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# Interactomics of key kinases in energy metabolism : Characterizing interactions of AMP-activated protein kinase & B-type creatine kinase

Anna Klaus (née Brückner)

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DOCTORAT DE L'UNIVERSITE DE GRENOBLE

THESE

- Biologie Cellulaire -

**Anna Brückner**



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# **Interactomics of key kinases in energy metabolism**

Characterizing interactions  
of AMP-activated protein kinase & B-type creatine kinase

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**Jury**

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Cécile Polge	Co-directeur
Uwe Schlattner	Directeur de Thèse

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# PART I



STATE OF THE ART

&

AIM OF THIS WORK

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AIM OF THIS THESIS

CHAPTER I :

*Regulation of cellular and whole body energy homeostasis*

CHAPTER II :

*Yeast Two-Hybrid, a Powerful Tool for Systems Biology*

## AIM AND STRUCTURE OF THE THESIS

A key property of complex biological systems is the presence of interaction networks crucial for all levels of cellular function, including the regulation of cellular energy. Two enzymes take center stage in the regulation of cellular and whole body energy metabolism to maintain energy homeostasis. Creatine kinase (CK) functions as an intracellular energy storage and transport system. AMP-activated protein kinase (AMPK) maintains cellular and whole body energy balance by regulating ATP supply and consumption at various levels. Both enzymes are supposed to require specific protein interactions for their cellular functions. Creatine kinase is partially localized in cellular microenvironments of high ATP turnover, providing a rapid energy transfer system based on creatine/phosphocreatine to use or restore local ATP pools. This requires direct interaction with ATPases or at least anchoring in their close vicinity. AMPK senses energy status and provides complex downstream signaling to specific key metabolic enzymes and genes. This requires substrate recognition and possibly also scaffolding and recruitment to specific pathways or subcellular sites to ensure specificity.

Thus, protein/protein interactions may be fundamental for the molecular mechanisms by which these two key kinases regulate energy homeostasis. The aim of this thesis was to apply state-of-the-art technology, in particular yeast two-hybrid (Y2H) systems, for a genome-wide, non-biased screening of cytosolic creatine kinase and AMPK protein interaction partners in the brain, and to perform initial characterization of candidates.

The thesis is structured in 5 different parts, most of them containing several chapters.

### PART I – INTRODUCTION.

The current state of knowledge on creatine kinase and AMPK is presented in Chapter I, together with a short overview on cellular and whole body energy metabolism. Chapter II then summarizes the methodological basis of protein interactomics, in particular recent technological developments in the field of high-throughput interactomics research, with an emphasis on yeast two-hybrid (Y2H) screening.

### PART II - AMPK

Chapter III summarizes all publically available data on interaction partners, but also on substrates of AMPK, a field that has experienced an explosive growth during the last few years. Chapter IV then describes application of a cytosolic, split protein Y2H approach to screen for novel protein interaction partners of AMPK in a human brain cDNA library, revealing several putative AMPK interactors and

associating the kinase, among others, to intracellular vesicle transport. Chapter V presents a second, new strategy combining a biophysical interaction approach (surface plasmon resonance) with an *in vitro* phosphorylation assay, which allowed identification of targets with a preference for specific AMPK isoforms. Finally, Chapter VI gives a detailed characterization of a new AMPK substrate, the glutathione S-transferase, revealing a link of AMPK to oxidative stress defense. All results on AMPK interaction partners are discussed together in Chapter VII.

### PART III – BRAIN-TYPE CREATINE KINASE

Chapter VIII provides an introduction to creatine kinase compartmentation in the brain, and Chapter IX summarizes some preliminary experiments to characterize subcellular distribution of brain-type creatine kinase (BCK). A cytosolic, split protein Y2H screen applied to BCK and a human brain cDNA library is described in Chapter X. Results on BCK compartmentation and interaction partners are discussed in Chapter XI.

### PART IV – FINAL SUMMARY, CONCLUSIONS AND OUTLOOK

This part summarizes the different experimental strategies, gives a synthetic discussion, provides main conclusions, and proposes some experimental strategies for future research.

### PART V – EXPERIMENTAL PROCEDURES

All materials and methods used and applied in the experiments presented in this thesis are described here.

### ANNEXES

Supplemental data, more detailed description of some materials (vectors, DNA constructs) and a CV are given here.

**- CHAPTER I -****REGULATION OF CELLULAR AND WHOLE BODY ENERGY HOMEOSTASIS**

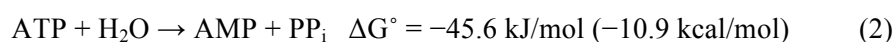
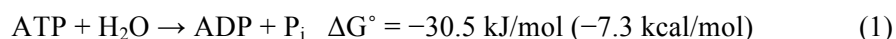
*„A living organism continually increases its entropy [...] and thus tends to approach the dangerous state of maximum entropy, which is of death. It can only keep [...] alive, by continually drawing from its environment negative entropy. [...] To put it less paradoxically, the essential thing in metabolism is that the organism succeeds in freeing itself from all the entropy it cannot help producing while alive.“*

Erwin Schrödinger – What is life?

**1. CELLULAR ENERGY METABOLISM*****1.1. General information***

Multicellular organisms are highly organized and mostly contain different organs with specific structure and function. Each organ is an ordered aggregate of communicating cells, and each cell contains a complex and dynamic network of macromolecules and chemical reactions. Considering this important role of organization, Erwin Schrödinger defined life as the struggle against disorder (defined by negative entropy change) (Schrödinger 1986). For this daily battle, each organism must take up, transform and distribute energy. Energy metabolism is therefore one of the fundamental prerequisites for life.

The cellular energy metabolism is mainly supported by the oxidation of carbohydrates (glucose) and lipids (fatty acids, FA) taken up with the food. Breakdown of glucose and FA results mainly in adenosine triphosphate (ATP) production, the universal cellular energy carrier. In cells, endergonic reactions are always coupled to exergonic reactions. Thus, metabolic processes use energy in form of “high energy” phosphate ( $\beta$ - and  $\gamma$ -phosphates in nucleoside triphosphates), converting ATP to ADP or AMP (Eq1 and 2). The latter are continuously reconverted into ATP during exergonic catabolic processes.



The initial step of cellular energy conversion consists in the transport of nutrients across the cell membrane, followed by their activation prior to entrance into the two major pathways of ATP

production, glycolysis and FA- $\beta$ -oxidation. Glucose transport is regulated by a family of 13 carriers (GLUT), which differ in kinetic characteristics and are translocated from a cytosolic vesicular storage pool to the plasma membrane in energy requiring situations (Joost et al. 2001, Ishiki et al. 2005). One of these carriers, GLUT4, is directly linked to energy status as its translocation is regulated by insulin (Chang et al. 2004) and AMP-dependent protein kinase (AMPK) (Ishiki et al. 2005). Similar mechanisms are implicated in FA transport across the plasma membrane. The FA carrier FAT/CD36 is also stored in cytoplasmic vesicles and its recruitment to the plasma membrane also involves active AMPK (Koonen et al. 2005).

In the cell, glucose is then phosphorylated by hexokinase and subsequent enzymatic steps convert glucose-6-phosphate into pyruvate, yielding 2 ATP and 2 NADH molecules. NADH is an important coenzyme in living cells that functions as an electron donor in its reduced (NADH) or as an electron acceptor in its oxidized (NAD<sup>+</sup>) state. Under aerobic conditions, pyruvate is further metabolized in mitochondria. Oxidative decarboxylation leads to acetyl-CoA formation which then enters the tricarboxic acid (TCA) cycle where it is fully oxidized to carbon dioxide and water yielding ATP and redox equivalents in form of NADH and FADH<sub>2</sub>. The latter enter the mitochondrial electron transport chain (or respiratory chain), which couples reactions between electron donors (such as NADH) and electron acceptors (such as O<sub>2</sub>) to the transfer of H<sup>+</sup> ions across the mitochondrial inner membrane. The created electrochemical (proton) gradient is then used by the mitochondrial ATP synthase complex to produce ATP, the last step in the process of oxidative phosphorylation (OxPhos, reviewed by (Leverve et al. 2007, Rigoulet et al. 2007)). Under anaerobic conditions (e.g. during extreme exercise) pyruvate is converted into lactate by reducing NADH to NAD<sup>+</sup>, again available for glycolysis.

The stepwise breakdown of fatty acids in mitochondrial  $\beta$ -oxidation also generates acetyl-CoA, which is then further metabolized in mitochondria by the TCA cycle and OxPhos as described above to generate ATP.

### ***1.2. Energy homeostasis***

While every living organism has to strive for minimizing disorder, it also faces a continuously changing and fluctuating external environment, including food supply, temperature etc., resulting in different energetic requirements. However, the internal environment of an organism or a cell is regulated in a sophisticated way to maintain a rather stable and constant energy balance (Eq3) (Flier et al. 2000).

$$\text{Energy intake} = \text{internal heat produced} + \text{external work} + \text{storage} \quad (3)$$

This energy homeostasis is ensured by adjustment of multiple dynamic equilibria and complex regulatory mechanisms. Indeed, intracellular metabolite and in particular ATP levels are maintained remarkably homeostatic despite changes in whole body energy expenditure (e.g. rest-work)

(Hochachka 2003). Disorders in energy homeostasis are related to diseases such as diabetes, obesity or cancer and can eventually lead to death.

### ***1.3. Brain energy metabolism***

During evolution, complex mechanisms have emerged to control whole body energy homeostasis of multicellular organisms. Paracrine, endocrine, neural and neuroendocrine signaling mechanisms work in concert to control energy metabolism and to coordinate behavioural aspects of energy balance, such as feeding and exercise. The central nervous system (CNS) is not only the main regulatory hub, but is itself a major energy consumer. The brain represents only 2 % of our body weight but receives 15 % of our cardiac output and even 20 % of total body oxygen and 25 % of total body glucose consumption (Ames 2000). Its energy is procured predominantly from carbohydrate (e.g. glucose) by an oxidative metabolism. Because of its tissue heterogeneity and rapidly changing energy demands, it remained difficult to monitor rates of energy generation and consumption of specific brain cells, and detailed organization of inter- and intracellular brain energy metabolism is still unclear. Generally it is assumed that the maintenance of the ionic concentration gradient *via* sodium pumps ( $\text{Na}^+\text{K}^+\text{ATPases}$ ) constitutes the major energy requirement in brain, followed by  $\text{Ca}^{2+}$ -handling (Table 1-1). Both processes are of primary importance for neurotransmitter release and thus for brain function.

TABLE 1-1. **Estimates of relative energy-demands in brain.**

<i>Energy-requiring processes</i>	<i>Share (%)</i>
Gated $\text{Na}^+$ influx through plasma membrane	40-50%
$\text{Ca}^{2+}$ influx from organelles and extracellular fluid	3-7%
Neurotransmitter processing	10-20%
Vegetative metabolism	5-15%
Intracellular signaling system	20-30%
Axonal and dendritic transport; other	20-30%

Principal energy-requiring processes with estimates of their relative energetic demands in brain. Energy requirements might however fluctuate between different cell types. (Table from (Ames 2000))

As mentioned above, one of the specificities of the CNS is its cellular heterogeneity. Brain cells differ in morphology, molecular composition or physiological activity. In the same way, energy metabolism seems to vary in the different cell types. There is for example evidence that glycolysis is separated from oxidative metabolism at some sites with lactate becoming an important substrate. Some glia are primarily glycolytic and the lactate they generate is transferred to adjacent neurons as substrate for their oxidative metabolism in what has been characterized as the astrocyte-neuron lactate shuttle (Bittar et al. 1996).

Beside this intercellular organization of energy metabolism, its intracellular compartmentation has also to be considered. Mechanisms to facilitate energy transfer within cells include the close spatial organization of ATP generating enzymes (Knull 1978, Campanella et al. 2005) and the juxtaposition of sites of ATP generation, like mitochondria, with sites of consumption (Wong-Riley 1989). In cases



where generation and consumption are separated, a transfer system for “energy-rich” phosphate moieties (~P) like creatine kinase (see below) maintains a rapid transfer between both sites.

## 2. CREATINE KINASE & OTHER PHOSPHOTRANSFER SYSTEMS IN ENERGY HOMEOSTASIS

### 2.1. Energy transfer systems

It has been well accepted that many biochemical processes are spatially confined within cells, leading to a locally increased demand for ATP and release of ADP. Some cell types as muscles and neurons can drastically increase their metabolic activity during short time periods resulting in highly fluctuating energy demands. ATP production by OxPhos or glycolysis does not obligatorily concur temporally and spatially with cellular energy requirements. To avoid such disequilibria, metabolic enzymes and mitochondria are able to relocate to sites of increased energy consumption. It has i.e. been reported that glycolytic enzymes are able to form functional complexes and to bind to  $\text{Na}^+\text{K}^+\text{ATPases}$  (Knull 1978, Campanella et al. 2005). In skeletal muscle and cardiac cells, in which an oxidative metabolism predominates, mitochondria are highly organized and form a regular arrangement with elements of the cytoskeleton (Kuznetsov 2007). In addition a relocation of mitochondria along microtubules and actin filaments has been observed (Hollenbeck et al. 2005, Boldogh et al. 2007). Beside the ability to couple primary ATP production sites physically to ATP consumption sites, cells also possess further energy transfer or buffering systems.

These phosphotransfer systems include three families of enzymes: the nucleoside diphosphate kinases (NDPK), the adenylate kinase (AK) and the phosphagen kinases, with creatine kinases (CK) as the most prominent sub-family.

NDPKs control intracellular nucleotide homeostasis, catalyzing the reversible exchange of the  $\gamma$ -phosphate between tri- and diphosphonucleosides:  $\text{N}_1\text{TP} + \text{N}_2\text{DP} \leftrightarrow \text{N}_1\text{DP} + \text{N}_2\text{TP}$ . Cellular concentrations of the different nucleotides suggest that reaction *in vivo* mainly proceeds in the direction of using ATP, e.g. formed by oxidative phosphorylation, to generate the other triphosphonucleosides necessary for specific reactions. Through the action of NDPK, high intracellular ATP concentration is used for buffering local NTP pools (Schlattner et al. 2009).

AK controls homeostasis among adenine nucleotides. The reaction:  $2\text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$  is also able to increase the energy gain per ATP molecule by using its  $\beta$ -phosphate (in ADP) as energy source. This reaction is also the major source of AMP by converting any drop in ATP into a rise in AMP concentration (Zelevnikar et al. 1995).

CK is the major enzyme in higher eukaryotes managing high and fluctuating energy demands and energy homeostasis (Wallimann et al. 1992). CK isoenzymes catalyze the reversible phosphate transfer from ATP to creatine (Cr) yielding phosphocreatine (PCr) and ADP:  $\text{ATP} + \text{Cr} \leftrightarrow \text{ADP} + \text{PCr}$  and will be described in more detail below.

## ***2.2. Tissue specific expression of creatine kinase isoenzymes***

Five different oligomeric isoforms of creatine kinase are expressed in mammals, encoded by four different genes (reviewed by (Wallimann et al. 1992)). Their tissue and compartment specificity is one of the important properties for their function in cellular energy metabolism. Most mammalian tissues express a combination of two CK isoenzymes, one cytosolic dimeric isoform, either muscle-type MMCK or brain-type BBCK, and one mitochondrial mostly octameric isoform, either sarcomeric sMtCK or ubiquitous uMtCK. The combination of MMCK and sMtCK is expressed in differentiated cardiac and skeletal muscle, whereas the combination of BBCK and uMtCK is more widely distributed and was detected in brain (in neuronal and retina photoreceptor cells, hair bundle of the inner ear and astrocytes), smooth muscle, kidney, endothelial cells, spermatozoa and skin. CK expression levels are very low or absent in liver. A fifth dimeric isoenzyme (MBCK), formed by one monomer of each, MMCK and BBCK, is only expressed in adult cardiac muscle and transiently during muscle differentiation.

## ***2.3. CK compartmentation and high energy phosphate channeling***

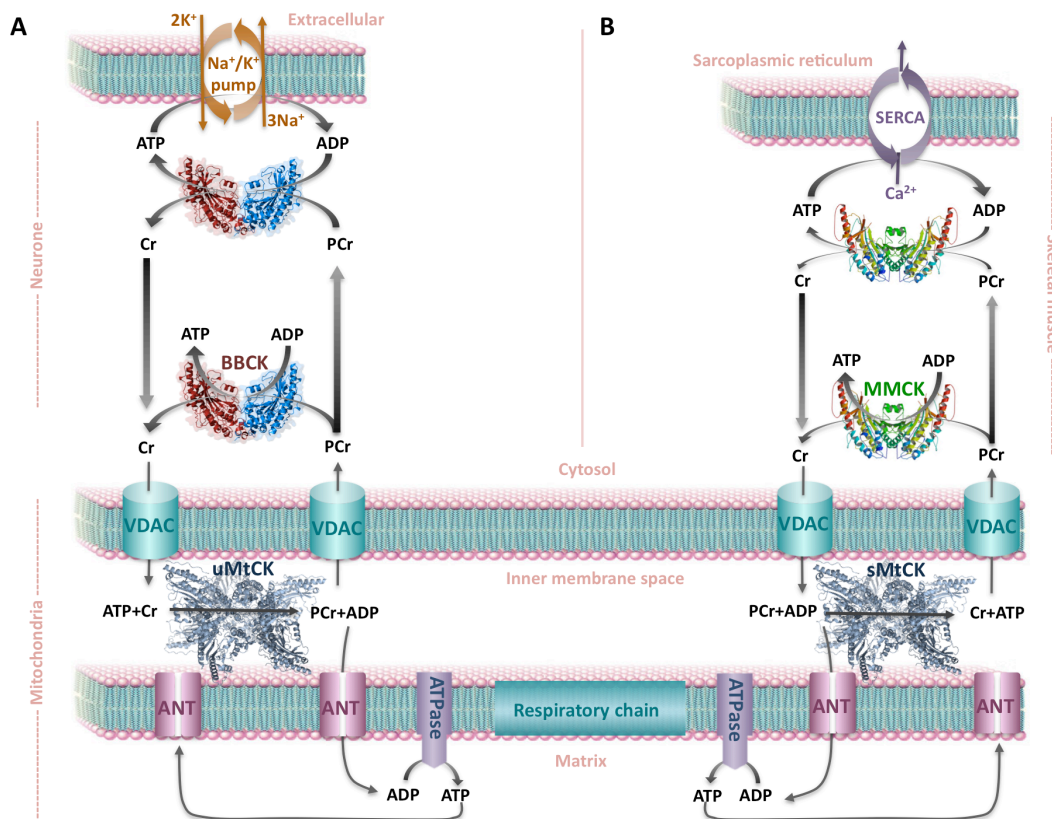
The different CK isoenzymes are not entirely soluble, but located at specific sites within their cellular compartments. MtCKs are located in the innermembrane space of mitochondria and are functionally coupled to the voltage dependent anion channel (VDAC) of the mitochondrial outer membrane and to the adenine nucleotide transporter (ANT) of the mitochondrial inner membrane. ATP generated by OxPhos is transported by ANT and, thanks to the functional coupling to MtCK, directly transphosphorylated into PCr and ADP. The latter can directly reenter into the matrix via ANT and activate OxPhos. PCr can exit and Cr enters the mitochondria via VDAC. Altogether, this leads to high-energy phosphate channeling from the mitochondrial matrix to the cytosol (Fig. 1-1, bottom part).

In the cytosol, BBCK or MMCK assume local ATP regeneration from PCr at cellular ATP consuming sites (Fig. 1-1, upper part). In fact, a fraction of cytosolic CK was found permanently bound or transiently associated to the plasma membrane (Wallimann et al. 1985) and to subcellular structures (i.e. to the myofibrillar M-band (Turner et al. 1973) or I-band (Kraft et al. 2000)) or to compartments, such as the sarcoplasmic reticulum (SR) (Rossi et al. 1990) or the Golgi apparatus (Burklen et al. 2007). Bound CK is supposed to provide required ATP and to be functionally coupled to membrane ATPases as the Na<sup>+</sup>K<sup>+</sup> pump (Guerrero et al. 1997) at the plasma membrane or the Ca<sup>2+</sup> pump at the SR (Rossi et al. 1990). The remaining soluble part of cytosolic CK would be involved in buffering the global ATP/ADP ratio (Fig. 1-1, central part).

