) Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care* **32 Suppl 2**, S157-163
37. He, J., and Kelley, D. E. (2004) Muscle glycogen content in type 2 diabetes mellitus. *Am J Physiol Endocrinol Metab* **287**, E1002-1007
38. Hage Hassan, R., Hainault, I., Vilquin, J. T., Samama, C., Lasnier, F., Ferre, P., Fofelle, F., and Hajdouch, E. (2012) Endoplasmic reticulum stress does not mediate palmitate-induced insulin resistance in mouse and human muscle cells. *Diabetologia* **55**, 204-214
39. Alberti, K. G., Zimmet, P., Shaw, J., and Group, I. D. F. E. T. F. C. (2005) The metabolic syndrome-a new worldwide definition. *Lancet* **366**, 1059-1062
40. Vitzel, K. F., Bikopoulos, G., Hung, S., Pistor, K. E., Patterson, J. D., Curi, R., and Ceddia, R. B. (2013) Chronic treatment with the AMP-kinase activator AICAR increases glycogen storage and fatty acid oxidation in skeletal muscles but does not reduce hyperglucagonemia and hyperglycemia in insulin deficient rats. *PLoS One* **8**, e62190
41. Chavez, J. A., Knotts, T. A., Wang, L. P., Li, G., Dobrowsky, R. T., Florant, G. L., and Summers, S. A. (2003) A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. *J Biol Chem* **278**, 10297-10303
42. Litherland, G. J., Morris, N. J., Walker, M., and Yeaman, S. J. (2007) Role of glycogen content in insulin resistance in human muscle cells. *J Cell Physiol* **211**, 344-352
43. Mott, D. M., Lillioja, S., and Bogardus, C. (1986) Overnutrition induced decrease in insulin action for glucose storage: in vivo and in vitro in man. *Metabolism* **35**, 160-165
44. Perseghin, G., Price, T. B., Petersen, K. F., Roden, M., Cline, G. W., Gerow, K., Rothman, D. L., and Shulman, G. I. (1996) Increased glucose transport-phosphorylation and muscle glycogen synthesis after exercise training in insulin-resistant subjects. *N Engl J Med* **335**, 1357-1362
45. Aragon, A. A., and Schoenfeld, B. J. (2013) Nutrient timing revisited: is there a post-exercise anabolic window? *J Int Soc Sports Nutr* **10**, 5
46. Dzamko, N., van Denderen, B. J., Hevener, A. L., Jorgensen, S. B., Honeyman, J., Galic, S., Chen, Z. P., Watt, M. J., Campbell, D. J., Steinberg, G. R., and Kemp, B. E. (2010) AMPK beta1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. *J Biol Chem* **285**, 115-122
47. Steinberg, G. R., O'Neill, H. M., Dzamko, N. L., Galic, S., Naim, T., Koopman, R., Jorgensen, S. B., Honeyman, J., Hewitt, K., Chen, Z. P., Schertzer, J. D., Scott, J. W., Koentgen, F., Lynch, G. S., Watt, M. J., van Denderen, B. J., Campbell, D. J., and Kemp, B. E. (2010) Whole body deletion of AMP-activated protein kinase (beta)2 reduces muscle AMPK activity and exercise capacity. *J Biol Chem* **285**, 37198-37209
48. Montori-Grau, M., Guitart, M., Garcia-Martinez, C., Orozco, A., and Gomez-Foix, A. M. (2011) Differential pattern of glycogen accumulation after protein phosphatase 1 glycogen-targeting subunit PPP1R6 overexpression, compared to PPP1R3C and PPP1R3A, in skeletal muscle cells. *BMC Biochem* **12**, 57
49. Cid, E., Cifuentes, D., Baque, S., Ferrer, J. C., and Guinovart, J. J. (2005) Determinants of the nucleocytoplasmic shuttling of muscle glycogen synthase. *FEBS J* **272**, 3197-3213
50. Nielsen, J. N., Derave, W., Kristiansen, S., Ralston, E., Ploug, T., and Richter, E. A. (2001) Glycogen synthase localization and activity in rat skeletal muscle is strongly dependent on glycogen content. *J Physiol* **531**, 757-769
51. Esbjornsson-Liljedahl, M., Bodin, K., and Jansson, E. (2002) Smaller muscle ATP reduction in women than in men by repeated bouts of sprint exercise. *J Appl Physiol* (1985) **93**, 1075-1083
52. Prats, C., Helge, J. W., Nordby, P., Qvortrup, K., Ploug, T., Dela, F., and Wojtaszewski, J. F. (2009) Dual regulation of muscle glycogen synthase during exercise by activation and compartmentalization. *J Biol Chem* **284**, 15692-15700
53. Mobbs, J. I., Koay, A., Di Paolo, A., Bieri, M., Petrie, E. J., Gorman, M. A., Doughty, L., Parker, M. W., Stapleton, D. I., Griffin, M. D., and Gooley, P. R. (2015) Determinants of oligosaccharide specificity of the carbohydrate-binding modules of AMP-activated protein kinase. *Biochem J* **468**, 245-257

54. Zaha, V. G., and Young, L. H. (2012) AMP-activated protein kinase regulation and biological actions in the heart. *Circ Res* **111**, 800-814
55. Scholz, R., Sidler, C. L., Thali, R. F., Winssinger, N., Cheung, P. C., and Neumann, D. (2010) Autoactivation of transforming growth factor beta-activated kinase 1 is a sequential bimolecular process. *J Biol Chem* **285**, 25753-25766
56. Li, J., Miller, E. J., Ninomiya-Tsuji, J., Russell, R. R., 3rd, and Young, L. H. (2005) AMP-activated protein kinase activates p38 mitogen-activated protein kinase by increasing recruitment of p38 MAPK to TAB1 in the ischemic heart. *Circ Res* **97**, 872-879
57. Wu, C., Ma, M. H., Brown, K. R., Geisler, M., Li, L., Tzeng, E., Jia, C. Y., Jurisica, I., and Li, S. S. (2007) Systematic identification of SH3 domain-mediated human protein-protein interactions by peptide array target screening. *Proteomics* **7**, 1775-1785
58. Koonen, D. P., Glatz, J. F., Bonen, A., and Luiken, J. J. (2005) Long-chain fatty acid uptake and FAT/CD36 translocation in heart and skeletal muscle. *Biochim Biophys Acta* **1736**, 163-180
59. Saha, A. K., Schwarsin, A. J., Roduit, R., Masse, F., Kaushik, V., Tornheim, K., Prentki, M., and Ruderman, N. B. (2000) Activation of malonyl-CoA decarboxylase in rat skeletal muscle by contraction and the AMP-activated protein kinase activator 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside. *J Biol Chem* **275**, 24279-24283
60. Huang, M. M., Bolen, J. B., Barnwell, J. W., Shattil, S. J., and Brugge, J. S. (1991) Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn, and Yes protein-tyrosine kinases in human platelets. *Proc Natl Acad Sci U S A* **88**, 7844-7848
61. Samovski, D., Sun, J., Pietka, T., Gross, R. W., Eckel, R. H., Su, X., Stahl, P. D., and Abumrad, N. A. (2015) Regulation of AMPK activation by CD36 links fatty acid uptake to beta-oxidation. *Diabetes* **64**, 353-359
62. Gollob, M. H., Green, M. S., Tang, A. S., Gollob, T., Karibe, A., Ali Hassan, A. S., Ahmad, F., Lozado, R., Shah, G., Fananapazir, L., Bachinski, L. L., and Roberts, R. (2001) Identification of a gene responsible for familial Wolff-Parkinson-White syndrome. *N Engl J Med* **344**, 1823-1831
63. Arad, M., Benson, D. W., Perez-Atayde, A. R., McKenna, W. J., Sparks, E. A., Kanter, R. J., McGarry, K., Seidman, J. G., and Seidman, C. E. (2002) Constitutively active AMP kinase mutations cause glycogen storage disease mimicking hypertrophic cardiomyopathy. *Journal of Clinical Investigation* **109**, 357-362
64. Light, P. E. (2006) Familial Wolff-Parkinson-White Syndrome: a disease of glycogen storage or ion channel dysfunction? *J Cardiovasc Electrophysiol* **17 Suppl 1**, S158-S161
65. Sidhu, J. S., Rajawat, Y. S., Rami, T. G., Gollob, M. H., Wang, Z., Yuan, R., Marian, A. J., DeMayo, F. J., Weilbacher, D., Taffet, G. E., Davies, J. K., Carling, D., Khoury, D. S., and Roberts, R. (2005) Transgenic mouse model of ventricular preexcitation and atrioventricular reentrant tachycardia induced by an AMP-activated protein kinase loss-of-function mutation responsible for Wolff-Parkinson-White syndrome. *Circulation* **111**, 21-29
66. Daniel, T., and Carling, D. (2002) Functional analysis of mutations in the gamma 2 subunit of AMP-activated protein kinase associated with cardiac hypertrophy and Wolff-Parkinson-White syndrome. *J Biol Chem* **277**, 51017-51024
67. Folmes, K. D., Chan, A. Y., Koonen, D. P., Pulinilkunnil, T. C., Baczko, I., Hunter, B. E., Thorn, S., Allard, M. F., Roberts, R., Gollob, M. H., Light, P. E., and Dyck, J. R. (2009) Distinct early signaling events resulting from the expression of the PRKAG2 R302Q mutant of AMPK contribute to increased myocardial glycogen. *Circ Cardiovasc Genet* **2**, 457-466
68. Yu, H., Hirshman, M. F., Fujii, N., Pomerleau, J. M., Peter, L. E., and Goodyear, L. J. (2006) Muscle-specific overexpression of wild type and R225Q mutant AMP-activated protein kinase gamma3-subunit differentially regulates glycogen accumulation. *Am J Physiol Endocrinol Metab* **291**, E557-565
69. Kalamidas, S. A., and Kotoulas, O. B. (2000) Glycogen autophagy in newborn rat hepatocytes. *Histol Histopathol* **15**, 1011-1018
70. Reichelt, M. E., Mellor, K. M., Curl, C. L., Stapleton, D., and Delbridge, L. M. (2013) Myocardial glycophagy - a specific glycogen handling response to metabolic stress is accentuated in the female heart. *J Mol Cell Cardiol* **65**, 67-75
71. Mellor, K. M., Varma, U., Stapleton, D. I., and Delbridge, L. M. (2014) Cardiomyocyte glycophagy is regulated by insulin and exposure to high extracellular glucose. *Am J Physiol Heart Circ Physiol* **306**, H1240-1245
72. Chandramouli, C., Varma, U., Stevens, E. M., Xiao, R. P., Stapleton, D. I., Mellor, K. M., and Delbridge, L. M. (2015) Myocardial glycogen dynamics: New perspectives on disease mechanisms. *Clin Exp Pharmacol Physiol* **42**, 415-425
73. Nishino, I., Fu, J., Tanji, K., Yamada, T., Shimojo, S., Koori, T., Mora, M., Riggs, J. E., Oh, S. J., Koga, Y., Sue, C. M., Yamamoto, A., Murakami, N., Shanske, S., Byrne, E., Bonilla, E., Nonaka, I., DiMauro, S., and Hirano, M. (2000) Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). *Nature* **406**, 906-910
74. Tagliabracci, V. S., Turnbull, J., Wang, W., Girard, J. M., Zhao, X., Skurat, A. V., Delgado-Escueta, A. V., Minassian, B. A., Depaoli-Roach, A. A., and Roach, P. J. (2007) Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen in vivo. *Proc Natl Acad Sci U S A* **104**, 19262-19266
75. DiPilato, L. M., and Zhang, J. (2010) Fluorescent protein-based biosensors: resolving spatiotemporal dynamics of signaling. *Curr Opin Chem Biol* **14**, 37-42
76. Zhang, J., and Allen, M. D. (2007) FRET-based biosensors for protein kinases: illuminating the kinome. *Mol Biosyst* **3**, 759-765
77. Allen, M. D., DiPilato, L. M., Rahdar, M., Ren, Y. R., Chong, C., Liu, J. O., and Zhang, J. (2006) Reading dynamic kinase activity in living cells for high-throughput screening. *ACS Chem Biol* **1**, 371-376
78. Tsou, P., Zheng, B., Hsu, C. H., Sasaki, A. T., and Cantley, L. C. (2011) A fluorescent reporter of AMPK activity and cellular energy stress. *Cell Metab* **13**, 476-486