These observations led to the model of the CK-PCr circuit detailed in Fig. 1-1. In particular in some large eukaryotic cells, diffusion-based adenylate transfer may become limiting, and fluctuating metabolic situations may lead to transient accumulation of ATP at the generating and ADP, Pi and H<sup>+</sup> at the consuming sites, provoking fatal inhibitory effects e.g. on ATPases. To overcome such diffusional limitations and local accumulation of adenylates, the high cellular concentrations of Cr and

PCr (up to 30 mM) and the higher diffusibility of the smaller PCr can be exploited to constitute a temporal PCr/CK shuttle (Fig. 1-1) (Wallimann et al. 2007).

It has also been proposed that a near-equilibrium network of CK enzymes transmits high-energy phosphoryl fluxes by rapidly equilibrating reactions. In this way the incoming ligand at one end may trigger propagation of a flux wave through the network without moving along the pathway. Beside its ability to work nearly without a concentration gradient, this type of energy transmission may be much faster (Dzeja et al. 2003). However, the complexity of the intracellular environment seems to be too high and CK enzyme concentrations too low to support this hypothesis for long distance shuttling. In contrast, in the vicinity of ATPases for example, such a model may apply in form of the so-called “functional coupling”.



**FIGURE 1-1. The CK-phosphocreatine circuit.** Models for a circuit in brain (A) or muscle (B). **LOWER PART:** In both tissues, mitochondrial MtCK (uMtCK in non-muscle tissues and sMtCK in muscle) is specifically located in the mitochondrial inner membrane space where it is functionally coupled to adenine nucleotide translocator (ANT) of the mitochondrial inner membrane and to the voltage dependent anion channel (VDAC) of the mitochondrial outer membrane. MtCK accepts mitochondrially generated ATP and phosphorylates it into PCr, which will then leave the mitochondria through VDAC whereas generated ADP enters the mitochondrial matrix to be rephosphorylated into ATP. **CENTRAL PART:** The cytosolic CK isoenzymes (MMCK in muscle and BBCK in non-muscle tissues) form an efficient ATP transfer system through the transmission of high-energy phosphoryl fluxes by rapidly equilibrating near-equilibrium reactions, buffering the global cellular ATP pool. **UPPER PART:** Cytosolic CK isoenzymes associated in the vicinity of ATP-consuming reactions maintain local ATP pools (e.g. in (A) the Na<sup>+</sup>/K<sup>+</sup> pump regulating neuronal K<sup>+</sup> fluxes across the plasma membrane, or in (B) the Ca<sup>2+</sup> pump SERCA at the sarcoplasmic/endoplasmic reticulum membrane of muscle). This shuttle system allows maintaining constant, elevated ATP concentrations in all cellular compartments.

### 3. A KEY-REGULATOR OF ENERGY METABOLISM: AMP-ACTIVATED PROTEIN KINASE

The PCr/CK shuttle constitutes the first safeguard to prevent sudden drops of ATP concentration. However, once the PCr pool is exhausted, the ATP concentration also declines, accompanied by a rise in ADP. Through the action of AK, some ATP can be generated from ADP, but this will generate at the same time a sharp increase in AMP concentration. This is the signal for AMP-activated protein kinase (AMPK) which, as indicated by its name, is sensitive to intracellular AMP levels, and is at the front line to sense any ATP decrease translated into a rise in AMP levels (Fig. 1-2).

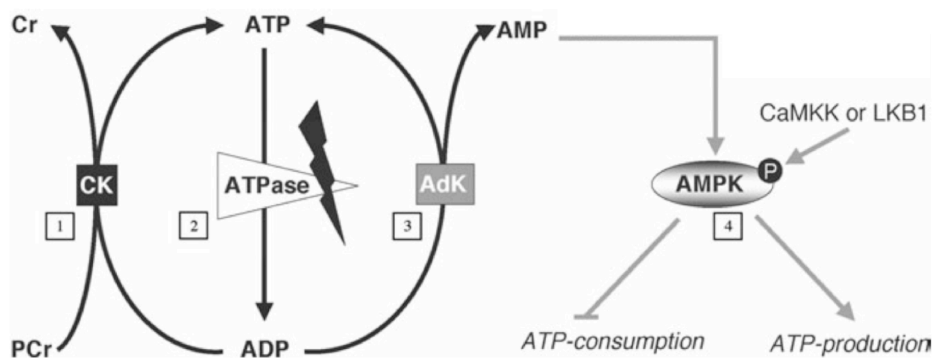


FIGURE 1-2. **Regulation of cellular energy state by an interplay of CK, AK and AMPK.** ATP is consumed by ATPases (2) and the energy is converted, among other purposes, for muscle contraction, ion pumping, and protein synthesis. The cellular ATP/ADP ratio is kept high by the action of two energy-related kinases: creatine kinase (CK) (1), and adenylate kinase (AdK) (3). The ADP generated upon hydrolysis by ATPases is recharged into ATP by the action of CK, which draws its energy from a phosphocreatine (PCr) pool, and by AdK, which uses two ADP molecules to regenerate one ATP and to generate one AMP molecule. AMP serves as a “second messenger” for cellular energy stress and stimulates AMPK (4). Phosphorylation by either of the two upstream kinases, i.e., LKB1 or CaMKK, at Thr172 in the  $\alpha$ -subunit causes its activation, with AMP inhibiting dephosphorylation. Activated and fully AMP-stimulated AMPK then upregulates catabolic pathways for ATP production and suppresses anabolic pathways that would consume ATP. Figure modified after (Neumann et al. 2007).

AMPK is an evolutionary conserved and ubiquitously expressed serine/threonine kinase. In response to diverse external (e.g. hormones, nutrients) and internal (e.g. AMP) stimuli signaling an impaired cellular energy state in diverse physiological and pathological situations, AMPK is activated to inhibit ATP-consuming (anabolic) processes (e.g. fatty acid and glycogen synthesis) and to stimulate ATP-generating (catabolic) processes (e.g. fatty acid oxidation, glucose uptake, glycolysis). Beyond the role of AMPK in the regulation of cellular energy homeostasis, the enzyme seems also to be a nutrient sensor and to play a role in appetite regulation and in energy-dependent regulation of cell shape and cancer signalling (Hardie 2007, Steinberg et al. 2009).

### ***3.1. Heterotrimer structure and expression***

AMPK is a heterotrimer composed of one catalytic subunit  $\alpha$  and two regulatory subunits  $\beta$  and  $\gamma$ . Each subunit includes different isoforms encoded by different genes ( $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ ,  $\beta 2$ ,  $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$ ). The AMPK  $\alpha 1$  and  $\alpha 2$  subunits are similar, including a conserved N-terminal catalytic domain and divergent C-terminal domains bearing an autoinhibitory sequence (AIS) and a  $\beta$  subunit interaction domain. The AMPK  $\beta$  subunits differ in the first 65 amino acids (aa) but are otherwise highly conserved, bearing a central glycogen binding domain (GBD) and a C-terminal scaffolding domain for binding both  $\alpha$  and  $\gamma$  subunits. The AMPK  $\gamma$  subunits differ in length but share a conserved C-terminal domain including four tandem repeat sequences, the cystathionine  $\beta$ -synthetase (CBS) domains. The CBS motifs are functionally organized in two pairs, called Bateman domains, known to bind AMP or ATP (Adams et al. 2004, Scott et al. 2004). The  $\gamma 2$  and  $\gamma 3$  isoforms contain N-terminal extensions which are subject to truncation by RNA splicing and whose function is currently unknown. The large number of subunit isoforms lead to at least 12 possible combinations within a given AMPK heterotrimer.

AMPK subunits show differential tissue-specific expression and activation. In rat, AMPK  $\alpha 1$  is equally expressed in heart, liver, kidney, brain, spleen, lung and skeletal muscle whereas AMPK  $\alpha 2$  is highly abundant in skeletal muscle, but less abundant in heart, liver, brain and kidney, and detectable in lung (Stapleton et al. 1996). The  $\beta 1$  subunit shows a similar widespread expression profile as  $\alpha 1$ , whereas  $\beta 2$  is highly expressed in skeletal muscle and heart and to lower extent in brain, placenta, liver and pancreas (Thornton et al. 1998). The  $\gamma 1$  isoform is ubiquitously expressed, whereas  $\gamma 3$  expression seems to be limited to skeletal muscle. The  $\gamma 2$  isoform is also widely expressed but most abundant in heart followed by brain, placenta and skeletal muscle (Cheung et al. 2000). The expression profile of the different subunits let to the conclusion that AMPK  $\alpha 1\beta 1\gamma 1$  is probably the predominant complex in most cell types. Interestingly the skeletal muscle is the only tissue that expresses all subunit isoforms including  $\gamma 3$ , and it has been proposed that the predominant heterotrimer in this tissue is  $\alpha 2\beta 2\gamma 3$  (Mahlapuu et al. 2004). The specific role of the different heterotrimer combinations is not yet clear. However it appears that the different AMPK isoform combinations may vary in some properties. In rat pancreatic  $\beta$ -cells,  $\alpha 2$  subunit displayed an equal distribution between the nucleus and the cytosol whereas  $\alpha 1$  subunit was much more abundant in the cytosol (Salt et al. 1998a). AMPK complexes purified from rat liver containing  $\alpha 2$  rather than  $\alpha 1$  isoform showed a greater dependence on AMP *in vitro* (Salt et al. 1998a). Finally, *in vitro* phosphorylation assays of variant peptides suggest a slight difference in AMPK  $\alpha 1$  or  $\alpha 2$  target recognition (Woods et al. 1996).

## **3.2. Regulation of AMPK**

### **3.2.1. Molecular regulation**

#### **3.2.1.1. Regulation by phosphorylation**

As the main regulatory mechanism indispensable for AMPK activation, phosphorylation at Thr172 located in the activation loop of the AMPK  $\alpha$  kinase domain was described (Hawley et al. 1996). Three mammalian AMPK upstream kinases have since been identified. The major upstream kinase in most cell types was shown to be the Ser/Thr kinase LKB1 in complex with its two accessory subunits STRAD $\alpha/\beta$  and MO25 $\alpha/\beta$  (Hawley et al. 2003, Woods et al. 2003a). LKB1 has originally been identified as a tumor suppressor whose inactivating mutations lead to the Peutz-Jeghers syndrome, an inherited susceptibility to different human cancers (reviewed by (Alessi et al. 2006)). LKB1 also functions upstream of 12 other AMPK-related kinases and it appears to be constitutively active (Lizcano et al. 2004). In cells with low or no expression of LKB1, the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinase  $\beta$  (CamKK $\beta$ ) mediates AMPK activation in a  $\text{Ca}^{2+}$ -dependent manner (Hawley et al. 2005, Hurley et al. 2005, Woods et al. 2005). CamKK is highly expressed in brain, and  $\text{K}^+$  depolarization followed by an increase of intracellular  $\text{Ca}^{2+}$  leads to a threefold activation of AMPK, independent of any change in the cellular AMP/ATP ratio (Hawley et al. 2005). CamKK mediated AMPK activation might thus anticipate increasing energy turnover which usually accompanies  $\text{Ca}^{2+}$  release in this tissue. Recently, the transforming growth factor- $\beta$  activated kinase-1 (TAK1) was identified as an Snf1 activator in yeast and an AMPK upstream kinase *in vitro* (Momcilovic et al. 2006). In addition it was shown that TAK1-deficient MEF cells displayed reduced oligomycin, AICAR or metformin mediated AMPK activation (Xie et al. 2006).

While the  $\alpha$  subunit Thr172 is the major AMPK activation site phosphorylated by the presented upstream kinases, both  $\alpha$  and  $\beta$  subunits have multiple phosphorylation sites, many of them targeted by autophosphorylation, the functional roles of which are poorly understood (Mitchelhill et al. 1997, Woods et al. 2003b). It was shown that the activation of the forskolin/isobutylmethylxanthine-mediated cAMP pathway led to increased  $\alpha 1$  Ser485 ( $\alpha 2$  Ser491) phosphorylation, necessary for reduced Thr172 phosphorylation (Hurley et al. 2006). Similar results were obtained in response to insulin where PKB mediated  $\alpha 1$  Ser485 ( $\alpha 2$  Ser491) phosphorylation inhibited ischemia-induced AMPK activation (Horman et al. 2006).

#### **3.2.1.2. Allosteric regulation**

Besides direct covalent activation of the  $\alpha$ -subunit Thr172 *via* upstream kinases by phosphorylation, AMPK is activated by allosteric binding of AMP to the  $\gamma$ -subunit as mentioned above. Two different mechanisms for the allosteric activation by AMP have been proposed. First, AMP binding to the  $\gamma$ -subunit seems to inhibit  $\alpha$  Thr172 dephosphorylation by the protein phosphatase 2C $\alpha$  (PP2C $\alpha$ ) and others (Suter et al. 2006, Sanders et al. 2007b) *via* a conformational change (Riek et

al. 2008). A second hypothesis is that an internal autoinhibitory sequence present in the first CBS domain of the  $\gamma$  subunit and which resembles the sequence of an AMPK target sites might sequester the catalytic  $\alpha$  subunit (Scott et al. 2007). However this latter hypothesis was not supported by *in vitro* phosphorylation assays, in which AMP did not affect  $\alpha$  Thr172 phosphorylation (Suter et al. 2006, Sanders et al. 2007b). Nevertheless, since the major AMPK kinase (LKB1) is constitutively active, the allosteric effect of AMP or other mechanisms like regulated dephosphorylation of Thr172 seem to play a major role in regulating AMPK activity.

The  $\beta$  subunit has also been implicated in AMPK activity regulation. Abolishing  $\beta$  myristoylation by a  $\beta$  G2A mutation caused a fourfold activity increase, and truncation of the first 63 amino acids even a fivefold increase in activity (Warden et al. 2001, Hudson et al. 2003). On the other hand glycogen as well as other synthetic branched oligosaccharides were found to inhibit AMPK activity by binding to the  $\beta$  GBD domain (McBride et al. 2009).

### 3.2.2. Cellular regulation

AMPK is activated in response to any metabolic situation that increases AMP/ATP ratios such as glucose deprivation (Salt et al. 1998b), muscle contraction (Winder et al. 1996), hypoxia (Evans et al. 2005a), oxidative stress (Choi et al. 2001) or treatment with metabolic poisons. However hyperosmotic stress (Fryer et al. 2002), several endocrine signals (e.g. leptin, adiponectin) or synthetic compounds (e.g. A769662, A23187) activate AMPK independent of cellular AMP levels (Hawley et al. 2010) either directly or via the upstream kinase or phosphatase pathways.

AMPK has emerged as a whole body energy sensor as its activity is modulated by cytokines and other hormones that regulate whole-body energy balance (Kahn et al. 2005). A summary of known AMPK endocrine regulators is given in Table 1-2. Regulation of AMPK by these factors partially depends on the tissue. While in peripheral tissues leptin activates and ghrelin inhibits AMPK in the regulation of FA oxidation and glucose uptake, their effects in hypothalamus are different, since they inhibit (leptin) or stimulate (ghrelin) AMPK-controlled food intake (for reviews see (Kahn et al. 2005, Steinberg et al. 2009)).

Some natural plant metabolites and synthetic compounds are also able to activate AMPK (reviewed in (Hwang et al. 2009, Steinberg et al. 2009)). Most of these activators function through an increase in intracellular AMP levels, mainly by targeting mitochondrial ATP generation. The synthetic anti-diabetic drug metformin activates AMPK mainly in liver through increase in intracellular AMP levels as a consequence of the inhibition of complex I of the mitochondrial respiratory chain (El-Mir et al. 2000, Hawley et al. 2010). Resveratrol, a polyphenol component of red wine, stimulates AMPK in liver, skeletal muscle, neurons and cancer probably also by an increase in AMP due to mitochondrial ATPase inhibition. (Steinberg et al. 2009).

Different other chemical compounds are currently used to study the AMPK pathway. These compounds include inhibitors of ATP synthesizing pathways (e.g. oligomycin, dinitrophenol, 2-deoxyglucose) and 5-aminoimidazole-4-carboxamide riboside (AICAR) that is converted into the

AMP mimick ZMP (Corton et al. 1995). Recently, a specific AMPK activator has been developed by Abbott Laboratories. A-769662 directly binds to the GBD of the  $\beta$ -subunit and has become a widely used activator in AMPK pathway research due to its few off-target effects (Sanders et al. 2007a). A summary of currently frequently used AMPK activators is presented in Table 1-3.

TABLE 1-2. AMPK regulation by different endocrine cytokines/hormones.

<i>Compound</i>	<i>AMPK regulation</i>	<i>Mechanism</i>	<i>Tissue</i>	<i>References</i>
leptin	+	indirect AMP increase	Muscle	(Minokoshi et al. 2002)
leptin	-	melanocortin receptor signalling ?	hypothalamus	(Minokoshi et al. 2004)
interleukin-6	+	?	Muscle	(Al-Khalili et al. 2006, Carey et al. 2006)
tumor necrosis factor $\alpha$ (TNF $\alpha$ )	-	increased PP2C expression	Muscle	(Steinberg et al. 2006b)
resistin	-	?	liver, muscle, adipose tissue	(Satoh et al. 2004)
ghrelin	+	G protein coupled receptor signalling (CamKK activation)?	hypothalamus, heart	(Nakazato et al. 2001, Kola et al. 2005)
ghrelin	-		liver	(Kola et al. 2005)
adiponectin	+	Adiponectin receptor 1 signalling?	muscle, adipose tissue, hypothalamus	(Yamauchi et al. 2002, Wu et al. 2003)
estrogen	+	?	muscle	(D'Eon et al. 2005)
dihydrotestosterone	-	?	adipocytes	(McInnes et al. 2006)

TABLE 1-3. Pharmacological AMPK activators.

<i>Compound</i>	<i>AMPK activation mechanism</i>	<i>Usage</i>	<i>References</i>
FA $\alpha$ -lipoic acid	?	natural compound	(Kim et al. 2004, Lee et al. 2005)
Resveratrol	AMP increase ( $\gamma$ -subunit)	natural component of red wine	(Steinberg et al. 2009)
Beberine	?	Chinese herbal medicine	(Steinberg et al. 2009)
Biguanides (metformin/phenformin)	AMP increase ( $\gamma$ -subunit)	Anti-diabetic drug	(Fryer et al. 2002, Hawley et al. 2010)
Thiazolidinediones	AMP increase ( $\gamma$ -subunit)	Anti-diabetic drug	(Fryer et al. 2002, Hawley et al. 2010)
Phenobarbital	AMP increase ( $\gamma$ -subunit)	Anesthetic	(Rencurel et al. 2005)
AICAR	ZMP (AMP mimick) ( $\gamma$ -subunit)	Research	(Sullivan et al. 1994)
A-769662	Direct AMPK binding ( $\beta$ -subunit)	Research	(Cool et al. 2006)
A-23187	Ca <sup>2+</sup> increase & CamKK activation ( $\alpha$ -subunit phosphorylation)	Research	(Hawley et al. 2005)
Oligomycin	AMP increase ( $\gamma$ -subunit)	Research	(Pan et al. 2002)
Dinitrophenol	AMP increase ( $\gamma$ -subunit)	Research	(Hayashi et al. 2000)
2-deoxyglucose	AMP increase ( $\gamma$ -subunit)	Research	(Rubin et al. 2005)



Sorbitol

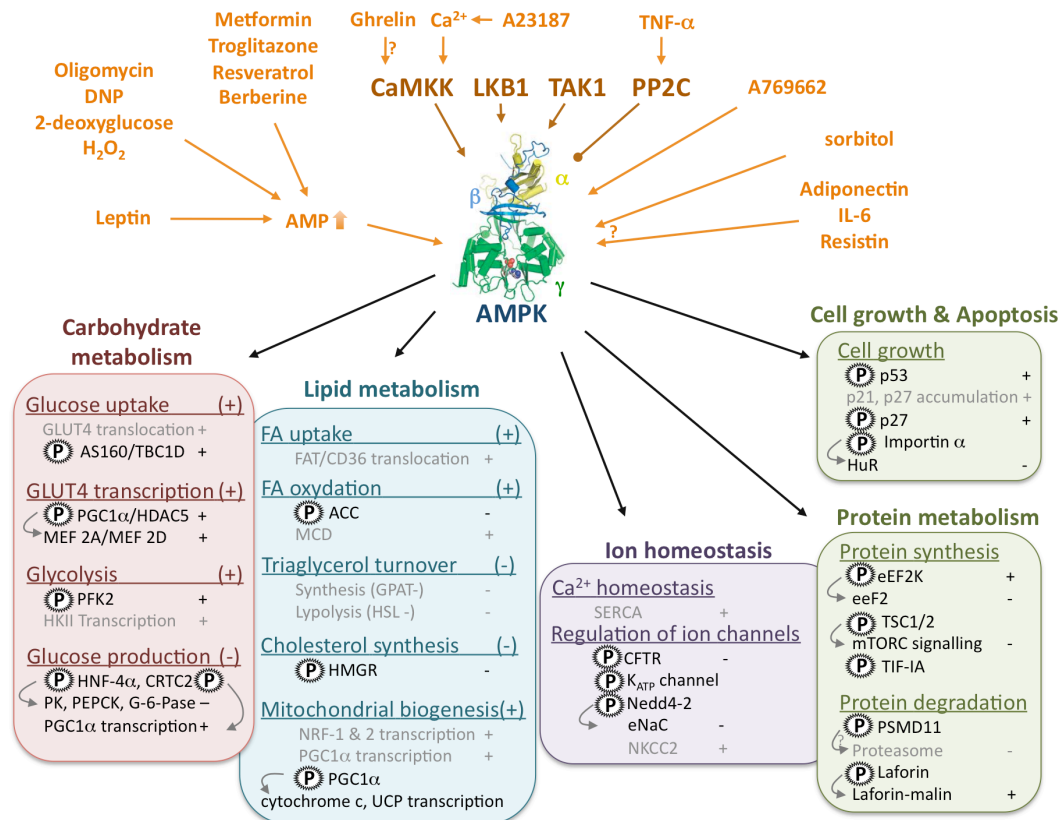
Osmotic stress

Sugar substitute

(Mao et al. 2004)

### 3.3. Downstream AMPK regulation

As already mentioned, AMPK activation stimulates ATP producing catabolic pathways and inhibits ATP consuming anabolic pathways, both by rapid effects via direct phosphorylation of metabolic enzymes and by long-term effects via regulation of transcription. The principal processes upstream and downstream of AMPK activation are summarized in Figures 1-3 and 1-4.



**FIGURE 1-3. AMPK activating pathways and downstream targets. UPPER PART:** Upstream signalling. AMPK is activated allosterically by pathways increasing cellular AMP/ATP ratios (top left) and covalently by phosphorylation (top center) via upstream kinases, or inactivated by dephosphorylation via protein phosphatases like PP1 or others. Other activators (A769662) act directly on AMPK, independent of AMP, or have unknown mechanisms (top right). **LOWER PART:** Downstream signalling. Phosphorylation by AMPK is indicated by (P), activation by (+) and inhibition by (-), arrows link primary phosphorylations to further downstream events, indirect AMPK effects are shown in grey. Activated AMPK stimulates e.g. glucose and FA uptake, glycolysis and FA oxidation, as well as transcription of mitochondrial proteins and mitochondrial proliferation in general. It inhibits energy requiring gluconeogenesis in liver, and FA/cholesterol biosynthesis. Other energy requiring processes such as cell growth, protein synthesis and degradation and regulation of intracellular ion concentrations are also regulated by AMPK. **Abbreviations:** AMPK kinases/phosphatases - LKB1, Liver kinase B1; CamKK, Ca<sup>2+</sup>/calmodulin dependent kinase kinase; TAK1, transforming growth factor-β activated kinase 1; PP2C, protein phosphatase 2C AMPK substrates - ACC, acetyl-CoA carboxylase; AS160, AKT substrate of 160 kDa; CFTR, cystic fibrosis transmembrane conductance regulator Cl<sup>-</sup> channel; CREB, CRE binding protein; EF2K, elongation factor 2 kinase; eeF2, endothelial elongation factor; eNaC, epithelial Na<sup>+</sup> channel; G-6-Pase, glucose 6-phosphatase; GLUT, glucose transporter; GPAT, glycerol-3-phosphate acyl transferase; HDAC, histone deacetylase; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HNF-4α, hepatic nuclear factor 4α; HSL, hormone-sensitive lipase; MCD, malonyl-CoA decarboxylase; MEF, myocyte enhancer factor; mTORC, mammalian target of rapamycin complex; NKCC, Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter; NRF, Nuclear respiratory factor; PEPC, phosphoenol-pyruvate kinase; PGC1α, PPARγ co-activator-1α; PFK2, 6-phosphofructo-2-kinase; PK, pyruvate kinase; SERCA, sarcoplasmic/endoplasmic reticulum

calcium ATPase; TSC, Tuberous Sclerosis Complex; UCP, uncoupling protein. AMPK structure adapted from (Townley et al. 2007).

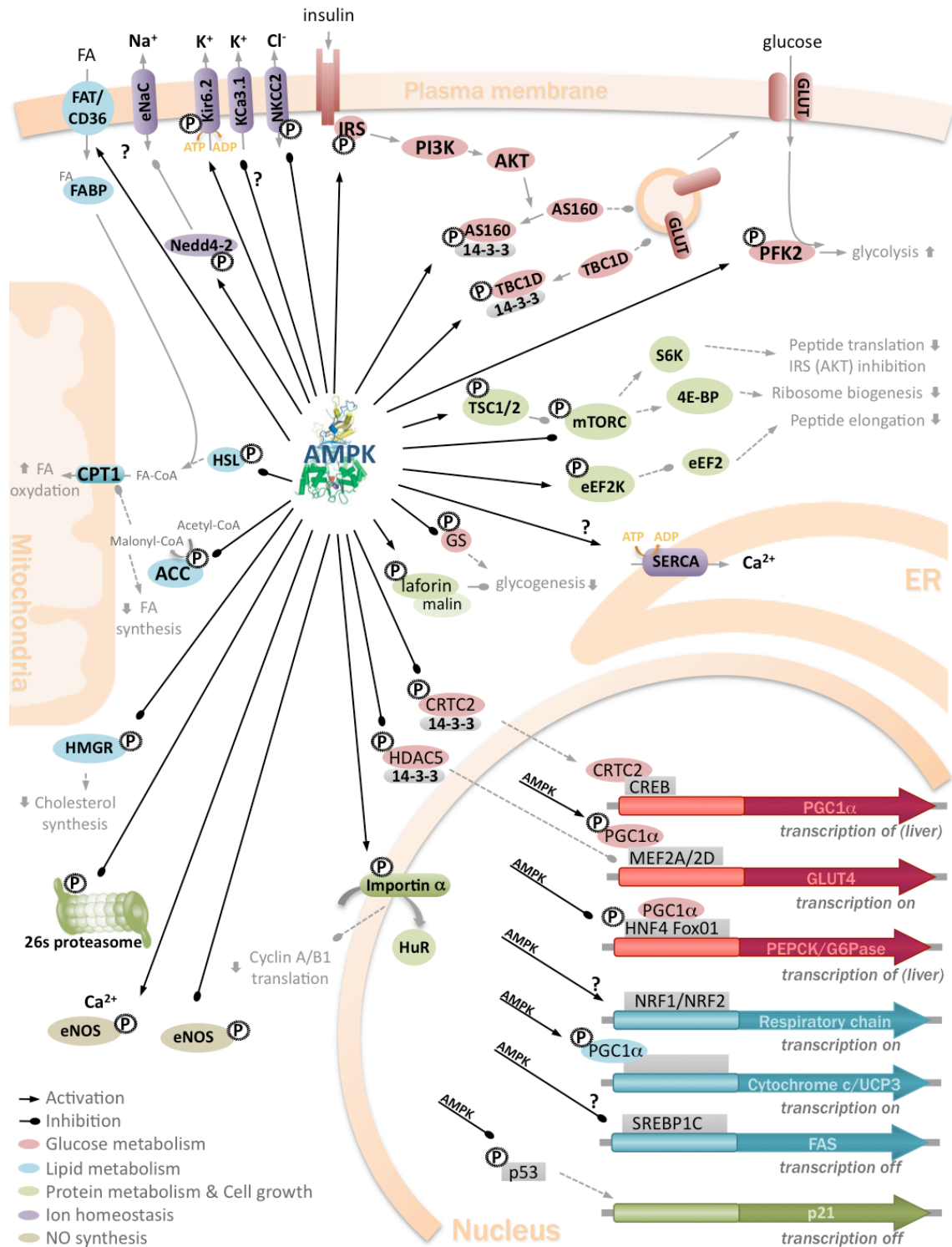


FIGURE 1-4. Cellular AMPK downstream signaling. Schematic representation of the AMPK downstream signaling processes mentioned in Fig. 1-3 and their subcellular localization.

### 3.3.1. Regulation of carbohydrate, lipid and whole body metabolism

Carbohydrates and lipids are the principal sources of energy for mammalian organisms. Supply, storage and processing of both, glucose and FA is crucial not only for cellular but also for whole body energy metabolism. An integrated metabolic control system is required to fine-tune the distribution between carbohydrate and lipid metabolism in response to a large number of physiological stimuli (e.g. exercise, fasting, feeding). AMPK controls both metabolic pathways by exerting a tight acute as well as transcriptional control.

#### 3.3.1.1. Regulation of carbohydrate metabolism

Cellular glucose uptake is mainly regulated by the glucose transporter (GLUT) family. These transporters are stored in intracellular vesicles and translocated to the plasma membrane mainly in response to insulin (reviewed in (Ishiki et al. 2005)). Activation of AMPK leads to increased GLUT4 translocation in muscle, cardiomyocytes and adipocytes (Kurth-Kraczek et al. 1999, Yamaguchi et al. 2005, Webster et al. 2010) and to increased GLUT3 translocation in neurons (Weisova et al. 2009). Recently, AMPK was shown to phosphorylate the AKT substrate AS160, regulating its binding to 14-3-3 and thus promoting vesicle translocation regulated by the monomeric G protein Rab (Treebak et al. 2006, Sakamoto et al. 2008). However, the exact molecular mechanism of AMPK-mediated GLUT translocation remains unclear. In addition to GLUT4 translocation, AMPK is also involved in GLUT4 expression by phosphorylation of both peroxisome proliferator-activated receptor gamma co-activator-1 alpha (PGC-1 $\alpha$ ) and histone deacetylase (HDAC) 5, which promote myocyte-enhancer factor (MEF)-dependent GLUT4 transcription (Steinberg et al. 2009) (Fig. 1-4).

Glycolysis is increased by activated AMPK either in short-term by 6-phosphofruktosekinase-2 (PFK2) phosphorylation (Marsin et al. 2000) or in long-term by stimulation of hexokinase II (HKII) transcription (Stoppani et al. 2002).

In liver, AMPK negatively regulates the transcription of gluconeogenic enzymes L-type pyruvate kinase (L-PK), phosphoenol-pyruvate carboxylase (PEPCK) and glucose-6-phosphatase (G-6-Pase) by phosphorylation of transcription factor HNF-4 $\alpha$  and transcriptional activator CREB (Steinberg et al. 2009).

#### 3.3.1.2. Regulation of lipid metabolism

Besides glucose, FA are the main substrates for cellular energy metabolism. As for glucose, the first step consists in FA uptake, which is regulated, similar to glucose uptake, by transmembrane transporters like FAT/CD36. CD36 is stored in intracellular vesicles similar to GLUT4, and AMPK activation by AICAR or oligomycin leads also to CD36 translocation (van Oort et al. 2009). However, the molecular mechanism remains unknown.

The longest known AMPK target is acetyl-CoA carboxylase (ACC). ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA. Malonyl-CoA is a precursor in FA synthesis and acts as an allosterical inhibitor of mitochondrial carnitine palmitoyltransferase 1 (CPT1) involved in FA

import, thus preventing FA  $\beta$ -oxidation. AMPK mediated phosphorylation inhibits ACC activity and therefore suppresses FA synthesis, while increasing at the same time mitochondrial FA  $\beta$ -oxidation (Munday 2002) (Fig. 1-4).

AMPK is suggested to inhibit both, triacylglycerol (TG) synthesis and degradation. One of the first steps of TG synthesis is regulated by glycerol-3-phosphate acyl transferase (GPAT) whose activity was reduced by AMPK *in vitro* and in AICAR-treated hepatocytes (Muoio et al. 1999). AMPK also phosphorylates hormone-sensitive lipase (HSL), preventing its activation by PKA and thus TG degradation (Watt et al. 2006).

Finally, AMPK phosphorylates and inhibits 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) preventing energy consuming cholesterol synthesis (Clarke et al. 1990).

### 3.3.1.3. Regulation of whole body energy metabolism

The idea that AMPK acts not only as a cellular energy sensor, but regulates the whole body energy status, has emerged from the findings that hypothalamic AMPK is regulated by food intake (Minokoshi et al. 2004) and that this regulation is mediated by the anorexigenic hormone leptin and the orexigenic hormone ghrelin (Andersson et al. 2004). Indeed, injection of adenovirus encoding a constitutive active AMPK mutant into the hypothalamus increased body weight and food uptake while a dominant negative mutant had the opposite effect (Minokoshi et al. 2004). However, the molecular mechanism by which AMPK regulates these processes remains unclear.

### 3.3.2. Regulation of protein metabolism, cell growth and other processes

Beside the regulation of major pathways in primary energy metabolism, AMPK seems to regulate other energy consuming processes, including protein synthesis, cell proliferation and cellular ion homeostasis.

First evidences for AMPK-mediated protein synthesis and cell growth came from the identification of tuberous sclerosis 2 (TSC2) as AMPK substrate (Inoki et al. 2003). TSC2 is a GTPase-activating protein which inactivates the small G-protein Rheb and thus exerts a critical negative regulation of downstream mammalian target of rapamycin complex 1 (mTORC1) (Yang et al. 2007). TSC2 is inhibited by AMPK, thus preventing mTORC1 downstream signalling involved in ribosome biogenesis, peptide translation and cell growth (Kwiatkowski et al. 2005). In addition, AMPK also inhibits mTORC1 assembly by phosphorylation of the TORC1 subunit raptor (Gwinn et al. 2008). In addition to protein synthesis, the mTORC1 pathway was shown to implicate AMPK also in autophagy (Meijer et al. 2007).

AMPK also phosphorylates and activates the eukaryotic elongation factor 2 kinase (eEF2K), which in turn inhibits eEF2 involved in peptide elongation (Browne et al. 2004). In addition, AMPK phosphorylates the RNA polymerase I-associated transcription factor TIF-IA, thus downregulating ribosomal RNA synthesis (Hoppe et al. 2009). AMPK activation also reduces the cytoplasmic level of the RNA-binding protein HuR, a mRNA stabilizing protein involved in regulation of cyclin A, cyclin

B1 and p21 expression (Wang et al. 2002). This might be due to increased AMPK-mediated importin  $\alpha$  phosphorylation which stimulates HuR nuclear import (Wang et al. 2004). AMPK also phosphorylates the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p27kip1, both contributing to cell cycle arrest (Jones et al. 2005, Liang et al. 2007).

AMPK seems not only to inhibit protein synthesis and cell growth, but also proteasome-dependent degradation (Viana et al. 2008). Indeed, AICAR treatment of MEF cells resulted in a 20% decrease of proteasome-dependent protein degradation, whereas no effect was observed in  $\alpha 1^{-/-}$ ,  $\alpha 2^{-/-}$  knock out MEF (Viana et al. 2008). AMPK was not involved in lysosomal-dependent protein degradation (Viana et al. 2008). AMPK phosphorylates and interacts with the 26s proteasome subunit PSMD11, indicating that AMPK might regulate the proteasome *via* direct phosphorylation of PSMD11 (Moreno et al. 2009). In addition, AMPK was shown to interact with and to phosphorylate laforin resulting in laforin-malin complex formation (Solaz-Fuster et al. 2008). The laforin-malin complex promotes protein-ubiquitination and degradation e.g. of type 1 protein phosphatase (PP1) which positively regulates glycogen accumulation (Solaz-Fuster et al. 2008).

In addition to protein metabolism and cell cycle progression, the maintenance of ion gradients across cell membranes and intracellular ion homeostasis are further very energy-demanding processes. Thus it is little surprising that AMPK might also regulate these processes. Indeed, different ion transporters seem to be regulated by AMPK. These include cystic fibrosis transmembrane conductance regulator  $\text{Cl}^-$  channel (CFTR) (King et al. 2009), ATP-sensitive potassium ( $\text{K}_{\text{ATP}}$ ) channel (Chang et al. 2009) and epithelial  $\text{Na}^+$  channel (eNaC) (Carattino et al. 2005) which are all inhibited by AMPK activation, whereas  $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$  cotransporter (NKCC2) (Fraser et al. 2007) seems to be activated. Recently AMPK activity has also been reported to stimulate the sarcoplasmic/endoplasmic reticulum calcium ATPase by preventing its oxidation (Dong et al. 2010a, Dong et al. 2010b).

## 4. CELLULAR ENERGY METABOLISM IN PATHOLOGIES

### 4.1. *The metabolic syndrome*

Impaired whole body energy balance is the major reason of poor human health worldwide. On the one hand, in 2006, malnutrition was responsible for almost 60% of total mortality worldwide. On the other hand, the industrialized world faces more and more problems related to overweight and obesity. In 2005, 66 % of the US American population and 38% of the French population were considered to have overweight (OECD 2005). The World Health Organization estimates that 1 billion adults worldwide have overweight and that 30 % of them are even clinically obese (body mass index > 30).

Obesity is often associated with a number of other health problems (type 2 diabetes, hypertension blood fat disorder, fatty liver and cardiovascular disease) that are summarized as the metabolic syndrome. Indeed, obesity raises the risk for developing type 2 diabetes by a factor of 30-40. Type 2 diabetes is characterized by insulin resistance and high blood glucose indicating impaired cellular glucose uptake. AMPK can bypass and replace insulin-signaling in many respects. It is recognized to

be a major energy metabolism-regulating enzyme whose activity has been implicated in both lipid and glucose metabolism, processes which are impaired in obesity and diabetes (Viollet et al. 2007, Hardie 2008, Viollet et al. 2009). Actually, AMPK activation with AICAR increases glucose uptake and FA oxidation in obese diabetic rodents (Bergeron et al. 2001) and humans (Koistinen et al. 2003, Steinberg et al. 2004, Steinberg et al. 2006a) suggesting a therapeutic potential of AMPK activators for bypassing skeletal muscle insulin resistance. Indeed, metformin, the major anti-diabetic drug that inhibits complex I of the mitochondrial respiratory chain, also activates AMPK (Musi et al. 2002, Zou et al. 2004). Chronic AICAR treatment was also shown to reduce blood pressure in obese Zucker rats by a still unknown mechanism (Buhl et al. 2002).

#### ***4.2. Energy metabolism in cancer***

Carcinogenesis is caused by mutations of the genetic material in normal cells, which disturbs the normal balance between proliferation and cell death. This process is accompanied by changes in energy requirements due to an increase in energy costly processes like biomass production and genome replication. Ninety years ago Otto Warburg observed that tumor cells change their glucose metabolism from OxPhos to aerobic glycolysis (Warburg effect) (reviewed in (DeBerardinis 2008)). Indeed, even if the latter provides ATP less efficiently than OxPhos, it generates at the same time metabolic intermediates important for cell growth and proliferation. It is thus not surprising that tumor suppressors and proto-oncogenes often affect metabolism. Mutations in these genes can promote a metabolic phenotype more supportive for accelerated cell growth and proliferation.