79. Miyamoto, T., Rho, E., Sample, V., Akano, H., Magari, M., Ueno, T., Gorshkov, K., Chen, M., Tokumitsu, H., Zhang, J., and Inoue, T. (2015) Compartmentalized AMPK Signaling Illuminated by Genetically Encoded Molecular Sensors and Actuators. *Cell Rep* **11**, 657-670
80. DiFranco, M., Quinonez, M., Capote, J., and Vergara, J. (2009) DNA transfection of mammalian skeletal muscles using in vivo electroporation. *J Vis Exp*
81. O'Neill, H. M., Maarbjerg, S. J., Crane, J. D., Jeppesen, J., Jorgensen, S. B., Schertzer, J. D., Shyroka, O., Kiens, B., van Denderen, B. J., Tarnopolsky, M. A., Kemp, B. E., Richter, E. A., and Steinberg, G. R. (2011) AMP-activated protein kinase (AMPK) beta1beta2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. *Proc Natl Acad Sci U S A* **108**, 16092-16097
82. Steensberg, A., van Hall, G., Keller, C., Osada, T., Schjerling, P., Pedersen, B. K., Saltin, B., and Febbraio, M. A. (2002) Muscle glycogen content and glucose uptake during exercise in humans: influence of prior exercise and dietary manipulation. *J Physiol* **541**, 273-281
83. Vorburger, S. A., and Hunt, K. K. (2002) Adenoviral gene therapy. *Oncologist* **7**, 46-59
84. Arad, M., Moskowitz, I. P., Patel, V. V., Ahmad, F., Perez-Atayde, A. R., Sawyer, D. B., Walter, M., Li, G. H., Burgon, P. G., Maguire, C. T., Stapleton, D., Schmitt, J. P., Guo, X. X., Pizard, A., Kupersmidt, S., Roden, D. M., Berul, C. I., Seidman, C. E., and Seidman, J. G. (2003) Transgenic mice overexpressing mutant PRKAG2 define the cause of Wolff-Parkinson-White syndrome in glycogen storage cardiomyopathy. *Circulation* **107**, 2850-2856

Summary

AMP-activated protein kinase (AMPK) is a metabolic master switch that plays a key role in maintaining energy homeostasis. This thesis has shed light into the functional role and regulation of AMPK in glycogen metabolism, advanced our understanding of AMPK activity at the subcellular level, and focused on finding a novel therapeutic strategy to shift AMPK away from glycogen, which may have beneficial impact on metabolic diseases such as type 2 diabetes. Further, this thesis introduces a new concept, the glycogen–cytoplasmic cycling of AMP-activated protein kinase, that is pictured as ‘*Tour d’AMPK*’.

Chapter 1 introduces the two key players of this thesis: AMPK and glycogen. AMPK is an enzyme that is only functional and stable upon formation of an obligatory $\alpha\beta\gamma$ heterotrimer. The α -subunit is a catalytic subunit that carries the threonine-172 phosphorylation site within the kinase domain. The γ -subunit allows AMPK to sense changes in adenine nucleotides, thereby regulating AMPK activity, whereas the regulatory β -subunit is responsible for regulating AMPK activity, and more importantly, localization to and from glycogen owing to a carbohydrate-binding module (CBM) being part of the β -subunit. Recent discoveries suggest that AMP is known to have three different effects on the activation of AMPK: (i) promotion of phosphorylation by upstream kinases to phosphorylate the enzyme at threonine-172, thereby activating AMPK, (ii) protection against the dephosphorylation of AMPK by protein phosphatases, and (iii) allosteric activation of AMPK. The introductory chapter further describes the coordination of myocellular glucose uptake and glycogen metabolism, discusses the structure of glycogen and provides insight into carbohydrate–protein interactions by specifying the role of the CBM in general. In more detail, this chapter presents the structure, activity, tissue-specific expression and function, and highlights the role and regulation of the AMPK β -CBM domain. Altogether, in this chapter, the interplay of AMPK with glycogen and/or its glycogen-binding proteins has been evaluated.

In **Chapter 2**, the molecular mechanism responsible for AMPK localization to and from glycogen was investigated. Although the β -subunit of AMPK carries a CBM, AMPK is known to localize to a variety of subcellular localizations, such as the cytoplasm or glycogen. Using cell-free carbohydrate-binding assays, our data revealed that AMPK autophosphorylation rather than AMPK activation is involved in the regulation of AMPK–glycogen binding. Furthermore, structural data disclosed the presence of the threonine-148 (Thr-148) residue, centrally situated in the carbohydrate-binding pocket. Substitution of Thr-148 to a phospho-mimicking aspartate (T148D) resulted in loss of carbohydrate-binding ability of the AMPK- β -CBM in cell-free assays. Immunofluorescence studies confirmed that the T148D mutant had lost its capability of binding to intracellular glycogen, whereas wild-type CBM localizes to glycogen particles. Breakdown of glycogen, which was induced by lowering glucose availability and/or pharmacological activation of AMPK, enhanced phosphorylation of Thr-148.

In contrast to overexpression of AMPK- β -T148D, the intracellular glycogen content further decreased upon overexpression of wild-type AMPK. Altogether, these data suggest that phosphorylation of Thr-148 of AMPK β is involved in regulating the synthesis and breakdown of glycogen.

Chapter 3 provides novel insight into the role of muscle-specific AMPK β 2 and the involvement of Thr-148 phosphorylation in regulating glycogen metabolism. In this chapter, yeast-two-hybrid analyses and co-immunoprecipitation assays in HEK293T cells showed protein-protein interaction between wild-type AMPK β 1 or AMPK β 2 and the protein phosphatase 1 (PP1)-glycogen targeting subunit R6 upon overexpression, an interaction that occurred via the substrate-binding motif of R6. AMPK β 2 interaction with R6 was weak upon culturing HEK293T cells and C2C12 myotubes in high glucose, whereas glycogen breakdown significantly induced the interaction. Substitution of Thr-148 to the phospho-mimicking aspartate (T148D) prevented the interaction from occurring, independently of the intracellular glycogen content. Treatment of cells cultured in low glucose with the known AMPK activator oligomycin not only increased Thr-148 phosphorylation, but also enhanced the interaction between muscle-specific AMPK β 2 and R6. These data indicate that R6 directly binds to AMPK β 2 presumably after leaving the degrading glycogen particle, and that this interaction occurs in conjunction with increased AMPK β 2 Thr-148 phosphorylation. This model suggests that AMPK β 2/R6 interaction is dynamically regulated by the intracellular glycogen content. Whether AMPK is under proper control by the protein phosphatase complex PP1-R6 under conditions of glycogen breakdown in muscle needs further investigation.

In **Chapter 4**, cytoplasmic AMPK activity was monitored using a novel optimized AMPK-specific Förster Resonance Energy Transfer (FRET) biosensor, referred to as ASS, based on Fluorescence Lifetime Imaging Microscopy (FLIM) technology, as the activity of (glycogen-) localized AMPK is understudied. Data revealed a strong increase in basal FRET efficiency upon nutrient starvation in HL-1 cardiomyocytes overexpressing wild-type ASS. However, this increase impaired further monitoring of changes in average cyan fluorescent protein (CFP) lifetime induced by the known AMPK activator oligomycin. Optimization of the experimental conditions revealed that glucose/serum-rich medium (non-starvation) minimized this effect. Although oligomycin increased the FRET efficiency of wild-type ASS, the effect was mainly attributed to the DMSO vehicle. In contrast, the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMP analog, enhanced FRET efficiency of wild-type ASS-expressing cells in a time- but not dose-dependent manner, whereas overexpression of a non-phosphorylatable mutant ASS biosensor prevented FRET from occurring. Altogether, these data revealed that the wild-type ASS biosensor is capable of detecting increased AMPK activity albeit that the dynamic range is limited,

suggesting that FLIM/FRET can be used as an approach to properly monitor fluctuations of intracellular AMPK activity.

Chapter 5 highlighted the importance of AMPK as therapeutic drug target and provided insight into a novel drug-targeting approach based on the CBM of AMPK. This strategy was based on three different methods (i) virtual ligand screening, (ii) carbohydrate-binding competition assay, and (iii) determination of the CBM subcellular localization pattern of the AMPK-CBM. Two of the top candidate compounds had intracellular micromolar affinities for cycling the AMPK-CBM away from glycogen, and direct binding of these compounds to the AMPK-CBM was observed using the surface plasmon resonance technique. Furthermore, in cellular assays, the most active and high-affinity compound induced increased intracellular AMPK activity and phosphorylation of the downstream target acetyl-CoA carboxylase (ACC). Moreover, this compound was capable of increasing glucose and long-chain fatty acid uptake in cultured HL-1 cardiomyocytes, an effect that was inhibited by the AMPK inhibitor Compound C. These data imply that compound-based targeting of the AMPK-CBM shifts AMPK away from glycogen and regulates AMPK activity, a dual effect that might have beneficial outcomes with respect to metabolic diseases such as type 2 diabetes.

Su

Chapter 6 shortly recapitulates the concept of the '*Tour d'AMPK*', recapitulated and elaborates on the novel findings described in the experimental chapters 2, 3, 4 and 5, and integrated these novel data with current knowledge in order to provide exciting future perspectives. Altogether, this chapter discussed the interplay of AMPK and glycogen metabolism and focused on the role of interacting glycogen-related proteins, in particular the PP1 glycogen-targeting subunit R6. Although the finish line of the '*Tour d'AMPK*' has not been reached yet, this chapter provides ample novel ideas and/or approaches to support the search for the metabolic impact of glycogen-cytoplasmic cycling of AMPK (e.g., on heart, skeletal muscle and liver) under both healthy and pathophysiological conditions. In summary, the assembly of generated knowledge, speculations and/or future directions described in this thesis allows for further elucidating the exact role and significance of AMPK in glycogen metabolism, with special reference to the heart and other relevant tissues strongly linked to metabolic diseases such as type 2 diabetes.

Samenvatting

Het eiwit *AMP-activated protein kinase* (AMPK) speelt een essentiële rol in de stofwisseling door te functioneren als sensor bij het behoud van de energiebalans, zowel in afzonderlijke cellen als in het gehele lichaam. In dit proefschrift is specifiek de functie bestudeerd van AMPK bij de regulatie van het glycogeenmetabolisme. Glycogeen is de vorm waarin met de voeding opgenomen koolhydraten in de cel worden opgeslagen. Omgekeerd hadden deze studies ook tot doel te achterhalen of de activiteit van het AMPK afhankelijk is van de plaats waar het zich bevindt in de cel. Daarnaast hebben we, door middel van een innoverende aanpak, getracht te onderzoeken of beïnvloeding van de AMPK-glycogeen interactie, dat wil zeggen het binden van AMPK aan glycogeen en het vervolgens weer loslaten, een gunstige werking zou kunnen hebben op metabole ziekten zoals type 2 diabetes. Kortom, dit proefschrift introduceert een nieuw concept, dat ik '*Tour d'AMPK*' heb genoemd.

Hoofdstuk 1 beschrijft de twee belangrijkste elementen van dit proefschrift, namelijk het eiwit AMPK en glycogeen. AMPK is een enzym dat alleen stabiel en dus werkzaam is wanneer het een complex vormt op basis van drie verschillende onderdelen, namelijk $\alpha\beta\gamma$. Het katalytische α -onderdeel bevat een kinase domein waarin het belangrijke threonine-172 residu (Thr-172) is gelegen. Het γ -onderdeel reguleert de activiteit van het eiwit AMPK door de binding van ribonucleotiden. Het derde, en in dit proefschrift belangrijkste, onderdeel is de β subeenheid. Ook dit onderdeel is betrokken bij het regelen van de activiteit van AMPK, maar bevat daarnaast ook een domein dat ervoor zorgt dat AMPK aan glycogeen kan binden, het zogeheten koolhydraat-bindend domein (*carbohydrate-binding module*, CBM).

Recente studies hebben aangetoond dat adenosinemonofosfaat (AMP) op drie verschillende manieren de activiteit van AMPK kan reguleren. (i) AMP kan enkele enzymactivatoren (kinases), die hogerop in de signaalcascade staan, stimuleren om Thr-172 te fosforyleren; hierdoor wordt AMPK geactiveerd. (ii) AMP beschermt AMPK tegen defosforylatie door specifieke fosfatases. (iii) AMP activeert AMPK op allosterische wijze; dit houdt in dat er een conformatieverandering van het enzym plaatsvindt waardoor het actief wordt.

In dit inleidend hoofdstuk wordt ook de communicatie tussen AMPK, glycogeen en andere betrokken eiwitten beschreven. Hiervoor gaat dit hoofdstuk dieper in op de structuur van glycogeen, het proces van glycogeenopbouw en -afbraak (glycogeenmetabolisme) in hart- en spiercellen, en op de algemene functie van het CBM domein. Om het verband tussen AMPK en glycogeen te verduidelijken worden de rol, structuur, activiteit en orgaan-specifieke functies van AMPK besproken en wordt in het bijzonder aandacht besteed aan de taak van het CBM domein van AMPK.

In **hoofdstuk 2** is gezocht naar het onderliggende moleculaire mechanisme dat bepaalt of AMPK zich verenigt met glycogeen of niet. Ondanks het feit dat het CBM domein, gelegen in het β -onderdeel van AMPK, ervoor zorgt dat AMPK kan binden aan glycogeen, blijkt uit onderzoek dat niet alle AMPK gebonden is aan glycogeen. Door middel van technieken waarbij *in vitro* de bindingscapaciteit van AMPK aan koolhydraten (zoals glycogeen) kan worden opgespoord, hebben we kunnen aantonen dat het niet de activatie maar de autofosforylatie van AMPK is die ervoor zorgt dat AMPK los komt van glycogeen.

In de literatuur recent beschikbaar gekomen gegevens over de eiwitstructuur van AMPK toonden aan dat het threonine-148 residu (Thr-148) centraal ligt in de 'binding-pocket' van het β -CBM domein. Op grond daarvan hebben we gebruik gemaakt van een AMPK mutant waarbij Thr-148 vervangen werd door een asparaginezuur (T148D), om zo het effect van constitutieve Thr-148 fosforylatie na te bootsen. We ontdekten dat door deze mutatie AMPK niet meer kon binden aan glycogeen, terwijl het oorspronkelijke (wild-type) AMPK-CBM domein wel kon binden. Met behulp van fluorescentiemicroscopie hebben we dit bevestigd.

Tenslotte hebben we in hartspiercellen gevonden dat de afbraak van intracellulair glycogeen resulteerde in een verhoogde fosforylatie van Thr-148. Door het glucosegehalte in het omringende medium te verlagen of door farmacologische activatie van AMPK werd deze cellulaire glycogeen-afbraak in gang gezet. Overexpressie van wild-type AMPK had een sterker effect op de afbraak van glycogeen dan de T148D mutant. Dit alles wijst er op dat de fosforylatie van het Thr-148 residu een prominente rol speelt in de regulatie van het glycogeenmetabolisme.

In **hoofdstuk 3** komt vooral AMPK β 2 aan bod. Deze AMPK isovorm komt voornamelijk voor in hart- en skeletspieren. In dit hoofdstuk wordt dieper ingegaan op de rol van β 2-Thr-148 met betrekking tot het glycogeenmetabolisme. Met behulp van methodes zoals de gist *two-hybrid* analyse en co-immunoprecipitatie hebben we kunnen aantonen dat zowel AMPK β 1 en AMPK β 2 interageren met het eiwit R6. R6 is een eiwit dat in staat is om het enzym *protein phosphatase 1* (PP1) naar glycogeen te begeleiden. Zonder aanwezigheid van het substraatbindingsdomein gelegen in R6 kan deze wisselwerking niet plaatsvinden. Wanneer HEK293T cellen en C2C12 spierbundels gekweekt werden in een kweekmedium met een hoog glucosegehalte was de interactie tussen AMPK β 2 en R6 zwak. Echter, wanneer het intracellulaire glycogeengehalte van deze cellen werd verlaagd, observeerden we een sterkere wisselwerking tussen de twee eiwitten.

Substitutie van Thr-148 door een asparaginezuur (T148D) zorgde ervoor dat de wisselwerking tussen AMPK en R6 niet kon plaatsvinden. Dit stond los van het intracellulaire glycogeengehalte. Wanneer echter wild-type cellen waren gekweekt in een kweekmedium met een laag glucosegehalte, en daarna blootgesteld aan de AMPK-activator oligomycine, zagen we zowel een toename in de fosforylatie van Thr-148 als een versterkte interactie tussen AMPK β 2 en R6.