The AMPK signaling network contains a number of such tumor suppressor genes including its upstream kinase LKB1 and its downstream targets p53 and TSC1/2. Rather rare LKB1 mutations lead to increased incidence of epithelial cancers and hamartomas, and its inactivation is estimated to occur in 30-50 % of lung adenocarcinomas, 20% of squamous cell carcinomas and 10% of lung large cell carcinomas (Ji et al. 2007). The role of LKB1 in the activation of AMPK suggests that pharmacological AMPK activation might be associated with reduced cancer incidence and progression. Indeed, AICAR-induced AMPK activation in breast cancer cell lines blocks their proliferation and reduced tumor growth in nude mice (Swinnen et al. 2005). In addition, diabetic patients treated with metformin show a 30% reduction in deaths from cancers versus those treated with either sulfonylurea or insulin (Evans et al. 2005b).

#### ***4.3. Neurodegenerative diseases***

A common feature of neurodegenerative disorders such as Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer disease (AD) are mutations in nuclear or mitochondrial DNA leading to impaired mitochondrial function and energy metabolism disturbance (Beal 2000). In AD, the cerebral glucose metabolism is decreased, accompanied by impaired OxPhos. In addition, AD is characterized by accumulation of pathological protein aggregates like extracellular

amyloid plaques and intracellular neurofibrillary tangles, as well as by neuronal loss (Burklen et al. 2006, Jellinger 2006, Bettens et al. 2010).

Frequently, oxidative alterations of proteins and lipids have been implicated in the progression of neurodegenerative disorders (Beal 2002). Some metabolic enzymes are particularly sensitive to such oxidative damage. These include the mitochondrial aconitase and adenine nucleotide translocase (ANT) as well as cytosolic glutamine synthase and creatine kinase (reviewed in (Beal 2002)). Indeed CK activity is decreased by 86 % in AD brain homogenates whereas the CK expression level is decreased by 18 % (David et al. 1998). In addition, Cr deposits have been found in brains of transgenic APP (amyloid precursor protein) mice (Gallant et al. 2006) corroborating a putative role of CK in neurodegeneration. Different creatine supplementation studies have shown that CK might have a positive role in protection of hippocampal neurons against amyloid  $\beta$ -peptide ( $A\beta$ ) toxicity, of GABA-ergic neurons in a HD model, and of dopaminergic neurons in a PD model (reviewed in (Burklen et al. 2006)).

In a transgenic mouse model of AD, brain vessel remodeling was also deregulated, which was associated to abnormal insulin-like growth factor I (IGF-I) and AMPK activity (Lopez-Lopez et al. 2007). AICAR treatment was found to protect hippocampal neurons against cell death induced by glucose deprivation, chemical hypoxia, glutamate and  $A\beta$  toxicity (Culmsee et al. 2001). Taken together, these data suggest a putative neuroprotective role of AMPK that, however, requires further investigations

#### ***4.4. Brain creatine kinase-/creatine deficiency-related diseases***

CK is expressed in every brain cells, but highest levels are detected in Bergman glia and Purkinje cells in the cerebellum that are important for movement coordination and control. CK is also prominent in neuronal cells in the hippocampus, where learning and memory functions reside, and finally in epithelial cells in the choroid plexus that is rich in ATP-dependent pumps for homeostasis of ions and metabolites at the ventricular fluid/brain interface (Wallimann et al. 1994, Kaldis et al. 1996). Accordingly, brain CK knock out mice display altered behavior, e.g. low nest-building activity, less exploratory activity, less grooming etc. and neurological difficulties in spatial learning and memory functions (Jost et al. 2002, in 't Zandt et al. 2004, Streijger et al. 2004, Streijger et al. 2005). In addition, they present reduced body weight, as well as altered brain morphology. The phenotypes of these knock out mice reveal the importance of CK activity for correct brain function.

The enzyme is very sensitive to oxidation and oxidative stress related disorders (aging, neurodegenerative and neuromuscular diseases), which lead to increased CK oxidation and inactivation (Wallimann et al. 2007). In addition, different disorders are associated with impaired creatine metabolism due to a deficiency in one of the two key enzymes of creatine synthesis, guanidinoacetate methyltransferase (GAMT) and arginine:glycine amidinotransferase (AGAT), or to a defect in creatine transporter (CrT1). These patients present developmental delay, mental retardation, and severe disturbance of their expressive and cognitive speech. The common feature of all creatine

deficiency syndromes is the severe depletion of creatine/phosphocreatine in the brain. Treatment with oral creatine supplementation is in part successful in GAMT and AGAT deficiency, whereas defective CrT1 it is not able to replenish cellular creatine stores in the brain (reviewed in (Schulze 2003)).

#### **4.5. AMPK deficiency related diseases**

Mutations in the AMPK  $\gamma 2$  subunit, expressed at particularly high levels in heart, impair AMP binding and AMPK activation causing low constitutive activity of the complex (Scott et al. 2004, Burwinkel et al. 2005). They are associated to heart disease of varying severity, involving cardiac hypertrophy, contractile dysfunction and arrhythmias. The major cause of such AMPK-related heart disease is the increased basal AMPK activity leading to higher glucose uptake, accumulation of glycogen in cardiac myocytes, and finally impairment of heart muscle development (Burdick et al. 2005).

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## - CHAPTER II -

### YEAST TWO-HYBRID, A POWERFUL TOOL FOR SYSTEMS BIOLOGY

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**Abstract:** A key property of complex biological systems is the presence of interaction networks formed by its different components, primarily proteins. These are crucial for all levels of cellular function, including architecture, metabolism and signaling, as well as the availability of cellular energy. Very stable, but also rather transient and dynamic protein-protein interactions generate new system properties at the level of multiprotein complexes, cellular compartments or the entire cell. Thus, interactomics is expected to largely contribute to emerging fields like systems biology or systems bioenergetics. The more recent technological development of high-throughput methods for interactomics research will dramatically increase our knowledge of protein interaction networks. The two most frequently used methods are yeast two-hybrid (Y2H) screening, a well established genetic *in vivo* approach, and affinity purification of complexes followed by mass spectrometry analysis, an emerging biochemical *in vitro* technique. So far, a majority of published interactions have been detected using an Y2H screen. However, with the massive application of this method, also some limitations have become apparent. This review provides an overview on available yeast two-hybrid methods, in particular focusing on more recent approaches. These allow detection of protein interactions in their native environment, as e.g. in the cytosol or bound to a membrane, by using cytosolic signaling cascades or split protein constructs. Strengths and weaknesses of these genetic methods are discussed and some guidelines for verification of detected protein-protein interactions are provided.

**Keywords:** interactomics; mass spectrometry; protein-protein interaction; systems bioenergetics; yeast two-hybrid

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## 1. INTERACTOMICS TAKE CENTER STAGE IN SYSTEMS BIOLOGY

### 1.1. A central role for protein interactions

The field of systems biology has achieved tremendous momentum during recent years. This development has been driven by: (i) a huge amount of genomic and proteomic data already available, (ii) the need to understand complex cellular systems or multifactorial diseases such as cancer or the metabolic syndrome, and (iii) emerging technologies which allow high-throughput screening of complex mixtures of biomolecules or non-invasive studies of live cells or entire organisms. In

addition, evolution in this field would have been impossible without the parallel development of bioinformatics tools to analyze the large amounts of data generated.

Multiprotein complexes, not individual proteins, are increasingly recognized as the molecular basis of cellular fluxes of molecules, signals and energy. Thus, technologies which enable us to decipher cellular interactions between biomolecules (interactomics) together with those measuring metabolite fluxes (metabolomics, fluxomics) and signaling cascades (phosphoproteomics and others dealing with secondary protein modifications) have taken center stage in systems biology [1].

Interactomics can be applied in a global, unbiased cell systems approach, or in a more targeted approach to study a specific set of proteins [2]. While the former may identify so-called “nodes” or “hubs” in cell signaling but is often prone to errors (see discussion below on false negatives and positives), the latter is able to reliably describe sub-networks in more detail, including biophysical constants of the interaction and their spatiotemporal organization [3].

To date, the cellular interactome has mainly been explored for interactions involving proteins in the fields of cell signaling and cell architecture to understand the wiring of cellular data processing. However, it is also becoming increasingly important in many other fields.

### ***1.2. Systems bioenergetics***

Bioenergetics has known several decades of intense research, starting with the discovery of the main biochemical pathways and energy conservation in a chemiosmotic gradient in the 60s and 70s of the last century. After being a quiescent field for more than a decade, several developments during the last 15 years have put bioenergetics and mitochondria back to the forefront of scientific development [4] (for an excellent book see [5]): There has been the description of the involved protein machines at an atomic level (like the respiratory complexes in mitochondria), the discovery of a close link between mitochondria and cell signaling (calcium, apoptosis), and the emerging relationship between a dysfunction of cellular energetics and a plethora of complex pathologies, including (neuro-)muscular and age-related diseases, metabolic and cardiovascular diseases and cancer. Currently, the field of bioenergetics is about to enter the era of systems biology [6]. In fact, ATP generation needs a precise interplay between proteins of glycolysis, TCA cycle, mitochondrial electron transport and energy transfer systems like creatine kinase, which often includes specific (micro)compartmentation of proteins or multiprotein complexes maintained by specific protein-protein interactions. These topologies then allow for more precise regulation or have further thermodynamic advantages like substrate channeling between active sites. Systems bioenergetics holds the promise of integrating the multiple aspects of cellular energetics in a holistic approach which: (i) extends our knowledge on protein complexes involved in metabolic control and cell signaling [7], (ii) considers cellular compartmentation particularly important in this field [8], and (iii) aims to understand the complex regulatory cellular network which governs homeostasis in cell energetics and which apparently fails in so many pathologies [9]. Inversely, manipulating energy metabolism holds promises for therapeutic

strategies. For example, it was surprising that inhibiting mitochondrial complex I in mitochondria is part of the molecular mechanism of the most successful antidiabetic drug, metformin [10]. Thus, the emerging field of systems bioenergetics does not only involve basic research, but is of prime importance for applied and clinical scientists.

For bioenergetics, interactomics goes far beyond cell signaling or cell structure, since it may uncover a new layer of regulation. The components of the mitochondrial redox chain or the ATPases are among the most complex protein assemblies, and understanding their regulation as well as the flux of protons and electrons will need intense work. Spatiotemporal organization of the long known pathways in primary metabolism is still incompletely understood [11], and the same applies to the systems of “energy-rich” intermediates like nucleoside triphosphates or phosphocreatine and mechanisms like metabolite channeling between different components in a complex [6,12,13].

### ***1.3. Interactomics tools***

This review gives an overview of several methods for global or targeted interactomics, with a particular emphasis on classical and emerging yeast two-hybrid (Y2H) systems. These Y2H tools now allow access to the almost entire cellular proteome for interaction screening, including membrane proteins, transcriptionally active proteins and proteins localized in different subcellular compartments. Massive application of such tools can be expected, since they are comparatively inexpensive as compared to others, do not need specialized large equipment and can be performed in any molecular biology laboratory with reasonable throughput.

## **2. SCREENING TECHNOLOGIES FOR PROTEIN-PROTEIN INTERACTIONS**

Protein-protein interactions are involved in all cellular processes. Mapping of these interaction networks to elucidate the organization of the proteome into functional units is thus of prime importance for systems biology. A large number of methods have been developed for screening protein interactions. The more classical biochemical approaches, such as copurification, affinity purification or coimmunoprecipitation of protein complexes require *in vitro* handling of protein extracts. Further limitations of these techniques include restricted sensitivity and bias towards high affinity interactions. Once a partner has been detected, identification by mass spectrometry (MS) is generally straightforward, although rather costly. Cloning of corresponding cDNAs may be time-consuming, but clone repositories such as RIKEN or IMACE can be a convenient alternative. More recently, surface plasmon resonance (SPR), a biophysical technology, has been adopted for screening protein-protein interactions. Purified cellular extracts are injected onto a sensor chip covered with an immobilized binding partner. The instrument setup combines capture of the binding partner with a quantitative readout of the binding event, such that putative partners can be eluted and identified by MS [14,15]. Another approach to interaction screening are “cDNA-expression” libraries (for a review

see [16]) such as phage display or Y2H methods, the latter detecting protein interactions *in vivo*. For studies on a genomic scale, highly parallel and automated processes are needed. However, only few detection methods for protein-protein interactions can be easily adapted for a high-throughput strategy. These include in particular yeast two-hybrid (Y2H) and affinity purification coupled to MS (AP/MS).

### **2.1. Yeast two-hybrid**

The Y2H technique allows detection of interacting proteins in living yeast cells [17]. As described in full detail in chapter 3, interaction between two proteins, called bait and prey, activates reporter genes that enable growth on specific media or a color reaction. Y2H can be easily automated for high-throughput studies of protein interactions on a genome-wide scale, as shown for viruses like bacteriophage T7 [18], *Saccharomyces cerevisiae* [19,20], *Drosophila melanogaster* [21], *Caenorhabditis elegans* [22] and humans [23,24]. Experimental Y2H data have been a crucial part in establishing large synthetic human interactomes [25,26] or to dissect mechanisms in human disease [27]. Two screening approaches can be distinguished: the matrix (or array) and the library approach.

In the matrix approach, all possible combinations between full-length open reading frames (ORFs) are systematically examined by performing direct mating of a set of baits versus a set of preys expressed in different yeast mating types (e.g. mating type *a* for baits and mating type  $\alpha$  for preys). This approach is easily automatable and has been used in yeast and human genome-scale two-hybrid screens. In yeast, 6,000 ORFs were cloned and over 5,600 interactions were identified, involving 70% of the yeast proteome [19,20,28]. The defined position of each bait in a matrix allows rapid identification of interacting preys without sequencing, but screens are usually restricted to a limited set of full length ORF's and will thus fail to detect certain interactors (called false negatives).

The classical cDNA-library screen searches for pairwise interactions between defined proteins of interest (bait) and their interaction partners (preys) present in cDNA libraries or sub-pools of libraries. An exhaustive screen of libraries with selected baits can be an alternative to a matrix approach. Here, preys are not separated on an array but pooled (reviewed in [29]), and libraries may contain cDNA fragments in addition to full length ORFs, thus largely covering a transcriptome and reducing the rate of false negatives. However, inherent to this type of library screening, the rate of wrongly identified proteins (called false positives) is increased. In addition, interaction partners have to be identified by colony PCR analysis and sequencing, making such screens more expensive and time consuming.

### **2.2. Affinity purification/mass spectrometry**

The value of MS for high-throughput screening of protein interactions has been recognized only more recently. This analytical technique is based on the determination of the mass-to-charge ratio of ionized molecules. Already introduced in 1948, sensitivity and implementation range of MS has been largely extended by technological advances. These include Nobel prize crowned methods for

ionization like electrospray ionization [30], generating ions from macromolecules in liquid medium without their fragmentation, soft laser desorption (SLD) [31] or matrix-assisted laser desorption/ionization (MALDI) [32], using a laser beam for ionization of macromolecules without breaking chemical bonds. MS is now routinely applied to identify proteolytic fragments of proteins or even entire proteins and protein complexes [33]. Coupled to classic biochemical methods like affinity purification or chemical cross-linking, MS has become also a powerful tool for large-scale interactome research, mainly in form of affinity purification-MS (AP/MS). In this approach, a protein mostly fused to an epitope-tag is either immunoprecipitated by a specific antibody (e.g. against the tag) or purified by affinity columns recognizing the tag. Affinity purification can make use of an individual tag (e.g. a Flag-tag) for single step purification. However, it is more efficient when using two subsequent purification steps with proteins that are doubly tagged (e.g. 6xHis- and Strep-tag) or carry either C- or N-terminally a fusion of two affinity tags separated by a protease cleavage site (e.g. protein A and calmodulin binding protein) where the first tag is cleaved off after the first AP step (tandem affinity purification, TAP). This results in an enrichment of native multiprotein complexes containing the tagged protein. Subsequent MS analysis then identifies the different constituents of the complexes [34]. Ho *et al.* [35] expressed 10% of yeast ORFs with a C-terminal flag-tag under the control of an inducible promoter in yeast. They were able to connect 25% of the yeast proteome in a multiprotein complex interaction network. With the TAP-tag approach, Gavin *et al.* [36,37] and Krogan *et al.* [38] purified 1,993 and 2,357 TAP-fusion proteins covering 60% and 72% of the yeast proteome, respectively. As compared to the single Flag-tag approach, combination of two different purification steps in TAP results in improved sensitivity and specificity (TAP is reviewed in more detail in [39,40]). Recent technical progresses in automation of complex purification and MS analysis, together with dedicated computational methods to increase accuracy of data analysis, have made this approach a powerful tool in interactome research.

### ***2.3. Comparison of Y2H- and MS-based methods***

MS is less accessible than Y2H due to the expensive large equipment needed. Thus, a large amount of the data so far generated from protein interaction studies have come from Y2H screening. For example, more than 5,600 protein interactions have been so far reported for yeast [19,20,28,41] and about 6,000 for humans [23,24], establishing extensive protein interaction networks. Approximately half of the interaction

data available on databases such as IntAct [42] and MINT [43] are coming from Y2H assays. Genome-scale Y2H screens have highlighted considerable cross-talk in the cell, even between proteins that were not thought to be functionally connected. However, Y2H and AP/MS are complementary in the kind of interactors that they are detecting. AP/MS may determine all the components of a larger complex, which not necessarily all interact directly with each other, while Y2H studies identify defined binary, interactions in this complex. In addition, some types of protein-protein interactions can

be missed in Y2H due to inherent limitations, like interactions involving membrane proteins, self activating proteins, or proteins requiring post-translational modifications, but this may also occur with AP/MS-based approaches. Given the strengths of both methods, considerable effort is invested to overcome the remaining drawbacks. Different Y2H systems have been developed to extend the coverage of the proteome of interest, as will be described in detail further below. Recently, also the sensitivity and robustness of AP/MS was improved by the development of an integrated workflow coupling rapid generation of bait-expressing cell lines with an increase in protein complex purification using a novel double-affinity strategy [44]. Only a combination of different approaches that necessarily includes bioinformatics tools, will eventually lead to a fairly complete characterization of physiologically relevant protein-protein interactions in a given cell or organism. This will be a fundamental requirement to use interactome data in a systems biology approach at the cellular or higher complexity level.

### **3. AIMING AT *IN VIVO* INTERACTIONS: THE YEAST TWO-HYBRID APPROACH**

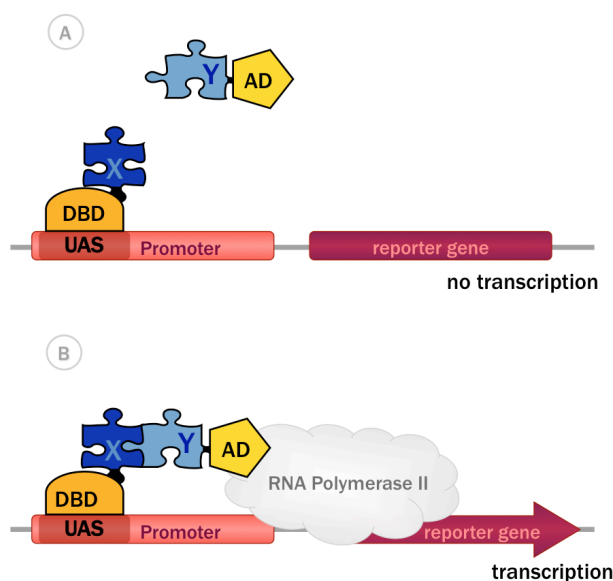
#### ***3.1. Historical perspective: The principles of the approach***

In 1989, Fields and Song revolutionized protein interaction analysis by describing a genetic system to detect direct protein-protein interactions in the yeast *Saccharomyces cerevisiae* [17]. Until then, interactions between two proteins had mostly been studied using biochemical techniques. The development of this completely new analytic tool was triggered by the molecular analysis of eukaryotic transcription factors. Only few years before, the Ptashne Laboratory had discovered the modular structure of Gal4, a transcriptional activator in yeast. They showed that Gal4 binds a specific DNA sequence (the upstream activation domain, UAS) and thus activates transcription in the presence of galactose. If separated into two fragments, the N-terminal fragment did still bind to DNA, but did not activate transcription in presence of galactose, while this latter function was mediated by the C-terminal fragment [45]. However, both fragments could interact and non-covalently reconstitute a fully functional Gal4. Thus, two different functional domains of Gal4 were identified: an N-terminal DNA binding domain (DBD) and a C-terminal (transcriptional) activation domain (AD), with both individual domains maintaining their function independent of the presence of the other.