Deze gegevens wijzen erop dat R6 een directe binding kan aangaan met AMPK β 2, waarschijnlijk nadat beide eiwitten loskomen tijdens het afbraakproces van glycogeen. Dit doet ons veronderstellen dat de wisselwerking tussen AMPK β 2 en R6 op dynamische wijze gereguleerd wordt door het intracellulaire glycogeengehalte. De vraag of AMPK gereguleerd wordt door het eiwit complex PP1-R6 vereist verder onderzoek.

In **hoofdstuk 4** onderzochten we de cytoplasmatische activiteit van AMPK onder verschillende omstandigheden. Het meten van deze activiteit was gebaseerd op het gebruik van een nieuwe, geoptimaliseerde AMPK-specifieke biosensor (hierna ASS genoemd). Om de cytoplasmatische AMPK activiteit te kunnen visualiseren hebben we *Förster Resonance Energy Transfer* (FRET) in combinatie met *fluorescence lifetime imaging* microscopie als techniek gebruikt. Onze experimenten zijn uitgevoerd op bepaalde hartspiercellen (HL-1 cellen) waarin de ASS-biosensor tot expressie is gebracht. Wanneer aan het kweekmedium van deze cellen zowel serum als glucose werd onttrokken, werd een verhoging in het basale FRET rendement gemeten; een waarde berekend op basis van gemiddelde lifetime van het cyaan fluorescentie eiwit (*cyan fluorescent protein*, CFP). Echter, de meting van een mogelijke, door oligomycine gestimuleerde, verdere stijging in FRET rendement werd belemmerd door deze onverwacht hoge toename. Door het eerder genoemde kweekmedium te vervangen met medium waaraan glucose en serum werden toegevoegd konden we de ongewenste stijging voorkomen. Hoewel oligomycine op basis van dit medium het FRET rendement leek te verhogen in de cellen, bleek dit effect echter te danken aan het oplosmiddel DMSO.

Wanneer we HL-1 cellen, die ASS tot overexpressie brengen, blootstelden aan een andere activator van AMPK, namelijk *5-aminoimidazole-4-carboxamide ribonucleotide* (AICAR), zagen we een duidelijke toename in FRET rendement. Deze toename bleek wel tijdsgebonden, maar niet concentratiegebonden te zijn. Dit FRET effect vond echter niet plaats wanneer HL-1 cellen een ASS mutant biosensor tot expressie brachten, die niet gefosforyleerd kan worden. Samengevat wijzen deze resultaten erop dat de (wild-type) ASS biosensor een hulpmiddel kan zijn om verhoogde AMPK activatie in levende cellen te detecteren. Toepassing van deze techniek zou het mogelijk moeten maken om schommelingen in de intracellulaire activiteit van glycogeen-gebonden AMPK nauwkeurig te kunnen waarnemen.

In **hoofdstuk 5** staat het loskomen van AMPK van zijn binding aan glycogeen centraal. Hierbij doelen we op de therapeutische mogelijkheden van AMPK en hebben we gescreend op kleine moleculen met het CBM domein als doelwit. Deze *drug-targeted* strategie bestond uit drie opeenvolgende methodes: (i) een virtuele *ligand screening*, (ii) een competitie assay gebaseerd op het CBM domein, en (iii) de waarneming van het intracellulaire glycogeen bindingspatroon van het CBM domein. Twee van de top-kandidaat moleculen hadden een hoge affiniteit (micromolair) voor het CBM domein waardoor AMPK gescheiden kon worden van glycogeen. Daarnaast werd met de *surface plasmon resonance* techniek bevestigd dat deze moleculen direct konden binden aan het CBM domein van AMPK.

Blootstelling van cellen aan het meest actieve molecuul resulteerde in een verhoging van de activiteit van AMPK. Tevens vonden we een toename in de fosforylatie van het eiwit acetyl-CoA carboxylase (ACC), dat in de signaleringscascade zich downstream bevindt van AMPK. Daarnaast zorgde dit molecuul voor een verhoogde opname van glucose en van vetzuren door HL-1 hartspiercellen, terwijl dit effect geremd werd door de enzymremmer Compound C. Onze studie suggereert dat de *drug-targeted* strategie die we hier beschrijven, dat wil zeggen, het onderbreken van de AMPK-glycogeen wisselwerking door middel van een klein molecuul specifiek gericht tot het CBM domein van AMPK, zou kunnen worden toegepast om zo de glycogeenvoorraad van hart- en skeletspiercellen van type 2 diabetes patiënten beter op peil te kunnen houden.

Tenslotte wordt in **hoofdstuk 6** een algemene beschouwing gegeven van de bevindingen beschreven in de experimentele hoofdstukken 2, 3, 4 en 5. Integratie van deze nieuwe onderzoeksresultaten en de huidige kennis van AMPK opent nieuwe wegen. Dit hoofdstuk bediscussieert de wisselwerking tussen AMPK en het glycogeenmetabolisme en richt zich op de essentie van glycogeen-bindende eiwitten, in het bijzonder het eiwit R6. Ondanks dat de eindstreep van deze *'Tour d'AMPK'* nog niet in zicht is, levert dit proefschrift een grote hoeveelheid nieuwe ideeën om verder onderzoek te stimuleren. Het doel is om completer inzicht te krijgen in de rol van de AMPK-glycogeen *cycling*, enerzijds in gezonde cellen, en anderzijds onder verschillende pathofysiologische omstandigheden. De nieuwe kennis en vooruitzichten in deze thesis dagen ertoe uit om de functie die AMPK heeft in het reguleren van het glycogeenmetabolisme verder uit te diepen. Zonder twijfel is dit van groot belang niet alleen voor het hart maar ook voor andere organen die betrokken zijn bij metabole ziektes zoals type 2 diabetes.

Valorization

Socioeconomic relevance

It is well recognized that diet and physical inactivity are major contributors to obesity and diabetes. Diabetes is one of the most common chronic diseases in the Netherlands, a disease that is for instance correlated with increased risk for cardiovascular disease. Especially for people with type 2 diabetes (T2D), regular exercise together with moderate caloric intake are important for maintaining health. Nevertheless, some people with T2D may also need diabetes medications, alternative therapies, dietary compounds or insulin therapy.

Epidemiological research shows that in the Netherlands around 1 million people have diabetes, with an incidence of 50.000 new cases a year. Approximately 3.12 billion euro is spent for taking care of diabetes patients, which is approximately 9% of the total health care costs (1-3). According to the World Health Organization, 350 million people suffer from diabetes, a number that may be doubled by 2025, and 90% of all diabetic patients have T2D (4). Because T2D affect an enormous amount of people, (*e.g.*, due to financial and intangible costs), it is of great importance to arrest this growing global epidemic and improve the quality of life of T2D patients. Thus in an era of epidemic obesity, diabetes and sedentary lifestyles, there is serious need for effective and potentially curative therapies.

The dysregulation of AMPK plays an important role in the development of T2D and activation of AMPK (*i.e.*, pharmacological or physiological) can prevent or ameliorate some of the pathologies. Nevertheless, the actions of activated AMPK have generated questions regarding its potential role at specific subcellular localizations such as glycogen. We know that activated AMPK improves glucose uptake, glycolysis and fatty acid oxidation to provide ATP, but what exactly occurs with and/or at glycogen, to our knowledge, has never been considered. Because phosphorylation of muscle glycogen synthase is known to inhibit the rate of glycogen synthesis, the present study aimed at investigating the molecular mechanism involved in the ‘cycling’ of AMPK to and from glycogen. We were excited to dissect a relatively unexplored area, the role and/or activity of glycogen-localized AMPK, with particular interest in glycogen metabolism, and employed a variety of biochemical, cell-biological and mutational studies. With the work described in this thesis, we envisage the novel concept that ‘cycling’ of AMPK might be another way of exercise to restore glycogen deficiency.

Our results can be considered as the first step towards clinical implementation (*‘Bench to Bedside’*). In a nutshell, we have shown the importance of the threonine-148 residue (Thr-148). Our data revealed that phosphorylation of this residue, both in cell-free assays and *in vitro*, prevents AMPK from binding to glycogen (5). Further investigation confirmed these findings, but also led to new mechanistic insight. The fact that the interaction between AMPK and other glycogen-binding proteins, such as the PP1 glycogen-targeting subunit R6 or glycogen synthase is dynamically regulated by glycogen, suggests a tight regulation of glycogen

metabolism, likely depending on the formation/collapse of an AMPK/R6/PP1 multi-protein complex. In order to decode the circuits linking fundamental cell biological processes to physiology, we further explored the role of ‘*cycling*’ of AMPK away from glycogen in cells that were made insulin resistant. Our data revealed that cytosolic AMPK was more active, had increased basal glycogen levels, enhanced glucose uptake, and was capable of preventing cells from becoming insulin resistant. Overall, the research described in this thesis presents a novel molecular mechanism that provides possibilities of utilizing it to combat T2D, which will be further elucidated below.

To gain insight into the possible clinical impact of shifting AMPK away from glycogen either on a healthy volunteer or on a T2D patient, the very first step to be undertaken is to translate the results presented into experimental animal models: investigate the *in vivo* (and/or *ex vivo*) regulation of AMPK, aiming at activity, subcellular localization, protein interaction and its role on glycogen metabolism. In other words, the question whether the ‘*cycling*’ of AMPK away from glycogen (by modifying the Thr-148 residue) does affect insulin-independent glucose uptake, and in particular intracellular glycogen levels, requires further investigation. The use of healthy rodent models, in combination with applying the toolbox and techniques established in the lab will provide detailed insight into the AMPK signaling cascade on transcriptional and translational level, intracellular localization, and the regulation of glycogen metabolism. Also studying important processes such as autophagy/glycophagy, fatty acid oxidation and gluconeogenesis will contribute to knowledge of the function of localized AMPK on whole-body level.