Inspired by these findings, Fields and Song exploited the modular properties of the transcription factor Gal4 to monitor protein-protein interactions. The basic idea was to fuse the two proteins of interest X and Y to DBD and AD of Gal4, respectively, such that interaction between X and Y reconstitutes a functional transcription factor that could then drive reporter gene expression (Figure 2-1). In the first construct called bait, protein X (e.g. the glucose-sensor SNF1) was fused to the N-terminal part of GAL4 containing the DBD (GAL4DBD). In the second construct, the prey, protein Y (e.g. the regulatory protein SNF4) was fused to the C-terminal part of Gal4 that contains the AD (GAL4AD). Expression of both fusion proteins in yeast and interaction between bait and prey indeed

reconstituted a functional Gal4 transcription factor from the two separate polypeptides. Gal4 then recruited RNA polymerase II, leading to transcription of a *GALI-lacZ* fusion gene. This reporter gene encodes the enzyme beta-galactosidase which labels the yeast cell when using a colorimetric substrate [17].

For a genome-wide screen for interactors of given baits, a cDNA library is used to construct an entire library of preys. From a methodological point of view, any such Y2H screen implies the transformation of yeast cells with bait and prey cDNA on different vectors under the control of yeast promoters. Expression levels will depend on the promoter used and may affect sensitivity and specificity of the screen. Once expressed in the cytosol, bait and prey must be able to enter the nucleus to activate transcription, a limitation of the original Y2H approach further discussed below.



**Figure 2-1.** The classical yeast two-hybrid system. (A) The protein of interest X, is fused to the DNA binding domain (DBD), a construct called bait. The potential interacting protein Y is fused to the activation domain (AD) and is called prey. (B) The bait, i.e. the DBD-X fusion protein, binds the upstream activator sequence (UAS) of the promoter. The interaction of bait with prey, i.e. the AD-Y fusion protein, recruits the AD and thus reconstitutes a functional transcription factor, leading to further recruitment of RNA polymerase II and subsequent transcription of a reporter gene.

This classical Y2H system has been extended to exploit different other DNA-binding proteins (e.g. DBD of *E. coli* repressor protein LexA), transcriptional activators (e.g. AD of Herpes simplex virus VP16) and various reporter genes. A suitable reporter gene must encode a protein whose function provides a simple readout. Thus, besides the colorimetric reaction with the *lacZ* gene, the most commonly used are auxotrophic markers (e.g. *LEU2*, *HIS3*, *ADE2*, *URA3*, *LYS2*) that allow growth on minimal media. In the current state-of-the-art, more than one reporter gene is assayed in parallel to increase the stringency of Y2H screens [46]. In fact, one of the common problems of Y2H is the generation of false positives due to non-specific interactions (as described in detail further below). Selection for two active reporter genes requires a more solid transcriptional activation and thus increases the stringency of the assay, but concomitantly penalizes detection of weak and transient



interactions. Another possibility to adjust the stringency of the assay is partial inhibition of the enzymatic activity encoded by the reporter gene. For example, the product of the *HIS3* reporter, imidazole glycerol phosphate dehydratase, is competitively inhibited by increasing concentrations of 3-aminotriazole.

**Table 2-1.** Overview of different Y2H systems and their specificities.

Year	Y2H method	Possible baits	Response	Cellular compartment *	Screen compatibility #
1989	Classic Y2H system [17]	Non-transactivating proteins capable of entering nucleus	Transcriptional activation	Nucleus	Yes [17]
1994	SOS recruitment system (SRS) [51]	Transactivating, cytosolic proteins	Ras signaling	Membrane periphery	Yes [52]
1994	Split-ubiquitin system [53]	Nuclear, membrane and cytosolic proteins	Uracil auxotrophy and 5-FoA resistance	Cytosol	Yes [54]
1998	Membrane split-ubiquitin system (MbY2H) [55]	Membrane proteins	Transcriptional activation	Membrane periphery	Yes [56]
1998	Ras recruitment system (RRS) [57]	Transactivating, cytosolic proteins	Ras signaling	Membrane periphery	Yes [57]
1999	Dual bait system [49]	Two non-transactivating proteins capable of entering nucleus	Transcriptional activation	Nucleus	Yes [49]
2000	G-protein fusion system [58]	Membrane proteins	Inhibition of protein G signaling	Membrane periphery	No
2001	RNA polymerase III based two-hybrid (Pol III) [59]	Transactivating proteins (in the RNA polymerase II pathway )	Transcriptional activation	Nucleus	Yes [59]
2001	Repressed transactivator system (RTA) [60]	Transactivating proteins capable of entering nucleus	Inhibition of transcriptional activation	Nucleus	Yes [60-62]
2001	Reverse Ras recruitment system (rRRS) [63]	Membrane proteins	Ras signaling	Membrane periphery	Yes [63]
2003	SCINEX-P system [64]	Extracellular and transmembrane proteins	Downstream signaling & transcriptional activation	Endoplasmic reticulum (ER)	No
2004	Split-Trp system [65]	Cytosolic, membrane proteins	Trp1p activity	Cytosol	Yes (Lentze & Auerbach, unpubl.)
2007	Cytosolic split-ubiquitin system (cytoY2H) [66]	Transactivating, cytosolic proteins	Transcriptional activation	ER membrane periphery	Yes [66]

\* Cellular compartment where the interaction occurs.

# Indicates whether a given Y2H system has been used for cDNA-library screening.

Compared to earlier interaction screens, the Y2H system was able to detect interactions *in vivo* in a true cellular environment. Since it is also relatively easy to implement and inexpensive, Y2H rapidly became the system of choice for detecting protein-protein interactions. Its principles were rapidly adopted for screenings involving interaction of more than two partners. To analyse ligand-receptor interactions, a synthetic heterodimer of two different small organic ligands is used as a third hybrid molecule together with two receptors fused to DBD and AD. In this case, binding of the hybrid

organic ligand to both receptors will force them together to reconstitute the DBD-AD complex [47]. This three hybrid system can also be used to identify inhibitors of protein-protein interactions [48]. Another extension of the classical Y2H system is the use of more than one bait, in particular to compare interaction specificities [49]. In the so-called Dual Bait system, protein  $X_1$  is fused to the LexA DBD, and protein  $X_2$  is fused to the DBD of the cI repressor from bacteriophage  $\lambda$ . Thus, each bait is directed to a different reporter gene. Positive interactions with  $X_1$  are registered through lexA operator activation of *LEU2* and *LacZ*, and positive interactions with  $X_2$  through cI operator activation of *LYS2* and *GusA*. *GusA* codes for beta-glucuronidase that can use a colorimetric substrate to report interactions. This system has been successfully used to identify proteins interacting with specific regions in larger proteins [50]. Further more recent expansions of Y2H to high-throughput applications, the so-called matrix or array approach, has been already discussed in the previous chapter.

In their original publication Fields and Song already mentioned some of the limits of their Y2H method: “The system requires that the interaction can occur within the yeast nucleus, that the Gal4-activating region is accessible to the transcription machinery and that the Gal4(1-147)-protein X hybrid is itself not a potent activator”. These limitations would exclude almost half of all proteins, explaining the great interest for developing alternative Y2H variants.

### ***3.2. Choosing the right strategy: Available Y2H systems and their advantages***

More recent Y2H-based techniques access almost the entire cellular proteome (see Table 2-1). Almost all of them rely on a similar principle, namely the modular structure of the protein reporting the interaction. Similar to DBD and AD reconstituting a transcription factor in the original Y2H system, they employ proteins containing two structural domains which can fold correctly independently of each other and which reconstitute the functional reporter system if brought together via bait-prey interaction. An exception of this principle is the recruitment-based Y2H, where the reporter cascade is activated by forced membrane localization of the bait-prey complex. The following chapter will present in more detail the currently available Y2H systems (Table 2-1, Figure 2-2).

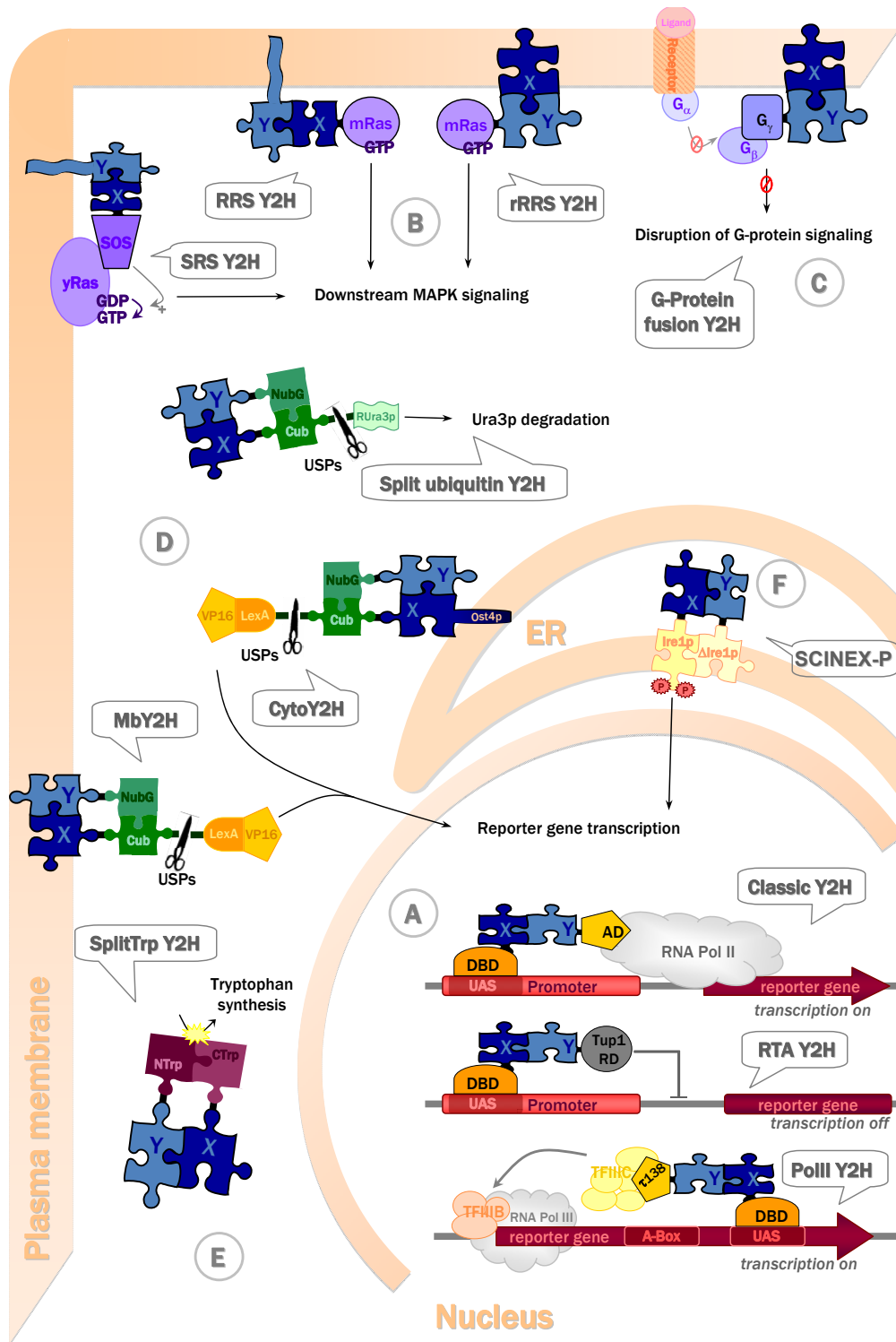
#### ***3.2.1. Y2H with transactivating proteins in the nucleus***

The classic Y2H system is based on reconstitution of a transcription factor and thus not adapted for interaction analysis with proteins that can directly activate transcription. Such transactive baits would trigger transcription in absence of any interaction with a prey. Two alternative Y2H systems have been developed to analyze the interaction network of such proteins. One is based on repression of transactivation, while the other uses the alternative polymerase III transcription pathway. Also methods mentioned under 3.2.2 (e.g. the split-ubiquitin systems) are suitable to screen transactive baits.

In the **repressed transactivator (RTA) system** (Figure 2-2A), inversely to the classic Y2H, the bait-prey interaction represses transcriptional activation of reporter genes [60]. The protein of interest X fused to the DBD of Gal4 is transactive, e.g. a transcription factor. If it interacts with another protein Y fused to the repression domain (RD) of a transcription repressor (e.g. Tup1p), the transcription of the reporter gene is repressed [60]. The RTA system has been used to demonstrate interactions between the mammalian basic helix-loop-helix proteins MyoD and E12, and between the protooncogenic transcription factor c-Myc and the putative tumor suppressor protein Bin1 [60]. It has also been applied to screen for novel interactions with a variety of transcriptional activators, including herpes simplex virus 1 (HSV-1) regulatory protein VP16 [60], c-Myc [62], and the androgen receptor [61].

**Figure 2-2 (on the right).** Yeast two-hybrid systems, their subcellular location within a yeast cell, and their operating mode (represented at the moment of bait-prey interaction). Protein X (dark blue puzzle piece, part of bait construct) and protein Y (light blue puzzle piece, part of prey construct) directly interact (fitting puzzle pieces), thus inducing reconstitution of split-proteins (puzzle pieces of different colors in A, D, E), membrane recruitment (B, C) or protein dimerization (F). Protein fusions in bait or prey constructs are shown as solid black lines between puzzle pieces. Bait-prey interaction activates further downstream events (arrows) that directly (A) or indirectly (B, C, D, F) lead to transcriptional activation, or are independent of transcriptional activation (D, E), finally yielding screenable readouts like growth on specific media or color reactions.

(A) **Nuclear Y2H systems** all require protein recruitment and bait-prey interaction at nuclear DNA. The classic Y2H and RTA Y2H both engage RNA polymerase II (RNA Pol II) transcription either by its activation or its inhibition. By contrast, the Pol III Y2H, involves RNA polymerase III (RNA Pol III) transcription. (B) **Ras signaling based Y2H at the plasma membrane.** The SRS Y2H, RRS Y2H, and rRRS Y2H are all based on protein recruitment to the plasma membrane via bait-prey interaction and subsequent activation of MAPK downstream signaling. While in the SRS and RRS Y2H the prey constructs harboring protein Y are anchored at the membrane via myristoylation to analyze interactions with cytosolic bait constructs harboring protein X, the rRRS is used to analyze interactions between soluble preys containing protein Y and partner X being a membrane protein. (C) **G-protein signaling-based Y2H at the plasma membrane.** In the G-protein fusion Y2H, bait X is a membrane or membrane-associated protein whose interaction with the prey construct disrupts protein G downstream signaling. (D) **Split-ubiquitin based Y2H systems** involve reconstitution of ubiquitin from two domains upon bait-prey interaction. Their subcellular localization depends on the nature of interacting proteins X or Y, and on the reporter proteins used. The Split ubiquitin Y2H uses non-transcriptional reporting of protein interactions in the cytosol, but can also be used for membrane proteins (not shown). The MbY2H is used for interaction analysis with membrane baits and thus occurs at the membrane location of protein X, e.g. the plasma membrane. The CytoY2H is used for membrane anchored cytosolic baits and occurs close to the ER membrane (E) **Split-protein sensor Y2H.** The Split-Trp Y2H is used to assay cytosolic bait-prey interactions based on reconstitution of an enzyme in tryptophan synthesis, allowing for non-transcriptional reporting. (F) **ER Y2H system.** The SCINEX-P Y2H allows bait-prey interaction analysis in the reducing environment of the ER, based on protein dimerization in unfolded protein signaling. ER, endoplasmic reticulum; for further abbreviations and details see chapter 3.2.



Recently, this system has been extended to screen for molecules which inhibit protein-protein interaction, for example between the immunophilin FKBP12 and the transforming growth factor  $\beta$  receptor (TGF $\beta$ -R) C terminus [67]. FKBP12 itself is not transactivating, but was fused to VP16-AD in addition to Gal4-DBD. In the absence of interaction with a RD-fusion protein, e.g. due to the presence of an inhibitor, transcription of reporter gene *HIS3* is activated. Strength of the inhibition is translated into expression levels of *HIS3* which can be probed by increasing amounts of 3-

aminotriazole, a competitive inhibitor of the *HIS3* gene product. Compared to the classic Y2H, this assay has the advantage that inhibition of interaction does not result in a loss but in a gain of reporter gene transcription and thus in a positive signal facilitating screening procedures. Thus, the RTA Y2H can not only be used to identify interaction partners of transcription factors, but also as a reversed Y2H to screen small molecule libraries e.g. for potentially novel therapeutic compounds acting as inhibitors of a given protein-protein interaction.

The **RNA polymerase III based two-hybrid (Pol III) system** (Figure 2-2A) is another alternative to screen for interaction partners of transcription factors activating RNA polymerase II-based transcription. As in the classic Y2H, a protein X is fused to a Gal4-DBD (bait), and this bait is able to bind DNA due to Gal4p binding sequence artificially introduced into the reporter gene *SNR6*. However, the prey construct is different, since the second protein Y is fused to  $\tau$ 138p. This protein is a subunit of the multimeric protein complex TFIIC, one of the two transcription factors involved in RNA polymerase III (PolIII)-mediated transcription. If now the bait interacts with the prey containing  $\tau$ 138p, the TFIIC complex is bound to DNA and recruits a second transcription factor (TFIIB) and Pol III. This will activate transcription of the *SNR6* reporter gene to produce U6 snRNA [68]. In a yeast strain harboring a temperature-sensitive U6 snRNA mutant [59], this reporter gene transcription will rescue the temperature-sensitive phenotype and allow yeast growth at 37°C. The system has been used to screen a mouse embryonic cDNA library using  $\tau$ 138p-mBRCA1 as a bait [59], but apparently has not been further adopted for screening assays.

### 3.2.2. Y2H with cytosolic and membrane proteins

The classic Y2H and the two alternative systems presented above require the translocation of the interacting proteins into the nucleus and are thus not suitable for membrane associated proteins, integral membrane proteins and many other soluble cytosolic proteins or proteins localized in other subcellular compartments. To circumvent these limitations, truncated versions of these proteins have been used for Y2H screens [69-71]. However, the use of such truncated proteins can lead to misfolding, and the problem remains that the nucleus is not the natural environment for most of these proteins. Such problems, probably leading to a high rate of false negatives in the past, would be circumvented by screening procedures where interacting proteins remain in their natural cellular compartment. Outside the nucleus, away from the transcription machinery, also the use of transactivating baits would no longer constitute a problem.

The **SOS- and the RAS recruitment systems (SRS and RRS)** (Figure 2-2B) are bypassing the transcriptional readout by using the Ras signaling pathway, which is homologous between yeast and mammals. Ras has to be localized at the plasma membrane to undergo GDP-GTP exchange by guanyl exchange factors, Cdc25 in yeast or Son of sevenless (SOS) in mammals. This activated Ras then triggers downstream signaling. For the Y2H systems described here, a Cdc25-2 temperature sensitive yeast strain is used which is unable to grow at a higher temperature (36°C) because Cdc25-2 becomes

inactive and fails to activate Ras signaling. The temperature-sensitive phenotype can then be rescued by alternative activation of Ras in the Y2H setup.

In the SOS recruitment system (Figure 2-2B: SRS Y2H), a soluble protein X is fused to mammalian SOS. If the SOS-X fusion interacts with a prey localized in the membrane (e.g. via myristoylation), SOS stimulates guanyl exchange on yeast Ras (yRas) and promotes downstream signaling [51].

In the Ras recruitment system (Figure 2-2B: RRS Y2H), the soluble protein X is directly fused to constitutively active mammalian Ras (mRas). Already active, this Ras only requires membrane location, bypassing the activity of Ras guanyl exchange factors (Cdc25 or SOS). The mRas-X fusion is recruited to the membrane by interaction with a membrane associated prey [57].

Both SRS and RRS allow the analysis of interactions between soluble baits and soluble or membrane preys. Specifically for the use of membrane localized baits, the reverse Ras recruitment system (Figure 2-2B: rRRS Y2H) has been developed. Conversely to the RRS, the prey is the Ras fusion protein, and the bait is membrane-anchored or itself a membrane protein [63]. Although the rRRs has been used for screening procedures [63], it has an important disadvantage. Preys containing membrane proteins are self-activating, since they localize Ras to the membrane even without bait-prey interaction. These false positive membrane proteins have to be eliminated by additional selection steps, rendering the method more laborious. Exclusion of membrane and membrane-associated proteins also represents serious limitation as compared to other more recent Y2H techniques.

The **G-protein fusion system** (Figure 2-2C) allows, similar to the rRRS, to study the interaction between integral membrane bait and a soluble prey. The latter is a fusion protein with the  $\gamma$ -subunit of a heterotrimeric G-protein. If the prey interacts with the membrane-located bait, it will sequester G-protein  $\beta$ -subunits, thus disrupting formation of heterotrimeric G-protein complex and subsequent downstream signaling [58]. The method has been used to identify neuronal Sec1 mutants unable to bind syntaxin1, a member of the SNARE complex [58]. Similar as with the RTA Y2H system (see above, Figure 2-2A), the authors suggest that G-protein Y2H may identify drugs disrupting protein-protein interactions. Both systems report disrupted interaction by a gain of signal which is easier to detect in a library screen as compared to a loss of signal.

The **Split-ubiquitin system** (Figure 2-2D) was designed by Johnsson and Varshavsky in 1994 [53] to allow detection of protein-protein interactions occurring between cytosolic proteins; it was later extended to membrane proteins. Ubiquitin is a small protein important for the turnover of cellular proteins. Proteins are labelled for proteasomal degradation by covalently attaching a poly-ubiquitin chain. This chain is then cleaved off prior to protein degradation by ubiquitin specific proteases (USP). The split ubiquitin Y2H technique is based on separation of ubiquitin into two independent fragments. It has been shown that ubiquitin can be split into an N-terminal (Nub) and a C-terminal half (Cub) and that these two parts retain a basic affinity for each other, thus allowing spontaneous reassembly of quasi-native ubiquitin. This spontaneous reassociation of Nub and Cub is abolished by point mutations

(I13G or I13A) in Nub (NubG, NubA) [53]. In these mutants, efficient association is only observed if the two moieties are brought into close proximity by interaction of two proteins fused to NubG/A and Cub respectively. Reconstituted split-ubiquitin is recognized by USPs, which then cleave off any reporter protein fused to the C-terminal end of Cub. The original system used dihydrofolate reductase as reporter protein, whose release was detected by SDS-PAGE [53]. However, this readout was not convenient, since it needed immunoprecipitation and electrophoretic separation.

Looking for a more direct readout, Ura3p protein has been used as reporter (Figure 2-2D: Split ubiquitin Y2H) [72]. Ura3p is an orotidine 5-phosphate decarboxylase (ODCase), an enzyme involved in the synthesis of pyrimidine ribonucleotides. ODCase activity leads to uracil auxotrophy and sensitivity to 5-fluoroorotic acid (5-FOA), because the latter is converted into the toxic compound 5-fluorouracil, causing cell death. As Y2H reporter, a variant of Ura3p is used, rUra3p, which is N-terminally modified for rapid degradation according to the N-end rule [73]. Interaction between bait and prey leads to ubiquitin reconstitution and subsequent cleavage of rUra3p, resulting in rapid degradation of rUra3p, inability to grow on minimal medium without uracil, and resistance to 5-FOA. This system is not based on a transcriptional readout and can therefore be applied to nuclear, cytoplasmic and membrane proteins [74-76].

In the membrane transactivator split-ubiquitin (MbY2H) system, an artificial transcription factor (LexA-VP16) has been used as a cleavable reporter protein to analyse interactions between membrane proteins of the endoplasmic reticulum (ER) (Figure 2-2D: MbY2H) [55]. Once ubiquitin is reassembled, LexA-VP16 is released to the nucleus, where it activates reporter gene transcription (i.e. *HIS3*, *LacZ*). Such a transcriptional readout leads to an amplification of the response following protein interactions and offers more sensitivity, more convenient for transient interactions. This system was successfully used to detect interactions involving different kinds of membrane proteins [56]. Split-ubiquitin based systems have become quite popular and have been successfully applied for cDNA library screens [77-81] and large scale matrix approaches [82].

Recently, an adaptation of the MbY2H system to screen cytosolic proteins has been published (Figure 2-2D: CytoY2H) [66]. Here, the bait construct contains both Cub and the transcription factor and is anchored to the ER membrane thanks to a fusion to the ER membrane protein Ost4p. This allows screening for interaction partners of a soluble protein among membrane and/or soluble proteins, as well as for proteins that are transcriptional activators or otherwise self-activating in nuclear Y2H.

**Other Split-protein sensors** (Figure 2-2E) have been developed, inspired by the split-ubiquitin system. While the cytosolic Y2H methods presented above are based on indirect readout that requires activation of signaling pathways or transcription, split-protein sensors can in principle also directly report their reconstitution. In 2004, Tafelmeyer *et al.* presented a combinatorial approach to generate split-protein sensors [65]. They used an enzyme in yeast tryptophan biosynthesis, N-(5-phosphoribosyl)-anthranilate isomerase (Trp1p), to perform activity selections of different combinations of fragment pairs. They identified C-terminal (CTrp) and N-terminal (NTrp) fragments

which reconstitute a quasi-native Trp1p only when fused to two interacting proteins that bring the CTrp and NTrp domains into close proximity. Thus, interacting fragments lead to Trp1p reconstitution and allow *trp1* deficient yeast strains to grow on medium lacking tryptophan (Figure 2-2E: SplitTrpY2H). This system has several advantages. The readout is direct and permutation-independent, i.e. independent of whether CTrp or NTrp were used for bait constructs. It is universally applicable to all types of proteins, because the interaction readout is entirely independent of cellular localization.

Recently, split enhanced green fluorescent protein has been used to monitor protein-protein interactions in yeast by confocal microscopy [53]. A variety of other split-protein sensors has been applied in eukaryotic cells (e.g. dihydrofolate reductase [54],  $\beta$ -galactosidase [55],  $\beta$ -lactamase [56]), but has not yet been used in Y2H screening.

### 3.2.3. Y2H with extracellular and transmembrane proteins

All Y2H systems presented so far detect interactions in the reducing intracellular environment, which is not necessarily ideal for extracellular proteins. However, interactions in the extracellular space, like between receptors and ligands or between antibodies and antigens, participate in a multitude of physiological processes, and their study is of particular interest for a better understanding of numerous pathologies.

The **SCINEX-P (screening for interactions between extracellular proteins) system** (Figure 2-2F) published by Urech *et al.* in 2003 allows the analysis of protein-protein interactions in the oxidizing environment of the ER [64]. This system exploits the signaling of the yeast unfolded protein response (UPR). Accumulation of incorrectly folded proteins in the ER induces dimerization of the yeast ER type I transmembrane protein (Ire1p), which induces production of transcriptional activator Hac1p that will activate transcription of chaperons. In the SCINEX-P system, proteins of interest are fused to mutated Ire1p proteins, one lacking its luminal, N-terminal oligomerization domain ( $\Delta$ Ire1p). The interaction between two hybrid proteins then reconstitutes Ire1p dimerization and thus activates UPR downstream signaling. To monitor protein interactions, the Hac1p UPR element is introduced into the promoter of reporter genes. This Y2H system was successfully used to analyze the interaction between the protein disulfide isomerase ERp57 and Calnexin, both involved in protein folding in the ER [83], as well as known interactions between antigens and antibodies [64].

### 3.3. Dealing with doubt: Limitations of Y2H systems and methods for its validation

Its relative methodical simplicity, its diversity, and its high-throughput capacity make the Y2H system the most popular analytic and screening method for interactomics. Nevertheless, all Y2H methods face the problem of false negatives and false positives.

**False negatives** in Y2H are protein-protein interactions which cannot be detected due to limitations of the screening method. In the classic Y2H, for example, protein interactions involving membrane



proteins are mostly undetectable. Thus, the Y2H strategy has to be chosen carefully, depending on the cellular sub-proteome of interest. Further, the interaction between the two proteins assayed in Y2H is often not symmetric (permutation-independent), meaning it depends on whether a given protein is used for fusion in the bait or the prey construct. The fused yeast reporter proteins or anchors may cause steric hindrance that impedes interaction, thus causing false negatives. Another reason for false negatives can be different or lacking post-translational protein modifications in the yeast system when analyzing interactions between proteins of higher eukaryotes. In this case, the modifying enzyme may be coexpressed in yeast together with bait and prey. This possibility has been used with success to identify tyrosine-phosphorylation dependent interactions [84]. Very transient interactions may also escape detection, as e.g. in case of substrate interactions of protein tyrosine phosphatase. Here, substrate-trap mutants have been used lacking phosphatase activity but retaining their affinity for the substrates to identify protein substrates of the phosphatase [85]. The expression of baits fused to their cognate modifying enzyme has been successfully used to identify acetylation dependent interactions with histones and interactions dependent on phosphorylation of the carboxy-terminal domain of RNA polymerase II [86]. The lack of more complex modifications, like complex glycosylation, appears to be more difficult to overcome. A humanized yeast strain has already been used to produce human glycosylated proteins in yeast *Pichia pastoris* [87], but it has so far not been used in Y2H.

False negatives mainly cause problems in reproducibility of Y2H screens. Two independent large-scale Y2H screens using the same Y2H method showed less than 30% overlap in the identified interactions and only 12,5% of known interactions were found in each of both [19]. These discrepancies may arise from a difference in selection stringency or a difference in the cDNA library used. Thus, false negatives represent a real limitation of the Y2H system in representing an entire protein interaction network. However, each screening system has to deal with false negatives. For example, MS of purified protein complexes reveals only few interactions involving transmembrane proteins due to their difficult purification [88]. AP/MS was also shown to be biased towards highly abundant proteins, whereas protein abundance appears not to influence Y2H [88]. While purification of protein complexes has to deal with mixtures of proteins showing very different abundance, depending on the used cell type, such differences are avoided in Y2H by overexpression of interacting proteins at similar levels. However, protein overexpression can provoke other artefacts such as false positives.

**False positives** in Y2H are physical interactions detected in the screening in yeast which are not reproducible in an independent system. They are of diverse origin and often depend on the Y2H system used. Among possible reasons for false positive interactions in yeast may be a high expression level of bait and prey and their localization in a compartment which does not correspond to their natural cellular environment. Another source of false positives is interaction of prey with the reporter proteins (e.g. LexA in the classic Y2H) or the membrane anchors (e.g. Ost4p in the cytoY2H) fused to the bait. Proteins which allow yeast to overcome nutritional selection when overexpressed are also

often scored as false positives. Finally, proteins that are known to be “sticky” or that are not correctly folded can show unspecific interactions. In general, for each Y2H system, a list of recurrent false positives can be established. A list created by Golemis and co-workers for the classic Y2H can be found at <http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>.

**Table 2-2.** Overview of different validation methods.

Method	Type	Description
Pull-down assay [89-91]	<i>in vitro</i>	Tagged bait (mostly expressed in <i>E. coli</i> ) is immobilized on a resin and subsequently “pulls down” target protein (prey) from lysates (of eukaryotic cells or of <i>E. coli</i> expressing proteins of interest). After washing steps, prey is detected by SDS-PAGE/immunoblot or MS.
Coimmunoprecipitation [80,89,90,92]	<i>ex vivo</i>	A specific antibody is used to precipitate the bait from cell lysates (see above). After washing steps, coimmunoprecipitated prey is detected as above.
Surface plasmon resonance (Biacore) [93]	<i>in vitro</i>	Bait immobilized on the surface of a sensor chip is probed by injection of prey onto the surface. Protein interaction is detected online via a biophysical principle (using the change in refractive index at the sensor surface in case of protein interaction). Protein is eluted and analyzed by MS.
<i>In situ</i> hybridization [90]	<i>in situ</i>	Hybridization of a labelled complementary DNA or RNA strand (i.e. probe) to a specific DNA or RNA sequence in a tissue section. Visualizes expression of specific genes to evaluate potential coexpression of proteins of interest in the same cell of a given tissue.
Immunohistochemistry, immunocytochemistry [80,89,90]	<i>in situ</i>	Proteins in fixed cells or tissue sections are detected by immune-labelling with fluorescently tagged antibodies, e.g. using confocal microscopy. Visualizes coexpression of proteins of interest in the same cell and potential subcellular colocalization.
Fluorescent detection in live cells [91]	<i>in vivo</i>	Proteins in living cells are detected with fluorescently tagged antibodies as above (using permeabilized cells) or after expression of fluorescently tagged protein variants. Visualizes colocalization of proteins of interest.
Fluorescence resonance energy transfer (FRET) [80]	<i>in vivo</i>	Bait and prey are fused to two different fluorescent tags with overlapping emission/excitation spectra. If both proteins are in close proximity, excitation of the first fluorophore (donor) leads to energy transfer to the second fluorophore (acceptor). Acceptor fluorescence can be observed <i>in vitro</i> (fluorimeter) or in living cells (confocal microscopy).
Bioluminescence resonance energy transfer (BRET) [92]	<i>in vivo</i>	Similar to FRET (see above), but with bait fused to bioluminescent luciferase, thus avoiding the external excitation step susceptible to generate background. Detection as with FRET.

Despite these limitations, the Y2H system remains a powerful tool for large-scale screening in interactomics. The comparative assessment of high-throughput screening methods by von Mehring *et al.* [88] revealed that Y2H has a lower coverage of the protein interaction network than the purification of protein complexes coupled to MS. But these authors only considered the classic Y2H, while the above presented diversity of Y2H systems may increase coverage considerably.

To evaluate the quality of a generated interaction data set, coverage and accuracy need to be considered together. In fact, a large interaction network cannot be a solid base for systems biology if confidence in the data is low. In a quantitative comparison of interaction data sets, von Mehring estimated the accuracy of a classic high-throughput Y2H screen to be less than 10%. Thus, the question remains how to increase accuracy of Y2H interaction data sets.

As mentioned before, there are two different screening approaches: the targeted library screening approach and the global matrix screening approach. To increase accuracy of a library screen, a bait-dependency test can be performed [66,94]. In this case, the previously identified preys are tested for interaction with unrelated baits. Preys interacting with others than the screening bait will be classified as false positives. This test helps to eliminate false positives resulting from non-specific interactions with the bait or other “sticky” interactions overcoming nutritional selection, but it cannot eliminate physical interactions, artificially occurring in the Y2H system without physiological meaning. For this reason, binary interactions detected in Y2H are nowadays published only if they are validated by other methods [80,89-91,93]. Different validation methods that can be used are listed in Table 2-2.

It is advisable to use more than one method to validate an identified protein-protein interaction, preferentially coupling biochemical methods (pull down assay, immunoprecipitation, Biacore surface plasmon resonance) with *in vivo/in situ* methods (colocalization, immunohistochemistry, *in situ* hybridization). The former methods allow the study of physical protein interactions, but pull-down assays require a certain stability of the protein complex or, in case of Biacore, even need purified interaction partners. It may be also difficult to validate transient protein interactions or protein interactions with transmembrane proteins in these assays. The *in vivo/in situ* methods allow insight into possible coexpression and colocalization of the two proteins involved, but generally do not provide conclusive evidence for direct interaction. However, an advantage of *in situ* hybridization would be its adaptability for high-throughput. The FRET method has been developed to go beyond protein colocalization *in vivo* to study the spatio-temporal occurrence of the interaction and its physiological significance. FRET can only occur when the distance separating the two different fluorophores is in the low nm-range, a condition that occurs if fluorophores are coupled to two directly interacting proteins [95]. However, many of these methods are relatively labor intensive and can only be applied to a small number of interactions detected in a larger screen.

Validation of results from high-throughput matrix studies is much more difficult to achieve. Using the mentioned validation methods would be experimentally extremely difficult. Given that both interaction partners are randomly selected, the large amount of generated interaction data would already render a bait dependency test impossible. To handle the problem of false positives in such large-scale approaches, help is coming from computational biology. Confidence scores can evaluate the biological significance and probability of a given interaction. One possibility is to relate screening results to known data like RNA expression levels (expression profile reliability (EPR) index), or interaction networks of protein paralogues (paralogous verification method (PVM)) [96]. Another score was calculated by combining data on sequence homology, known interacting Pfam domains and Gene Ontology annotations [97]. Even if these methods allow creation of higher confidence scores, they are limited by the number of existing data from other screens and experiments. Another possibility is thus the creation of a statistical model only based on screen data and topological criteria

[98]. These scores will not replace experimental validation of detected interactions, but may provide a tool to select proteins for further experiments.

#### 4. FURTHER CONFIRMATION: PPIs WITHIN A BIOLOGICAL SYSTEM

Once a protein-protein interaction has been identified and validated, the physiological function of a given interaction remains to be established in a biological system. The main questions in this respect are: (i) Where and when in the system the interaction does occur? (ii) Which parameters influence the interaction? (iii) What is the effect of the interaction? To answer these questions, the main strategy relies on varying different system parameters that mainly affects the proteins of interest. Combination of a panel of complementary methods is generally able to unveil the physiological significance of an interaction identified in a targeted approach.

Colocalization experiments in cell culture under different conditions can give information about the spatiotemporal dynamics of the protein-protein interactions. For example, choosing different time points during the cell-cycle may reveal transient colocalizations. In the case of the reported interaction between brain type creatine kinase (BCK) and the cis-Golgi matrix protein (GM130), a transient colocalization during early prophase was observed [91]. The authors suggest that BCK would facilitate GM130 phosphorylation by ATP-requiring protein kinases and thus play a role in initial fragmentation of the Golgi apparatus prior to cell division. Many other endogenous or external parameters influencing protein-protein interaction can be varied, including activation of signaling cascades or changes in the cellular environment. To analyse the impact of given protein-protein interactions on the cellular phenotype, the interaction may be either disturbed, e.g. by RNA silencing of one interaction partner, or favoured by addition or overexpression of one protein partner. More specifically, the interaction domains of both interaction partners can be mapped to inhibit the interaction *in vivo* by expressing interaction-deficient mutant proteins or using inhibitory peptides.

These experiments can be carried out for defined interactions of a small number of proteins, but again it would be quite difficult to transfer them to the large interaction network generated by global screens. So far, interactome approaches concentrate on a characterization of the nodes in the interaction network, which may be the major determinants of a phenotype.

#### 5. CONCLUSIONS

Since systems biology aims at a complete representation of cellular complexity, thus avoiding any reductionism, the applied experimental strategies have to provide non-biased, complete data sets. In this context, the yeast two-hybrid technologies presented here are a starting point rather than a complete solution to the elucidation of interaction networks. However, Y2H has demonstrated its power by its methodological diversity and technical simplicity to rapidly generate a large amount of

reliable protein-protein interaction data. More recent Y2H technologies, in particular those based on split proteins, allow to probe protein-protein interactions in their native cellular compartment and to access almost the entire cellular proteome. Y2H is rather complementary in respect to emerging AP/MS techniques, since it identifies direct interactions and also detects interaction of lower affinity that are rather transient.