Although addressing this kind of questions will certainly lead to novel insights mimicking physiological situations, many clinically relevant questions would still remain unanswered. Since AMPK is rapidly activated during exercise/contraction, caloric restriction, or in response to pharmacological agents (*e.g.*, metformin) sustained cytoplasmic AMPK activation may prove beneficial. However, does the shift of AMPK away from glycogen lower blood glucose levels in T2D conditions? Does it prevent the possible inhibitory effect of AMPK on glycogen synthesis? Or, alternatively, does it inactivate cytoplasmic glycogen synthase and thus negatively affect glycogen synthesis? The use of established disease models (*e.g.*, diabetic *ob/ob* mice) would allow for addressing such kinds of questions and would ultimately help in understanding whether the deficiency in glycogen-storage observed in heart and skeletal muscle of T2D patients is preventable and/or reversible by modulating the subcellular localization of AMPK. Today it is the challenge of the scientist, in collaboration with different target groups, to find these answers, explain them to the patient, and translate them into the clinic.

Target groups

The involvement of patients, healthcare providers and society, *i.e.*, target groups, is fundamental to the quality care for metabolic diseases such as T2D. From a scientific point of view, this list of people/groups, being interested in our work or being important for knowledge development, can be further extended for instance with other scientists and/or collaboration partners, patient associations, foundations, and pharmaceutical industry.

To make other scientists aware of our recent discoveries, and also importantly, to gather novel insights from different research angles, thereby facilitating scientific progress and contribution, the studies described in this thesis were presented at relevant scientific conferences by means of oral presentations at national (Genetics Retreat, Rolduc, Kerkrade, the Netherlands, 2012; NVDO Annual Dutch Diabetes Research Meeting, Oosterbeek, the Netherlands, 2013) and international conferences (1st European AMPK Workshop, Maastricht, the Netherlands, 2013; Society for Heart and Vascular Metabolism (SHVM) Conference in Tromsø, Norway, 2014; FASEB Science Research Conference on AMPK, Lucca, Italy, 2014) and poster presentations (FASEB Science Research Conference on AMPK, Asilomar, Pacific Grove, California, 2012; 2nd European AMPK Workshop, Maastricht, the Netherlands, 2015). In addition, our findings were published in scientific journals relevant to the research field (*Journal of Biological Chemistry*). In the near future, it might be of interest to participate in and present our current knowledge on conferences such as the World Congress on Interventional Therapies for Type 2 Diabetes, as it is a comprehensive, multidisciplinary forum of worldwide specialists whose aim is discuss on research priorities and health policy initiatives, and gain further insight into how the study of increasing intracellular glycogen content may improve our understanding of T2D and provide direction for future treatments of curative intent.

To further encourage knowledge utilization and development, there is strong interest for collaboration with people directly connected to our research/the field; in other words, networking with current collaborators or colleague scientists that are willing to cooperate and/or provide for instance relevant mouse models related to our research. Further, approaching gene targeting companies (for example, GenoWay), as they may be involved in the production of highly physiologically relevant transgenic mouse models (*e.g.*, wild-type AMPK β 2 vs. AMPK β 2-T148D transgenic mice) might be of serious interest. In future, such mice may be used to explore the above mentioned physiological impact of Thr-148 phosphorylation on glucose and glycogen metabolism.

In the era of advanced therapeutic approaches, we established a new screening procedure for the identification of small molecules specifically targeting the carbohydrate-binding module (CBM) of AMPK. Based on this strategy, we aimed at shifting AMPK away from glycogen by means of small molecules, for instance, to modulate cardiac and/or skeletal muscle glycogen content. Besides targeting AMPK to prevent it from binding to glycogen, the top candidate molecule has been shown to activate the AMPK signaling pathway and induce nutrient (*i.e.*, glucose and long-chain fatty acid) uptake. However, up until now, there was no isoform-specificity found (AMPK β 1 vs. AMPK β 2), underlining that optimization (*e.g.*, chemical modification) is necessary to obtain isoform-specificity. Thus, the manufacturing of innovative molecules/drugs for clinical use must involve our pharmaceutical friends.

In order to make sure that innovative findings and discoveries result into improvements for patients, investment must be put in big national and international collaborations. In essence, one should keep in mind that it is the total assembly of knowledge, from scientists, clinicians, health care providers, human subjects, pharmaceutical industry and foundations that creates the value. Therefore, we will need to extensively discuss, interact and collaborate in order to valorize. An example of such national collaboration is the CVON (Dutch Cardiovascular Research), which supports large-scale national research projects in which researchers, universities, and industry work together. Being involved in grants such as CVON would provide an excellent opportunity for further knowledge development (6). Moreover, European grants such as Horizon2020 Projects or European Framework for Research Program should create and further support such international collaborations and sharing of expertise (7).

Activities and products

Products of great relevance for the society may be the awareness of diabetes as an epidemic. To make the knowledge described in this thesis more transparent, accessible and useful for the benefit of the society, one of the best target groups to reach would be foundations such as the Diabetes Fonds or Hartstichting (1,6). Their function is to improve access to medicines and healthcare by disseminating knowledge to all layers of the population, making use of brochures and campaigns, and providing education, counseling and care. One of the aims/challenges could be to get more connected to such organizations. By giving them regularly comprehensible updates on the status/progress of the research, the transfer of knowledge to society will surely be facilitated and thus increased. In such way, the society (including the patient, relatives, generally interested people, contributors to the foundation, etc.) might better understand, realize, discuss or wonder about what kind of research (stepwise) is conducted in the lab for the benefit of the patient. Thus, by providing scientifically relevant information regarding the syndrome, which is intelligible for layman, we may create opportunities for improving the health status and quality of

life of the patient. Further, the organization Hart&Vaatgroep is an important association for patients or their relatives, which organizes regional or national meetings, workshops, courses and mutual patient connection (6). Making such organizations aware of the scientifically/clinically relevant questions being addressed will foster the exchange of knowledge for the benefit of the patient.

One of the products of great importance for future is based on the drug-targeted strategy presented in this thesis and thus may include novel molecules/drugs. After optimization of the top candidate molecule(s), the most potent molecule(s) would need to undergo pharmacodynamics, pharmacokinetics, ADME and toxicity testing through *in vivo* approaches before going into clinical trials. Although on average, only one in every 5,000 compounds that makes it through lead development to the stage of pre-clinical development becomes an approved drug these steps are of high value, thus determining the safest dose for first-in-human studies and evaluating the product's safety profile. Ultimately, the drug should have selective capacity to shift AMPK away from glycogen, and similar safety and bioavailability as for instance metformin does. Ideally, the drug should have the capacity of stimulating glucose uptake, and finally, it should not cross the blood brain barrier to prevent influence on hypothalamic AMPK.

Innovation and future directions

It is of vital importance to invest in patient care and prevention, and thus in the pioneering research that is underlying, also to reduce for instance the risk of heart failure. In other words, identification of the molecular mechanisms involved in the onset and/or progression of the metabolic dysregulation ultimately allows for new insights and therapeutic developments for the regression and cure of T2D, and this is where our fundamental molecular-biology-oriented research contributes. Obviously, we do not provide here any evidence for a potential new medication that would lead to the cure of the type 2 diabetic patient. However, interestingly, this thesis provides important knowledge and *state-of-the-art* techniques that is needed as basis for the development of therapeutic strategies and/or techniques that may lead to novel treatment options for T2D. As described in the paragraphs above, our findings provide ample of possible opportunities (*i.e.*, further exploring the novel drug-targeting approach, translational research by means of animal experimentation, pre-clinical testing, etc.) not only to address the importance of AMPK as metabolic master switch of the cell, but also to emphasize the relevance of subcellular localization of AMPK. This research will only be innovative if the ideas described here will ultimately become reality; in other words, if we can finally extrapolate all gathered knowledge to human subjects, aiming at the cure of the patient.

Conversely, from another interesting point of view, we might be able to further extract and apply this potentially advance knowledge to other diseases, such as the Wolff-Parkinson-White syndrome, a congenital cardiac abnormality associated with abnormal glycogen accumulation. In such disease, it might be interesting to study whether the shift of AMPK towards glycogen, in contrast to T2D, would lower cardiac glycogen content. Moreover, AMPK is also emerging as a possible metabolic tumor suppressor. Although there is no evidence yet, it might be worthy to further investigate whether the development of new drugs specifically aiming at shifting AMPK away from glycogen may have a potential beneficial role in cancer prevention and/or treatment.

References

1. Retrieved from <https://www.diabetesfonds.nl/>; Sept. 2015.
2. Retrieved from <http://www.nationaalkompas.nl/gezondheid-en-ziekte/>; Sept. 2015.
3. Retrieved from www.changingdiabetesbarometer.com/; Oct. 2015.
4. Retrieved from <http://www.who.int/mediacentre/factsheets/fs138/en/>; Sept. 2015.
5. Oligschlaeger, Y., Miglianico, M., Chanda, D., Scholz, R., Thali, R. F., Tuerk, R., Stapleton, D. I., Gooley, P. R., and Neumann, D. (2015) The Recruitment of AMP-activated Protein Kinase to Glycogen Is Regulated by Autophosphorylation. *J Biol Chem* **290**, 11715-11728
6. Retrieved from <https://www.hartstichting.nl>, Oct. 2015.
7. Retrieved from <http://ec.europa.eu/programmes/horizon2020/>; Oct. 2015.

List of abbreviations

ACC	acetyl-CoA carboxylase
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ASS	AMPK-specific substrate – biosensor
ATGL	adipose triacylglycerol lipase
ATP	adenosine triphosphate
CaMKK2/CaMKK β	Ca ²⁺ /calmodulin-dependent protein kinase kinase β
CBM	carbohydrate-binding module
CBS	cystathionine- β -synthase
CFP	cyan fluorescent protein
Cidea	cell death-inducing DFFA-like effector a
CK1/2	casein kinase1/2
E _F	FRET efficiency
EGCG	epigallocatechin gallate
ERK1/2	extracellular signal-regulated kinase 1/2
EV	empty vector
FLIM	Fluorescence Lifetime Imaging Microscopy
FRET	Förster Resonance Energy Transfer
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
GABA	gamma-aminobutyric acid
GABARAPL1	autophagy protein GABA(A) receptor-associated protein-like 1
Gal83	Snf1 β -subunit ortholog
GB8	generalized Born model 8
GBE	glycogen branching enzyme
GDE	glycogen debranching enzyme
GP	glycogen phosphorylase
GS	glycogen synthase
GSK-3	glycogen synthase kinase-3
HiG	High glucose
HSL	hormone sensitive lipase
IC50	half maximal inhibitory concentrations
iFCS	heat-inactivated calf serum
IL-6	interleukin-6
Ins	insulin
IP	immunoprecipitation
K _D	equilibrium dissociation constants
LKB1	liver kinase B1
MAPK	(ERK1/2)-mitogen-activated protein kinase

MCD	malonyl-CoA decarboxylase
MetS	metabolic syndrome
MT	mutant
MTFA	mitochondrial transcription factor A
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NRF-1	nuclear respiratory factor-1
PEPCK	phosphoenolpyruvate carboxykinase
PGC1 α	peroxisome proliferator-activated receptor γ coactivator 1 α
PIASy	protein inhibitor of activated STAT
PKA	protein kinase A
PKC	protein kinase C
PNK	Polynucleotide Kinase
PP	protein phosphatase
PP1	protein phosphatase type 1
PPAR	peroxisome proliferator-activated receptor
PPP1R3C	PTG/R5
PPP1R3D	R6
PTG	protein targeting to glycogen
RVXF	N-terminal PP1-binding motif of R6
R6	PP1-glycogen-targeting subunit PPP1R3D
Reg1	PP1 ortholog
SBD	starch binding domain
Snf1	sucrose-non-fermenting 1; Reg1, yeast targeting subunit of PP1
STAT	Signal Transducer and Activator of Transcription
STBD1	starch-binding domain-containing protein 1
SUMO	E3-small ubiquitin-like modifier
TAK1	(TGF- β -activated kinase 1
TGF	transforming growth factor
Thr-148 / T148	threonine-148
Thr-172 / T172	threonine-172
TZDs	thiazolidinediones
T2D	type 2 diabetes
WPW	Wolff-Parkinson-White
WXNXGNYX(L/I)	C-terminal substrate-binding site of R6
WT	wild-type
Y2H	yeast two-hybrid
YFP	yellow fluorescent protein
β -CD	β -cyclodextrin
$\Delta G_{\text{cal.}}$	calculated binding free energy
ΔG_{exp}	experimental binding free energy

Dankwoord

Dit proefschrift is het resultaat van mijn PhD traject binnen de afdeling Moleculaire Genetica, Maastricht University. De afgelopen vier jaar was een hektische periode. De tijd is omgevlogen. Het waren niet de makkelijkste vier jaar van mijn leven, maar ik ben enorm trots op mezelf en het resultaat dat er nu ligt. Als ik terugkijk naar mijn promotietraject, ben ik ontzettend blij dat ik zóveel heb geleerd, zóveel kennis heb opgedaan en dat ik zóveel leuke mensen heb mogen leren kennen. Het was net een achtbaan. En alles is mij bijgebleven.....deel uitmaken van een geheel nieuwe groep, technieken opzetten, fellowship schrijven, nieuwe assays leren en opzetten, celkweken, racletten, koffie leuten, vlaai proeven, labchats, 11e van de 11e, Journal Clubs, Sinterklaas vieren, kerstborrels, I'M CARIM representative, California, Golden Gate, Napa Valley, FASEB AMPK Conference Asilomar, poster presentations, PBL systeem, tutoren, progress meetings, lab-uitjes, high-tea TeaZone, paaseitjes tietsjen, etentjes, Goed-Fout-Feest, AMPK Workshop Maastricht, oral presentations, organiseren lab-uitje 2013, Bierfeesten, promotie-feestjes, CARIM Course Week & Social Events, SHVM Norway, EIC awardee, hiking, barbecue, Ridderronde Maastricht, Carnaval, borrels, FASEB AMPK Conference Lucca, Pisa, musical Dreamgirls, cursussen, CoffeeLovers, pretparken, Swentibold tocht, papers schrijven, JBC, weekendje Monschau, Solar, en.....dit proefschrift schrijven. Dit alles had ik allemaal nóóit willen missen, en daarom wil ik graag iedereen bedanken die hierbij betrokken is geweest!

Omdat je wetenschappelijk onderzoek natuurlijk niet alleen doet, ga ik de volgende mensen speciaal bedanken.

Allereerst wil ik de coach van Team Neumann bedanken om mij deel uit te laten maken van de selectie. Dear Dr. Neumann, dear Dietbert, first of all I would like to thank you for your confidence and support during this period of time. I have great admiration for you as a scientist. Your critical view, passion and perfectionism have guided me to this great end result. Thank you for giving me the opportunity of presenting our work on a variety of conferences and thanks for all knowledge and skills I have acquainted during this four-year trajectory. Also I would like to thank your wife Anja, your twins Alwin and Bero and your au-pair Romina for the warm welcome and great dinners we had at your home.

Ten tweede wil ik mijn promotor Prof. dr. Glatz bedanken. Beste Jan, dank dat ik op uw afdeling - Moleculaire Genetica - mijn promotie-onderzoek heb mogen uitvoeren. Ook in woelige tijden was u mijn vaste baken. Dank voor uw roestvaste vertrouwen in mij, uw ondersteuning, hulp en alle positieve feedback tijdens mijn hele promotie-traject.

Ten derde wil ik graag mijn co-promotor Dr. Luiken bedanken. Beste Joost, hoewel je misschien zelf het gevoel had dat je te weinig als co-promotor voor mij hebt kunnen betekenen, wil ik dit hier graag weerleggen. Dank voor alle feedback gedurende de Joost-Luiken/Progress Meetings, labchats en 1-op-1 gesprekjes. Daarnaast wil ik je extra bedanken voor je support tijdens het schrijven van mijn proefschrift.

I would like to thank the members of the reading committee, Prof. dr. Ellen Blaak, Prof. dr. Terje Larsen, Prof. Dr. Casper Schalkwijk, Dr. Blanche Schroen and Dr. Ramon Langen for being part of my reading committee and for their critical reading and proficient evaluation of this doctoral thesis.

Also I would like to thank all the members of the corona.

Ten vijfde wil ik mijn twee allerliefste paranimfen bedanken.

Lieve Armand, gedurende je stage bij ons wist ik dat er een klik was. Ik ben super trots dat jij mijn paranimf bent! Je bent naast een goede vriend, ook een intelligente en gezellige collega om mee samen te werken. Ik wil je enorm bedanken voor al je hulp in het lab, alle wetenschappelijke en niet-wetenschappelijke gesprekken, en alle leuke momenten die we samen, met Famke, hebben gehad en nog steeds hebben. Eén ding is zeker, ik miste je echt in ons team, maar ik ben ontzettend blij dat je zelf een leuke plek hebt gevonden bij Biochemie en wens je ook heel veel succes voor de komende tijd. Daarnaast: dankjewel voor je ondersteuning in de laatste sprint van dit traject. Natuurlijk doe jij dit paranimf-schap niet alleen!

Lieve Petra, ik voel me echt vereerd dat jij tijdens mijn promotie naast mij staat. Ik vergeet nooit het moment dat ik jou, samen met Bert, onder het genot van een lekker hapje en drankje mijn kaartje overhandigde waarin ik je vroeg of je misschien mijn paranimf wilde zijn. Heel erg bedankt voor alle Eerste Hulp Bij Onderzoek, zowel als secretaresse als buiten het werk om. Dank ook voor alle mooie momenten die we samen hebben gedeeld. Deze keer sta jij, samen met mij, aan deze kant. We zullen er samen van genieten!

Ten zesde wil ik graag Team Neumann bedanken. A word of thanks goes to the whole team! Marie, Dipanjan, Xiaoqing, Yilin, Jieyi, Ricardo, Armand, Jairo, Laura, Özge, Jurriaan, Hacer, Kenrick, Kwang, Daphne, Elianne, Magdalena, Christian and Milou, thanks for being my colleagues and friends! Thanks for collaborating and having lots of fun in and out of the lab. Thanks for all your help, sharing knowledge with me and having lots of scientific conversations. I am very glad that I have been one of the players in a team consisting of all of you, guys!

Dr. Miglianico, dear Marie, I am super proud of you, you have done an awesome job! It was an honour for me to be your paranimf. Thanks for the close connection in and outside the lab! I will never forget your lessons about the vegetable/fruit seasons.

Dr. Chanda, dear Dip, I have no words for all your insights, efforts and critical readings. Our coffee moments at the coffee house, library and at CoffeeLovers are unforgettable. I hope, that once, Joël and me are able to see your hometown!