Developing high throughput approaches at the cellular level and further progress in bioinformatics will be necessary to make interactomics a fully integral part of a systems biology approach. Major efforts will be necessary for the challenge of modelling the large and dynamic interaction network of a cell. Only a combination of different approaches (e.g. Y2H, MS, bioinformatics) will eventually lead to an accurate description of large interaction networks.

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# PART II



## ANALYSIS OF THE AMPK INTERACTION NETWORK BY COMBINED INTERACTOMIC APPROACHES

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### CHAPTER III :

*AMPK substrates and interaction partners – an overview*

### CHAPTER IV :

*A cytosolic Y2H approach reveals physical association  
between AMPK and intracellular Vesicle Transport*

### CHAPTER V :

*A two-dimensional screening strategy reveals  
novel isoform-specific substrates of AMP-activated protein kinase*

### CHAPTER VI :

*High affinity interaction of AMP-activated protein kinase  
with glutathione transferases Mu and Pi  
leads to their phosphorylation and Pi-1 enzymatic activation*

### CHAPTER VII :

*Discussion*

## AIM OF PART II

AMPK is an emerging signaling hub in detecting and responding to cellular energy stress. It mediates insulin-independent metabolic effects like those of exercise, and is also involved in regulating cell growth, proliferation and morphology. Since the anti-diabetic drug metformin was shown to activate AMPK and to reduce cancer risk of patients with type 2 diabetes, interest into AMPK signaling has further increased. Major efforts are focused on identification of new AMPK targets and interacting proteins. The application of high-throughput screening procedures revealed many new putative AMPK targets and interacting proteins.

The aim of Chapter III was to give an overview on putative AMPK targets and interacting proteins reported in literature and their distribution within different cellular compartments and biological functions.

The aim of the following experimental work was to further explore the AMPK interaction and signaling network. In contrast to published large-scale high throughput screens, multiple assays have been combined to obtain more reliable data. Two different strategies have been adopted:

(i) The first strategy was aiming at the identification of new AMPK interaction partners, substrates or regulators, by using Y2H screening. This is a powerful technique for detecting binary protein-protein interactions (Chapter IV).

(ii) The second strategy combined a biophysical interaction approach (surface plasmon resonance) with an *in vitro* phosphorylation assay to identify new AMPK substrates based on the interaction with specific AMPK isoforms (Chapter V).

Finally, an interaction partner that emerged during these studies, glutathione S-transferase, was selected for a more detailed characterization (Chapter VI). This included its *in vitro* phosphorylation and the consequences of interaction and phosphorylation for the function of both interaction partners.

The last Chapter VII discusses all identified protein-protein interactions in a more general context and focuses on putative implications for AMPK signaling.



## - CHAPTER III -

### AMPK SUBSTRATES AND INTERACTION PARTNERS – AN OVERVIEW

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**Abstract:** AMPK is a central signaling hub detecting and responding to cellular energy stress. It mediates exercise-dependent metabolic effects and is also involved in controlling cell growth, proliferation and morphology. Since the anti-diabetic drug metformin was shown to activate AMPK and in addition to reduce cancer risk of patients with type 2 diabetes, interest into AMPK signaling has further increased. While identification of new AMPK substrates/targets has attracted major research efforts, less has been done so far concerning AMPK interacting proteins. However, recent high-throughput screening procedures have proposed many new putative AMPK targets and interacting proteins. Here we give an overview on putative AMPK targets and interacting proteins reported in literature and public databases, and their distribution within different cellular compartments and biological functions. Some AMPK interacting proteins that were confirmed by multiple, independent methods are presented in more detail.

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#### 1. INTRODUCTION

Control of global cellular and body energy status is crucial to avoid that energy demands exceed energy generation. One of the key regulators of cellular metabolism is AMP-activated protein kinase (AMPK), a heterotrimeric serine/threonine kinase that evolved to sense AMP levels as an intracellular “second messenger” of cellular energy and nutrient status.

In response to energy depletion, AMPK is allosterically activated by a rise in intracellular AMP/ATP ratio and in addition by covalent activation *via* phosphorylation by the upstream kinases LKB1, CaMKK $\beta$  and probably also TAK1 (see Chapter I or recent reviews (Hardie 2007, Steinberg et al. 2009) for more detail). This activation leads to stimulation of ATP-producing (catabolic) and inhibition of ATP-consuming (anabolic) pathways. Activated AMPK then works either through phosphorylation of metabolic enzymes as an acute stress response or through phosphorylation of enzymes involved in gene transcription and protein synthesis as a long-term effect.

Since the discovery of the first AMPK targets, acetyl-CoA carboxylase (ACC) and hydroxymethyl-CoA (HMG-CoA) reductase, two enzymes involved in lipid metabolism (Carling et al. 1987), major efforts have been focused to identify additional substrates. Many more such AMPK targets have been reported since then, revealing a role of AMPK that exceeds simple regulation of energy metabolism (i.e. carbohydrate and lipid metabolism), e.g. with targets involved in protein synthesis, cell cycle regulation and cell proliferation. This has put AMPK signaling at the center stage of numerous pathologies such as cancer, type II diabetes and the metabolic syndrome (reviewed in (Viollet et al.

2007, Hardie 2008, Steinberg et al. 2009, Viollet et al. 2009)), and has made the kinase an attractive target for pharmacological intervention. However, the broad spectrum of AMPK downstream signaling makes it a difficult task to identify highly specific drug compounds, which will be able to activate or inhibit only a subset of pathways without affecting others. An approach to selectively manipulate specific pathways could be the selective inhibition of protein-protein interactions (PPI) between AMPK and its downstream target. PPIs are in fact the prerequisite for cell signaling. In addition to AMPK interactions with its substrate, there may be also formation of higher order complexes via scaffolding proteins. However, out of the AMPK PPIs reported in literature that were confirmed by at least two different interaction assays, only two correspond to non-substrates (Qi et al. 2008, Song et al. 2010) and seven to AMPK substrates (Annex 3).

The aim of this study was to collect and analyze all data available on putative AMPK substrates and interacting proteins, whether they were obtained by exhaustive individual studies or by large-scale phosphoscreening/interactomic approaches. Although the quality of these data is very heterogeneous and thus the level of confidence into these data is variable, an overall statistical analysis may yield interesting insight into the AMPK signaling network. Thus, identified proteins are not discussed individually with only some exceptions, but overall statistics are given on their cellular localization and physiological function.

## 2. METHODS

### 2.1. *In silico search for AMPK substrates*

AMPK substrates were identified by searching the MEDLINE database (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda MD, USA, <http://www.ncbi.nlm.nih.gov/pubmed/>). Putative AMPK substrates identified by large-scale phosphoscreening approaches are exclusively from two studies (Tuerk et al. 2007, Thali et al. 2010). A complete list of putative substrates can be found in Annex 2, including PubMed ID (PMID) referring to the original publication in MEDLINE and the SwissProt ID referring to the entry in the UniProt Knowledgebase (UniProtKB, <http://www.uniprot.org/>).

### 2.2. *In silico search for AMPK interacting proteins*

AMPK interacting proteins were identified by searching the MEDLINE database and the following protein-interaction databases: IntAct (<http://www.ebi.ac.uk/intact>) (Aranda et al. 2010), Mint [t (<http://mint.bio.uniroma2.it/mint>) (Ceol et al. 2010), BioGRID (<http://www.thebiogrid.org>) (Breitkreutz et al. 2008) and HPRD (<http://www.hprd.org/>) (Keshava Prasad et al. 2009). A complete list of putative AMPK interacting proteins can be found in Annex 3, including the PubMed ID (PMID)



referring to the original publication in MEDLINE and the SwissProt ID referring to the entry in the UniProt Knowledgebase.

### 2.3. Data analysis

No further selection or filtering was performed on the obtained protein lists, except the elimination of AMPK subunits in the list of interacting proteins. AMPK substrates and interacting proteins were classified in different categories according to their major subcellular localization and physiological function reported in literature and/or the UniProtKB.

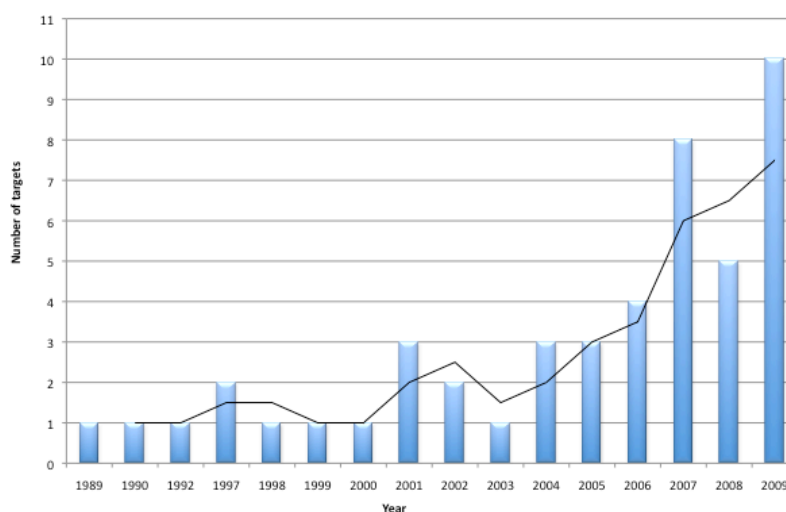
## 3. RESULTS

### 3.1. Experimental approaches to AMPK substrates and interacting proteins

Since the discovery of AMPK in 1987 (Carling et al. 1987) major efforts were concentrated to elucidate its signaling network, mainly by the identification of AMPK downstream targets. Since 1989, 47 substrates have been described in targeted studies (Fig. 3-1) with an important increase during recent years. In addition, two recent large-scale non-biased *in vitro* phosphoscreens have identified additional 31 putative AMPK substrates (Tuerk et al. 2007, Thali et al. 2010), i.e. 2 of 5 putative substrates were identified by these large *in vitro* approaches (Fig. 3-2A). Further investigations have to show whether these are *bona fide in vivo* substrates of AMPK, and further description of AMPK substrates in this analysis rather refers to the notion of “putative” substrates.

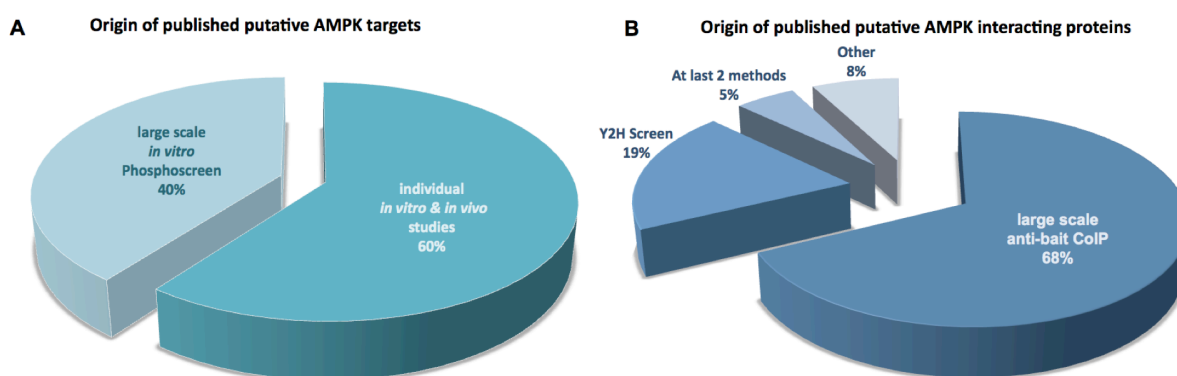
Only a minority (14 %) of referred AMPK substrates was also reported to interact directly with AMPK. However, many interactions may escape detection due to their low affinity, corresponding rather to “kiss and run” type of kinase-substrate recognition. Substrate recognition may also be rather indirect, involving higher order signaling complexes, although clear evidence for such complexes is missing so far.

**FIGURE 3-1. Number of AMPK substrates reported by targeted studies.** During the last 20 years, 47 AMPK substrates were identified by targeted studies mostly providing extensive data on *in vitro* and/or *in vivo* phosphorylation



Due to the massive application of high throughput screenings, much more putative AMPK interacting proteins than AMPK substrates are reported in literature (Annex 3). However, these data have to be considered with caution. Only 5 % of published AMPK-protein interactions have been detected by at least two complementary methods, generally considered as necessary to reach sufficient confidence in a given interaction. Almost 70 % of all reported interactions come from a single large-scale non-biased immunoprecipitation study using Flag-tagged AMPK  $\beta$ 1-subunit, followed by LC-ESI-MS/MS analysis (Ewing et al. 2007)(Fig. 3-2B). The putative interactors resulting from this study were neither inspected with a second method nor do they include published interaction partners or targets except other AMPK-subunits. As for AMPK substrates from non-biased studies, it is currently difficult to estimate the rate of false-positive identifications. Moreover, in principle, co-immunoprecipitation experiments do not allow discrimination between binary AMPK-protein interaction and formation of higher order protein complexes. Thus, many of the identified proteins listed in this analysis may not directly but rather indirectly bind to AMPK.

Only 5 % of putative AMPK interacting proteins are known AMPK targets. Even if one assumes that AMPK phosphorylates also other isoforms of known substrates (e.g. 6-phosphofructokinase type C, importin-9 and ubiquitin carboxyl-terminal hydrolase 5) this number does not exceed 7%. Thus, possibly further AMPK substrates may be found in the AMPK interactome.

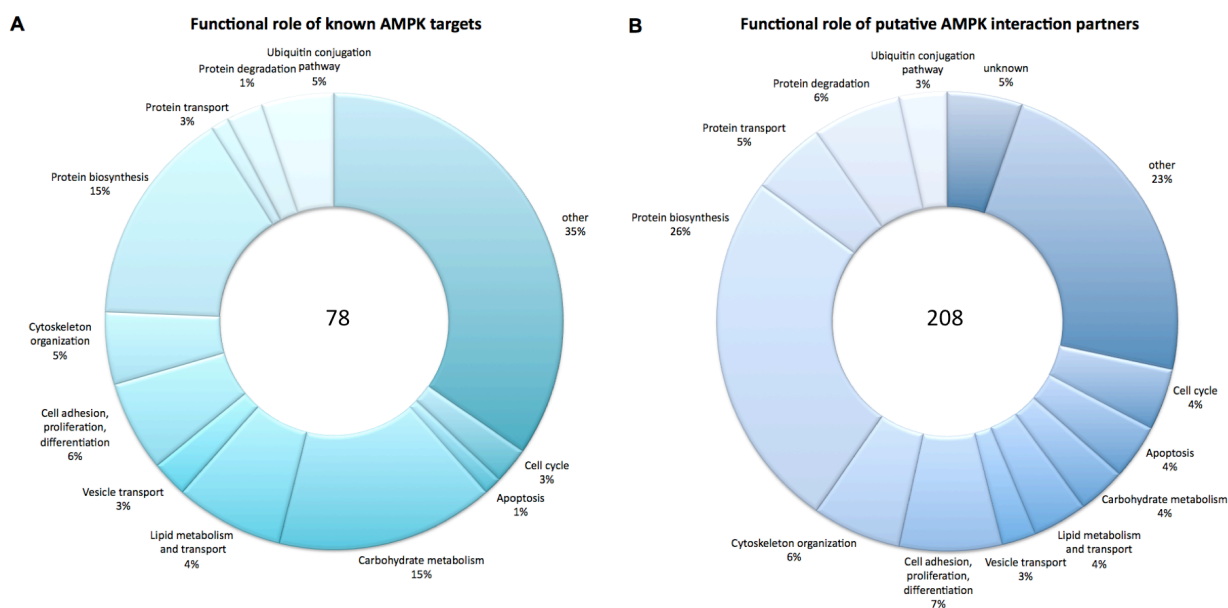


**FIGURE 3-2. Methods applied to identify AMPK targets/substrates or AMPK interaction partners.** (A) Putative AMPK targets/substrates. 40% were identified by only two large-scale *in vitro* studies (Tuerk et al. 2007, Thali et al. 2010). (B) Putative AMPK interaction partners. 68% were identified a single co-immunoprecipitation study (Ewing et al. 2007). Only 5% of interaction partners have been identified by more than one method.

### 3.2. Physiological role and subcellular localization of AMPK substrates and interactors

The multiple roles of AMPK signaling are reflected by the distribution of putative AMPK substrates and interacting proteins within functional groups (Fig. 3-3). Overall, the referred putative interactors and substrates both involve AMPK in the same cellular processes, albeit to a different degree. AMPK interactors in carbohydrate and lipid metabolism represent only 8 % of total

interactions, while they involve 19 % of published substrates. In contrast, only 19 % of the targets are involved in protein metabolism (i.e. biosynthesis, transport, degradation) as compared to 37 % of AMPK interactors (Fig. 3-3). This suggests that AMPK's role in protein synthesis and cell growth, which are processes of high energy demand, might be even more important than its role in direct regulation of energy metabolism.



**FIGURE 3-3. Cellular processes involving AMPK targets/substrates or interaction partners.** (A) Distribution of 77 putative AMPK targets/substrates to different cellular processes (as percentage of total targets). (B) Distribution of 207 putative AMPK interacting proteins to different cellular processes (as percentage of total interactors). Some details on the included proteins can be found in Annexes 2 and 3.

The subcellular localization of both, AMPK substrates and interactors is quite similar (Fig. 3-4). The majority is predicted to be located in the cytosol or in the nucleus, which is in accordance with the known AMPK cellular localization (see Chapter I). A small moiety associates AMPK to the plasma membrane or other subcellular structures like ER, Golgi apparatus, mitochondria, proteasome or transport vesicles. Certainly, these localizations deserve further analysis. Finally, putative interactions with 5 different ribosomal and ribosome-associated proteins suggest a direct link of AMPK with translation.

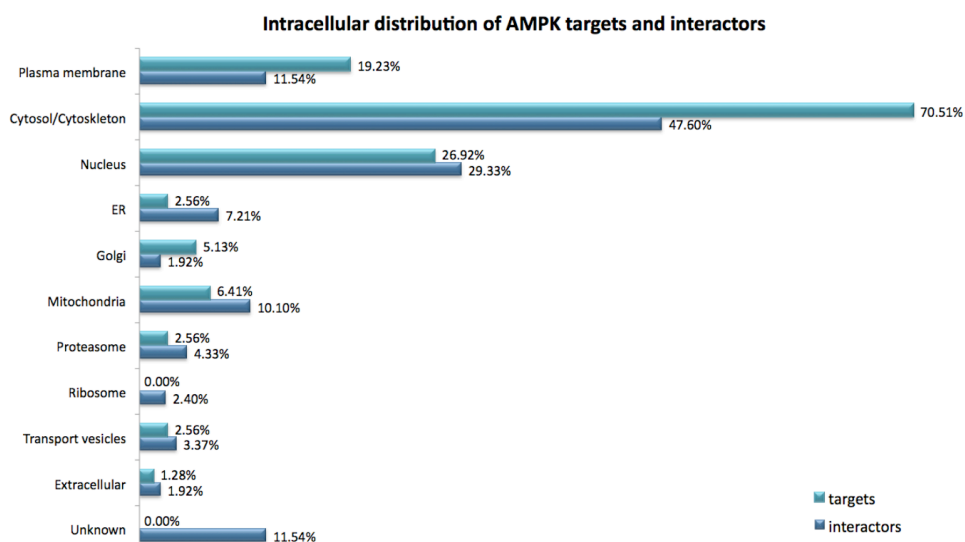


FIGURE 3-4. **Intracellular localization of AMPK targets and interacting proteins.** The vast majority of AMPK targets (green) and interacting proteins (blue) display cytoplasmic and nuclear distribution. Some are located at the plasma membrane or at mitochondria and a minority is associated with other subcellular structures. Note that some proteins can display more than one location, thus leading to a total percentage exceeding 100%.