Dear Xiaoqing, Yilin and Jieyi, thanks for the delicious Chinese dishes I have been able to taste. I wish you lots of good luck with finishing your doctoral theses!

Dr. Rodriguez-Calco, dear Ricardo, thanks for being my friend and thanks for the great time we had in the lab.

Ook zijn er een aantal studenten die ik heb mogen begeleiden. Laura en Jairo, dank jullie wel voor de prettige samenwerking! Last but not least, Milou, onze wervelwind, altijd vroeg aanwezig op het lab. Jouw gedrevenheid zal je nog heel ver brengen; héél veel succes in Basel en we'll keep in touch!

Daarnaast wil ik graag alle PI's en ervaren onderzoekers bedanken: Willem, Marjo, Ronit, Guillaume. Dank jullie wel voor jullie kritische blik en wetenschappelijke input gedurende de afgelopen vier jaar. Willem, jouw alertheid gedurende all kind of meetings waardeer ik enorm. Dank je wel voor je kennis, sparren, 'nasty questions' en dus kritische maar waardevolle feedback. Ook heb ik onder andere van jou geleerd om vragen te durven stellen aan collega-wetenschappers.

Marjo, ik vond het jammer dat jullie groep na ongeveer de helft van mijn PhD traject onze afdeling ging verlaten. Dank je wel voor alle gezellige, maar ook wetenschappelijke gedachtewisselingen die we hebben gehad.

Dear Ronit, your constructive and positive feedback was always very helpful. Even in times I felt a bit lost in science, you knew how to cheer me up. Your warm words will stick in my mind forever.

Guillaume, bedankt voor je niet aflatende belangstelling, koffie-momentjes en de heerlijke groenten uit jullie eigen moestuin. Jouw kracht en positiviteit waardeer ik enorm.

Dan wil ik graag het ondersteunend personeel bedanken. Will, Vivian, Patrick, Marion, Nicole, Chantal en Hanneke. Dank jullie wel voor alle organisatie, zowel binnen als buiten het lab om. Will, dank voor de fijne samenwerking op het lab. Ik zal altijd terugdenken aan 'het zuchtend – uh – briesend paard'. Dank voor alle bestellingen die je keer op keer voor me deed. Je warme belangstelling voor privé-omstandigheden heb ik altijd zeer op prijs gesteld.

Vivian, zonder jou had ik nóóit zoveel kunnen celkweken. Ik heb veel profijt gehad van jouw geordende en betrokken werkwijze. Een éxtra woord van dank voor je fanatieke hulp en inzet gedurende de laatste paar maanden rondom het AMPK β 2/R6-project!

Patrick en Marion, ik vond het een eer om samen met jùllie het labuitje te mogen organiseren. Wat een geweldige ervaring, met die kickbikes door Maastricht. Zóveel lol. Die avondjes samenzitten om uit te dokteren hoe we de routes zo spannend mogelijk konden maken waren echt de moeite waard. We hadden een top dag met een heerlijke barbecue en wijnproeverij als afsluiter bij Marion thuis.

Nicole, van jou heb ik ook ontzettend veel geleerd, van confocale microscopie tot het isoleren van cardiomyocyten. Jammer dat je weg moest van onze afdeling!

Chantal, kersverse mama, het was een plezier om met jou op het lab te zijn en zo'n Goed-Fout-feestje is voor herhaling vatbaar.

Hanneke, fijn dat jij in alle drukte rustig en kritisch door mijn thesis heen hebt willen bladeren voordat het naar de beoordelingscommissie werd gestuurd. Dank je wel daarvoor.

Ten zevende bedank ik graag de volgende dames van MolGen, Vivian, Petra, Marion, Nadine, Peggy en Sofie, waar ik de meeste culinaire uurtjes na werktijd mee heb doorgebracht.

Dr. Jetten, lieve Nadine, lachen, gieren, brullen, dat kunnen we samen goed. Bedankt dat jij zo'n lieve kamergenoot voor me was. Ik heb je erg gemist vanaf het moment dat je weg bent, maar ik ben blij dat jij ook zo van Sushi-eten houdt. We'll keep in touch!

Dr. Prikaerts, lieve Peggy, zat ik eindelijk gezellig in jullie lab, was jij er niet meer...wat een gemis! Ondanks alles ben ik blij dat je maar een kattensprong van me vandaan woont zodat ik jou en je gezinnetje af en toe eens kan treffen.

Dr. Walenbergh in spe, lieve Sofie, ook op jou ben ik ontzettend trots, want ook jou is het in 4 jaar tijd gelukt om een prachtig proefschrift te schrijven! Dank je wel voor de sportieve en gezellige uitjes die we samen hebben gehad, en hopelijk herhalen we deze nog vaak in de toekomst.

Ten achtste gaat een woord van dank uit naar mijn lieve (ex-)collega-AIO's/post-docs op de afdeling MolGen: Tim, Sofie, Nadine, Guus, Emiel, Tom, Mike, Ellen, Veerle, Ine, Peggy, Laura, Laurant, Yeliz, Veronika, Miranda. Dank jullie wel, allemaal, voor de geweldige tijd die ik heb ervaren op deze afdeling! In het bijzonder wil ik dan graag nog even een van mijn kamergenoten bedanken.

Dr. Hendriks, lieve Tim, ook jou heb ik sinds dat je naar Wenen vertrok moeten missen. Dankjewel voor alle humoristische momenten en voor het organiseren van de wekelijkse vrijdagmiddag-borrels van kamer 5.120.

Lieve Tom, ook jij zijt sinds een klein half jaartje mijn kamergenoot geworden, daar ben ik enorm fier op. Dank u voor de fijne en inzichtelijke gesprekjes en Belgische grapkes.

Dr. Stöger, beste Laurant, jammer dat ik maar zo kort je buurvrouw ben geweest.

Daarnaast wil ik ook Kosta, Bart, Martijn en Nathalie bedanken voor de gezellige tijd op het lab en in het pretpark.

Ten negende bedank ik de mensen van I'M CARIM, Timo, Emiel, Siamack, Elke, Tom en Armand, voor de leuke activiteiten die we samen georganiseerd hebben. Van social events tijdens de CARIM Course Week tot Young Investigator Rounds en het organiseren van Workshops. Fijn dat we samen zo'n actief team gevormd hebben. Een speciaal woord van dank gaat naar Timo en Emiel, die vertrouwen in mij hadden en

mij deel uit lieten maken van I'M CARIM. Elke, Tom en Armand, ik weet zeker dat jullie goed zullen opvolgen. Siamack en Tom, ook jullie wens ik veel succes met de laatste loodjes.

Daarnaast wil ik ook graag de CARIM EPC board bedanken. Beste Marc, Matthijs, Hans, Adriaan, Eline, Simone, Esther, Riet, Tara en prof. dr. Unger. Bedankt dat jullie mij de kans gegeven hebben om kritisch een steentje te kunnen bijdragen aan de benadering van verschillende zaken die gerelateerd zijn aan onderwijs, monitoring en begeleiding van AIO's. Ik ben er trots op dat we er samen voor gezorgd hebben dat er een nieuwe CARIM Talent Fellowship van de grond komt.

Ten tiende wil ik alle congresorganisatoren bedanken voor het feit dat ik mijn wetenschappelijke ondervindingen mondeling heb mogen delen op de FASEB AMPK Conference in Lucca, SHVM in Tromsø, European AMPK Workshop in Maastricht en NVDO Diabetes meeting in Oosterbeek.

Een woord van dank gaat ook uit naar de supervisors van de stages gedurende de Bachelor- en Master-fase.

Dear Prof. dr. Maria Flavia di Renzo, I would like to thank you for giving me the great opportunity to join your Cancer Genetics lab at the IRCC in Candiolo (Turin). Almost seven years ago I got inspired from the amazing work you performed in your lab and the scientific knowledge you have and shared with me.

Geachte Prof. dr. van Schooten, beste Frederik-Jan, dank u voor de mogelijkheid die ik kreeg om op uw afdeling stage te mogen lopen.

Dear Dr. Chiu and Dr. Godschalk, beste Roland en beste Roger. Dank jullie wel voor jullie supervisie tijdens mijn stage die ik bij jullie liep. Roger, ik vond het geweldig dat ik na mijn stage bij jou de kans kreeg om me verder te ontwikkelen, dat ik aangesteld werd als student-assistent en dat hier nog een publicatie uitgekomen is.

Dr. Schults, beste Marten, dank je wel voor je directe supervisie op het lab en voor alles wat ik van jou geleerd heb.

Geachte Dr. Theys, beste Jan, dank voor de warme begeleiding die ik kreeg gedurende de periode dat ik senior stage liep op het NKI in Amsterdam. Ik heb in alle opzichten veel geleerd gedurende deze periode, mede dankzij u.

Ten twaalfde bedank ik graag al mijn vrienden & vriendinnen. Voor alle gezellige uitjes, etentjes en drankjes samen, tussen alle bedrijven door. Vandaar ook dank jullie wel voor jullie begrip! In het bijzonder bedank ik dan ook graag Evelyne, Eva, Linda en Jessica, en natuurlijk jullie lieve partners. Ook wil ik graag mijn maatjes-door-het-ganse-land bedanken. Lieve Kirsten, Sanne, Sandrine, Julia, Brenda, Ryan en Niels, thanks!

Gedurende mijn studententijd heb ik ook lieve mensen leren kennen, Helma, Kathleen, Evelyn, Niki, Emma, Leonie and Sara, with whom I still have a very special relationship, because out of sight is not out of mind... Thanks e grazie mille for being my friends and for all the good times we had and have together!

Paul & Nicole, mijn liefste broer en zus. Ik heb er respect voor hoe jij, lieve Nicole, als hard werkende vrouw, maar ook als liefste mama, door het leven gaat. Coachen, dat kun jij als geen ander! Bedankt voor al jouw goede input en positieve energie. Ik koester alle momenten die ik samen met jou, maar ook met mijn schone broer en lieve papa Marcel van Ilyano en Elysia heb kunnen, en nog steeds, kan delen.

Lieve Paul, ik sta versteld van de uitdagingen die jij keer op keer weet aan te gaan en van de avonturen die je daarin opzoekt. Je bent niet alleen een harde werker en diplomaat, die veel kennis heeft en dit ook goed weet over te dragen op anderen, maar gelukkig ook een levensgenieter! Ik vind het kei-gaaf dat jij al zoveel van de wereld hebt gezien! Geloof het of geloof het niet, er zijn van die momenten, dan ben je echt een voorbeeld voor me.

Ik ben trots op jullie!

Hans en Liliane, mijn lieve pap en mam. Als eerste wil ik zeggen dat ik veel bewondering voor jullie heb! Onvoorwaardelijke steun en liefde, onvoorwaardelijk geloof en vertrouwen in mij. Mede dankzij jullie kundige opvoeding, ben ik geworden wie ik ben. Dank jullie wel voor al jullie interesse in mijn onderzoek de afgelopen jaren; voor alle begrip, ondersteuning, luisterende oren en adviezen. En voor het zelfvertrouwen als ook het relativeringsvermogen dat jullie me hebben meegegeven. Hoe dan ook, wanneer dan ook, jullie stonden altijd voor me klaar en lieten blijken hoe trots jullie op me waren. Dat heeft me altijd veel kracht en moed gegeven. Ik heb mijn doel bereikt. Paps en mamsie, dank jullie wel voor alle mooie en waardevolle momenten, samen, met ons allen! Ik hoop dat er nog veel zullen volgen. Ik hou van jullie.

Dan mijn liefste Joël & onze Gioia. Lieverd, 'wachten' was een van mijn *key words* denk ik zo...dankjewel voor jouw engelengeduld! Je hebt te pas en te onpas interesse getoond in mijn onderzoek. Dat je begrijpt dat mijn onderzoek gaat over een eiwit dat *AMPK* heet, en dat ik probeer te onderzoeken waarom, wanneer en hoe dit eiwit bindt aan glycogeen, vind ik heel bijzonder. Dat tekent jouw karakter; jouw belangstelling, jouw interesse, in mij, en in de dingen die ik belangrijk vind. Je stond en staat altijd voor me klaar. Dankjewel voor je ultieme geloof en vertrouwen in mij. Jij bent een onuitputtelijk bron van energie! Jouw geduld en support hebben mij gebracht waar ik nu sta. De wandelingen met Gioia, samen of alleen, gaven me positieve energie en de ruimte om alles in mijn hoofd te kunnen ordenen. Dankjewel voor de liefde die jij me gegeven hebt. Zonder jou was dit nooit zo gelukt. Nu zijn we samen klaar voor een nieuw avontuur. *Every day I love you more.*

Last but not least; het was niet altijd even gemakkelijk. Ondanks het feit dat niet alle elementen gedurende dit traject zijn gelopen zoals ik gehoopt had, ben ik dankbaar dat ik deze leerweg ben ingeslagen. Een ding is zeker: wetenschap is onvoorspelbaar, bestaat uit vallen en opstaan, bergen en dalen, negatieve en positieve (of soms geen) data.

Na veel hoop, moed, ploeteren, wilskracht, energie en vooral doorzettingsvermogen, ben ik ontzettend trots op het onderzoek dat ik hier beschreven heb, en spreek de hoop uit dat dit proefschrift bijdraagt aan de huidige kennis van AMPK en al haar relevante biologische processen. Daarnaast hoop ik dat mijn onderzoek wetenschappers uitdaagt om nog beter inzicht te krijgen in de regulatie van glycogeen metabolisme in (hart)spieren van zowel gezonde mensen als mensen met diabetes type 2, of van mensen die kampen met een glycogeen-opstapelingsziekte. Het uiteindelijke doel is de levenskwaliteit van deze patiënten te verbeteren.

Curriculum Vitae

Curriculum Vitae

Yvonne Oligschläger was born on December 6, 1987 in Velsen, the Netherlands. In 2005, she acquired her high school diploma (Science & Health) at S.G. Sophianum in Gulpen, the Netherlands. She started her Bachelor's education in Life Sciences at Hogeschool Utrecht (University of Applied Sciences), the Netherlands in February 2006. In 2008, she performed an internship for 9 months at the department of Cancer Genetics, IRCC Candiolo, Università degli Studi di Torino (Turin, Italy), where she investigated ways to sensitize ovarian cancer cells to chemotherapeutic agents. She finalized her Bachelor study in 2009. In September 2009, she started her Master's study in Molecular Life Sciences at Maastricht University, the Netherlands. During this study, she performed a 4-months internship at the department of Health Risk Analysis and Toxicology of Maastricht University, focusing on the role of the tumor suppressor gene von Hippel-Lindau in renal-cell carcinomas in response to genotoxic agents. Subsequently, she performed an 8-months internship at the department of Cell Biology I at the Netherlands Cancer Institute (NKI) in Amsterdam, the Netherlands. Here, she investigated the role of binding protein SPIN90 in WAMP complex-mediated ruffle formation and cell motility. In July 2011, she graduated for her Master's degree and in September of the same year started her PhD trajectory at the department of Molecular Genetics in the Faculty of Health, Medicine and Life Science, and (FHML) of Maastricht University, within the School for Cardiovascular Diseases (CARIM), under the supervision of Dr. Dietbert Neumann. Her research addressed the molecular mechanisms involved in AMPK localization changes to and from glycogen, localization-dependent AMPK activity, and aimed at deriving novel therapeutic strategies to shift AMPK away from glycogen. During this 4-year period, she actively participated in teaching and supervising students and she was a PhD representative from CARIM (I'MCARIM). Additionally, she took part in the Education Program Committee (CARIM) and Interfaculty Program Committee (FHML), in which education and monitoring of PhD candidates is central. She actively attended national (NVDO Meeting in Oosterbeek) and international conferences (FASEB AMPK Conference in Asilomar (California) and in Lucca (Italy), Society for Heart and Vascular Metabolism (SHVM) Conference in Tromsø (Norway), 1st and 2nd European AMPK Workshop in Maastricht (Netherlands). During these conferences, she presented her work by means of oral or poster presentations. In October 2013, she received the Best Oral Presentation award at the 1st European AMPK Workshop. Moreover, in June 2014, she was awarded an Early Investigator Commendation by the SHVM organizing committee.

List of publications

1. **Oligschlaeger Y**, Miglianico M, Chanda D, Scholz R, Thali RF, Tuerk R, Stapleton DI, Gooley PR, Neumann D. The recruitment of AMP-activated protein kinase to glycogen is regulated by autophosphorylation. *J Biol Chem.* 2015 May 1;290(18):11715-28.
2. Linschooten JO, Verhofstad N, Gutzkow K, Olsen AK, Yauk C, **Oligschläger Y**, Brunborg G, van Schooten FJ, Godschalk RW. Paternal lifestyle as a potential source of germline mutations transmitted to offspring. *FASEB J.* 2013 Jul;27(7):2873-9.
3. Schults MA, **Oligschlaeger Y**, Godschalk RW, Van Schooten FJ, Chiu RK. Loss of VHL in RCC Reduces Repair and Alters Cellular Response to Benzo[a]pyrene. *Front Oncol.* 2013 Oct 28;3:270.
4. Lorenzato A, Martino C, Dani N, **Oligschläger Y**, Ferrero AM, Biglia N, Calogero R, Olivero M, Di Renzo MF. The cellular apoptosis susceptibility CAS/CSE1L gene protects ovarian cancer cells from death by suppressing RASSF1C. *FASEB J.* 2012 Jun;26(6):2446-56.

Submitted/in preparation

- **Yvonne Oligschlaeger**, Marie Miglianico, Vivian Dahlmans, Carla Rubio-Villena, Dipanjan Chanda, Maria Adelaida Garcia-Gimeno, Yilin Liu, J. Willem Voncken, Joost J.F.P. Luiken, Jan F.C. Glatz, Pascual Sanz, Dietbert Neumann. The interaction between AMPK β 2 and the PP1-targeting subunit R6 is dynamically regulated by intracellular glycogen content. *Submitted*
- Marie Miglianico, **Yvonne Oligschlaeger**, Dipanjan Chanda, Kanin Wichapong, Will A. Coumans, Armand Jaminon, Ricardo Rodriguez-Calvo, Özge D. Özçete, Roy Schrijver, Xiaoqing Zhu, Ivo Bleylevens, Nynke M.S. van den Akker, Daniel G.M. Molin, Jan F.C. Glatz, Gerry A.F. Nicolaes, Dietbert Neumann. Displacing AMPK from glycogen by pharmacological targeting of its carbohydrate-binding module. *In preparation*
- Dipanjan Chanda, Jieyi Li, **Yvonne Oligschlaeger**, Mike Jeurissen, Sofie M. A. Walenbergh, Ronit Shiri-Sverdlov, Dietbert Neumann. MSP is a negative regulator of inflammation and lipogenesis in ex vivo models of NASH. *Submitted*
- Dipanjan Chanda, **Yvonne Oligschlaeger**, Yilin Liu, Xiaoqing Zhu, Jieyi Li, Marie Miglianico, Will Coumans, Joost J.F.P. Luiken, Jan F.C. Glatz, Dietbert Neumann. 2-Arachidonoylglycerol reverses inflammation-induced insulin resistance in cardiomyocytes. *In preparation*