### 3.3. A closer look on AMPK interacting proteins

Different AMPK subunit-isoforms coexist in a single cell and all the resulting heterotrimeric AMPK combinations are regulated in the same manner by upstream kinases and phosphatases. Although AMPK- $\alpha$ 1 and - $\alpha$ 2 may slightly differ in their preferential target phosphorylation consensus site (Woods et al. 1996), AMPK isoform-specific targets have not been reported so far. A single AMPK isoform specific interaction has been reported in a recent study. Secreted protein acidic and rich in cysteine (SPARC) interacted in a Y2H assay only with AMPK- $\alpha$ 1 but not with AMPK- $\alpha$ 2 (Song et al. 2010). Thus, interactions may play a role in AMPK isoform-specific signaling at least in a subset of pathways.

Only ten reported AMPK-protein interactions were confirmed by at least two different methods and thus reach a confidence level that warrants further discussion (Table 3-1). Eight out of these ten interacting proteins were also shown to be phosphorylated by AMPK and are thus likely kinase substrates. These include four transmembrane proteins that otherwise are rather scarce among AMPK targets: channels or transporters like the cystic fibrosis transmembrane conductance regulator (CFTR) (Hallows et al. 2000), a voltage-independent potassium channel (Klein et al. 2009) and an Na-K/Cl symporter (Fraser et al. 2007), as well as a subunit of the plasma membrane GABA receptor (Kuramoto et al. 2007). From the soluble proteins, the thyroid receptor-interacting protein 6 relays signals from the cell surface to the nucleus to weaken adherent junction and promote actin cytoskeleton reorganization and cell invasiveness (Solaz-Fuster et al. 2006). Three further cytosolic proteins are linked to the ubiquitin-proteasome pathway: in addition to a subunit of the proteasome (Moreno et al. 2009), these are the dual specificity protein phosphatase laforin and the glycogen

targeting subunit R5/PTG of protein phosphatase 1 (PP1). AMPK interaction with and phosphorylation of laforin enhances its interaction with malin, an E3 ubiquitin ligase, leading to R5/PTG degradation (Solaz-Fuster et al. 2008). In turn, lowering R5/PTG-PP1 activity reduces dephosphorylation of glycogen synthase and glycogen phosphorylase, resulting in reduced synthesis and increased degradation of cellular glycogen. This process is reinforced by additional AMPK interaction with and phosphorylation of R5/PTG, which accelerates laforin-malin-dependent degradation of this protein (Vernia et al. 2009).

Two further interactions involving SPARC and CIDE-A do not involve target phosphorylation but were reported to affect rather AMPK itself. Increased SPARC expression led to increased AMPK activation, which in turn, stimulated SPARC expression (Song et al. 2010). CIDE-A was shown to interact with AMPK- $\beta$  and to regulate its ubiquitination and degradation (Qi et al. 2008). However, the exact mechanism and role of these regulations is still unknown. Taken together, convincing data on AMPK interaction partners are still scarce.

TABLE 3-1. **Published AMPK interactors detected by at least two independent interaction assays**

<i>Protein</i>	<i>SwissProt Entry</i>	<i>AMPK target</i>	<i>Interaction</i>	<i>Interaction methods used</i>	<i>Reference</i>
Cell death activator CIDE-A	O70302	No	Direct with $\beta$	Co-IP, Colocalization	(Qi et al. 2008)
Cystic fibrosis transmembrane conductance regulator	P13569	Yes	Direct with $\alpha$	Co-IP, Y2H	(Hallows et al. 2000)
GABA type B receptor subunit 1	Q9Z0U4	Yes	Direct with $\alpha$	Co-IP, Colocalization, Y2H	(Kuramoto et al. 2007)
Intermediate conductance calcium-activated potassium channel protein 4	O15554	Yes	Direct with $\gamma(1)$	Co-IP, Colocalization	(Klein et al. 2009)
Kidney-specific Na-K-Cl symporter (NKCC2)	Q13621	Yes	?	Co-IP, Colocalization	(Fraser et al. 2007)
Laforin	O95278	Yes	Direct with $\alpha(2)$ & $\beta(2)$	Co-IP, Y2H	(Solaz-Fuster et al. 2008)
Proteasome 26S non-ATPase subunit 11	O00231	Yes	Direct with $\beta(2)$	Co-IP, Y2H	(Moreno et al. 2009)
R5/PTG	O95278	Yes	Direct with $\alpha(2)$ & $\beta(2)$	Co-IP, Y2H	(Vernia et al. 2009)
SPARC	P09486	No	Direct with $\alpha1$	Co-IP, Y2H, Co-pull down	(Song et al. 2010)
Thyroid receptor-interacting protein 6 (TRIP6)	Q15654	Yes	Direct with $\alpha(2)$	Co-IP, Y2H	(Solaz-Fuster et al. 2006)

Where determined, the interacting AMPK subunit is mentioned. Isoforms are noted in parenthesis, if interaction with the other isoform was not excluded. A question mark indicates that an indirect interaction with AMPK cannot be excluded.

#### 4. CONCLUSIONS

Based on the available data summarized here, although individually many of them require further confirmation, global statistics can still give interesting insight into AMPK signaling, e.g. on intracellular distribution and relative importance of different metabolic pathways. In summary, 78 putative AMPK substrates have been reported in literature, with 31 of them resulting from large-scale phosphoscreen approaches, thus requiring further confirmation. At least 208 putative AMPK/protein

interactions, direct or indirect, have been reported in literature and interaction databases. However, convincing data is available only for a minority of these interactions. Still, these numbers may reflect the existence of a large number of AMPK interaction partners, warranting further investigation in this field. Since the number of such putative interaction partners exceeds by far the number of known targets, many more AMPK substrates involved in further physiological processes may await their full description.

AMPK substrates have also to be analyzed in the context of AMPK interaction, which may help to extend the AMPK signaling network and to assist in the development of new drugs. Indeed, it might be possible, that the different AMPK isoforms can also function in higher protein complexes together with scaffolding proteins, targets and signal terminators as it has been described for protein kinase A (PKA) (Beene et al. 2007). PKA forms signaling complexes together with multiple PKA-binding A-kinase anchoring proteins (AKAPs), PKA substrates and signal terminators such as phosphatases.

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**- CHAPTER IV -****A CYTOSOLIC Y2H APPROACH REVEALS PHYSICAL ASSOCIATION  
BETWEEN AMPK AND INTRACELLULAR VESICLE TRANSPORT**

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**Abstract:** AMPK is a heterotrimeric Ser/Thr protein kinase, composed of the catalytic  $\alpha$  and the two regulatory  $\beta$  and  $\gamma$  subunits. The  $\alpha$ -subunit contains the catalytic domain bearing Thr172 whose phosphorylation is required for AMPK activity. The regulatory  $\gamma$ -subunit is crucial for allosteric AMPK activation as it contains the AMP binding sites. The  $\beta$ -subunit seems to be a scaffold for the other two subunits ( $\alpha$  and  $\gamma$ ). In addition, the latter contains a glycogen-binding domain and an N-terminal domain proposed to be involved in subcellular localization. In yeast and plants,  $\beta$ -type subunits of AMPK orthologues SNF1 and SnRK1 were proposed to regulate both, the localization of the kinase complexes within the cell and the interaction of the kinases with their targets. This chapter presents novel, putatively AMPK-interacting proteins identified by a genome-wide screen using cytosolic Y2H assays with the N-terminal AMPK  $\beta$ -domain as bait. One of the identified interacting proteins is member of the vesicle-associated membrane protein (VAMP) family, VAMP3. VAMPs are required for endo- and exocytosis. Two VAMP members, VAMP2 and VAMP3 interacted with AMPK  $\beta$ -subunits in yeast. VAMP2 also interacted with native, heterotrimeric AMPK as shown by co-immunoprecipitation from rat brain synaptic vesicles. Neither VAMP2 nor VAMP3 were phosphorylated by AMPK. However, preliminary results indicate that AMPK activation reduces neurotransmitter release, suggesting that VAMPs recruit AMPK to vesicles for regulation of endo- and exocytosis.

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**1. INTRODUCTION**

AMPK is a heterotrimeric Ser/Thr protein kinase, composed of the catalytic  $\alpha$  and the two regulatory  $\beta$  and  $\gamma$  subunits. Different studies using truncated and/or mutated AMPK subunits revealed specific functions for each of them (Hudson et al. 2003, Iseli et al. 2005, Oakhill et al. 2009). The  $\alpha$ -subunit contains the catalytic domain bearing Thr172 whose phosphorylation is required for AMPK activity. The regulatory  $\gamma$ -subunit is indispensable for binding of AMP, an important allosteric activator of AMPK. The  $\beta$ -subunit includes  $\alpha$ - and  $\gamma$ -subunit binding sites at its C-terminal domain. In addition, this subunit contains a glycogen-binding domain and an uncharacterized N-terminal extension.

At least the  $\beta$ 1-subunit is posttranslationally modified by myristoylation (Warden et al. 2001). Removal of the myristoylation site by mutation of glycine 2 to alanine led to nuclear redistribution of AMPK and increased AMPK activity (Warden et al. 2001). In addition to the myristoylation site, the  $\beta$ -subunits contain multiple N-terminal phosphorylation sites of unknown function (Warden et al. 2001). In yeast and plants it has been proposed that the  $\beta$ -type subunits of corresponding AMPK

isoenzymes (SNF1 and SnRK1) regulate both the localization of the kinase complexes within the cell and the interaction of the kinases with their targets (Polge et al. 2008). In case of mammalian AMPK, the  $\beta$ -subunit can apparently localize the kinase to glycogen bodies and membranes (Bendayan et al. 2009). However, no scaffolding proteins analogous to PKA-binding A-kinase anchoring proteins (AKAPs; reviewed by (Beene et al. 2007)) have been described that could recruit AMPK into signaling complexes with specific subcellular localization. Multiple AKAP scaffold proteins form signaling complexes with PKA, PKA substrates and signaling terminators such as phosphatases. It might be possible that the mammalian AMPK  $\beta$ -subunits plays a similar role as their yeast and plant homologues in AMPK-protein interactions and that these interactions define different AMPK signaling complexes especially in cells expressing multiple isoforms of the  $\alpha\beta\gamma$  subunits.

The aim of this study was to identify such specific AMPK-protein interactions, regardless of the type of the interacting protein (i.e. target, regulator or scaffold). To this end, a modified cytosolic split-ubiquitin based Y2H was used to screen for interaction partners of the AMPK  $\beta$ -subunits in brain. The resulting putative candidates are presented and compared to the existing panel of AMPK substrates and interaction partners (see Annex 2 & 3). Interaction with vesicle-associated membrane proteins (VAMPs) involved in surface expression of nutrient transporters and neurotransmitter release were further characterized by complementary interaction methods and functional assays.

## 2. RESULTS

### ***2.1. Identification of novel interaction partners of AMPK in human brain***

#### ***2.1.1. Application of a cytosolic split-ubiquitin based Y2H system***

A new unpublished variant of the cyto-Y2H (Mockli et al. 2007) has been applied for screening. The cytosolic, split-ubiquitin-based system is coupled to a transcriptional read-out of protein-protein interactions (PPIs) that allows growth on nutrient-deficient medium. Such transcriptional read-out is very sensitive and can detect also weak or transient interactions. In contrast to the cyto-Y2H previously used, here the bait is anchored to the plasma membrane *via* the transmembrane  $\alpha\beta$ -domain of type 1 amyloid precursor protein (APP), an integral membrane protein. Since overall protein concentration is supposed to be lower at the plasma membrane than at the ER membrane, and elevated local concentration of proteins can contribute to false positives in Y2H screens, it was suggested that anchoring of the bait to the plasma membrane would reduce the number of false positives (Möckli 2007).

As a bait we used the N-terminal domains of AMPK- $\beta$ 1 and - $\beta$ 2 subunits ( $\Delta\beta$ , aa 1-54) to screen a NubG-fused human brain cDNA library (preys). This domain was chosen to avoid other AMPK subunits among the detected interactors, and because it is a good candidate for an AMPK interaction domain (see above). About  $6.2 \times 10^6$  and  $3.4 \times 10^6$  clones were screened, yielding 69 and 33 primary interacting clones, for AMPK- $\Delta\beta$ 1 and - $\Delta\beta$ 2 subunits respectively (Table 4-1). Plasmids containing

the cDNA sequence of putative interaction partners were extracted and reintroduced together with the corresponding  $\Delta\beta$ -encoding bait vector into the reporter yeast strain in order to confirm the interaction. In addition, control yeast was co-transformed with the prey-encoding plasmids and the unrelated bait encoding Large T antigen (LT) in order to verify bait-dependency. Only 40 clones interacted reproducibly with  $\Delta\beta$  but not with LT. They were sequenced and sequences were analyzed by the Basic Local Alignment Sequence Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/>). Twenty eight plasmids were excluded as they contained no coding sequence (CDS), their cDNA was out of frame, or the encoded proteins were already identified in several unrelated Split-ubiquitin based Y2H screens (Dualsystems, personal communication) and thus correspond to false positives. The remaining positive clones encoded in-frame cDNA inserts and are listed in Table 4-2.

**TABLE 4-1. Cyto-Y2H Screen of a human brain cDNA library with Plasma membrane anchored AMPK  $\beta 1$  or  $\beta 2$  N-terminal domain (aa 1-54),  $\Delta\beta 1$ ,  $\Delta\beta 2$ .**

	$\Delta\beta 1$	$\Delta\beta 2$
Library	human brain cDNA library	human brain cDNA library
3AT-concentration (mM) <sup>1</sup>	2.5	2.5
Transformation efficiency	2.2x10 <sup>5</sup> clones/ug library	1.2x10 <sup>5</sup> clones/ug library
Total number of clones	~6.2x10 <sup>6</sup>	~3.4x10 <sup>6</sup>
Clones selected on SD-AHTL	69	33
Clones after plasmid isolation	54	23
Bait dependent clones <sup>2</sup>	26	12
Clones discarded <sup>3</sup>	1(no cds), 2(not in frame), 17 (false positive)	1(no cds), 7(false positive)
Selected clones	4 different	4 different

<sup>1</sup>) 3-aminotriazole (3-AT) concentration was determined by a pilot screen testing autoactivation by using empty library vector. <sup>2</sup>) Clones interacting with  $\Delta\beta$  but not with LT were judged as bait dependent. <sup>3</sup>) After sequencing and Blast analysis, clones lacking CDS (coding DNA sequence) or CDS in the wrong reading frame as well as supposed false positives were eliminated.

**TABLE 4-2. Positive clones identified in Y2H screening of a human brain cDNA library using  $\Delta\beta 1$  or  $\Delta\beta 2$  as bait.**

Bait	Number of clones	Gene Name	Encoded Protein	SwissProt Entry	Peptide (amino acids)
$\Delta\beta 1$	1	RNF41	E3 ubiquitin-protein ligase Nrdp1	Q9H4P4	136-316
$\Delta\beta 1$	1	JWA or ARL6IP5	JWA protein or PRA1 family protein 3	O75915	1-188
$\Delta\beta 1$	1	CLDND1	Claudin domain-containing protein 1	Q9NY35	203-253
$\Delta\beta 1$ / $\Delta\beta 2$	2	ITM2B	Integral membrane protein 2B	Q9Y287	1-266 ( $\Delta\beta 1$ ) 23-266 ( $\Delta\beta 2$ )
$\Delta\beta 2$	1	C14orf1	Probable ergosterol biosynthetic protein 28	Q9UKR5	1-140
$\Delta\beta 2$	1	APLP2	Amyloid-like protein 2	Q06481	607-763
$\Delta\beta 2$	1	VAMP3	Vesicle-associated membrane protein 3	Q15836	22-80

Overall our results indicate that anchoring of the bait to the plasma membrane did not reduce non-specific interactions. About 37% of detected clones in both screens interacted also with LT during the bait dependency assay. In addition, a certain number of apparently specifically interacting proteins was classified as false positives based on information from screens with unrelated proteins (Dualsystems Biotech, personal communications). Even if the  $\Delta\beta 1$ -screen produced more interacting clones than the  $\Delta\beta 2$ -screen, finally they both yielded the same number of putative specific candidates. Among those, only one was identified in both screens, while three additional new interaction candidates were revealed by each of the two screens.

### *2.1.2. Truncated AMPK $\beta 1$ -subunit as bait*

In the AMPK- $\Delta\beta 1$  screening, we identified two plasma transmembrane proteins, claudin domain-containing protein 1 (CLDND1) and integral membrane protein 2B (ITM2B) whose function is not yet clearly defined. If the first is supposed to play a role in cell differentiation and cellular junction (Findley et al. 2009), the second was reported to interact with APP and to be involved in the generation of  $a\beta$  peptides (Matsuda et al. 2005). The clone encoding CLDND1 presented the C-terminal 50 aa which seem to be cytosolic (Findley et al. 2009) (Table 4-1). The clone encoding ITM2B presented the whole coding sequence. We also identified a clone encoding a full length sequence of PRA1 family protein 3 (JWA), which is supposed to play a role in the regulation of glutamate and cysteine transporter EAAC1 (Lin et al. 2001, Watabe et al. 2008), but also in F-actin cytoskeleton rearrangements mediated by the MAPK cascade during cancer cell migration (Chen et al. 2007). Finally we also identified the E3 ubiquitin-protein ligase Nrdp1, among others essential for degradation of receptor tyrosine-protein kinase ErbB3 (Diamonti et al. 2002, Yen et al. 2006). The clone identified contained the C-terminal domain of Nrdp1, which associates also to ErbB3 (aa 136-316) (Table 4-1).

### *2.1.3. Truncated AMPK $\beta 2$ -subunit as bait*

As in the AMPK- $\Delta\beta 1$  screen, ITM2B was identified as interactor. Another APP associated protein detected in this screen was APP family member amyloid-like protein 2 (APLP2). The clone included the C-terminal 156 aa of APLP2 which seem to cover a potential cytosolic domain (as predicted by the UniProt KB (UniProtConsortium 2010)) (Table 4-1). An interacting clone corresponding to C14orf1 encodes a protein without confirmed function that has been proposed as probable ergosterol biosynthetic protein 28 (Erg28p) and integral part of an enzyme complex in sterol biosynthesis (Gachotte et al. 2001). Finally, we identified vesicle associated membrane protein (VAMP), family member 3, playing a key role in the molecular machinery that drives fusion of membranes in exo- and endocytosis (Lin et al. 2000, Hong 2005).

## 2.2. Analysis of candidate interaction with both truncated $\beta$ -subunits

ITM2B and APLP2 have to be considered with caution. Both proteins are known to interact with APP (Matsuda et al. 2005, Soba et al. 2005). Since the transmembrane domain of APP has been used as membrane anchor in the Y2H screen, it is possible that this peptide is involved in the observed PPIs. Although no interactions were detected between these proteins and the membrane-anchored negative control LT, it is highly recommended here to verify these interactions by using an alternative membrane-anchor. Since for these and proteins like CLDND1 and Erg28, information on the physiological functions is scarce, these candidate proteins were not retained for further investigation.

From the remaining candidates, JWA and Nrdp1 were identified as putative interaction partners of the AMPK  $\beta$ 1-subunit whereas VAMP3 was found to interact with AMPK- $\beta$ 2. To investigate whether these interactions are really specific for a particular  $\beta$ -isoform, all three proteins were tested for interaction with each of the two  $\beta$ -isoform N-terminal domains (Fig. 4-1).

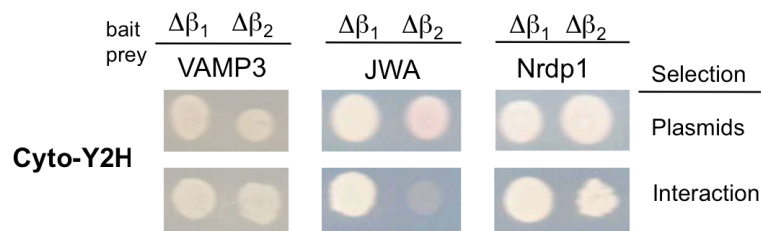


FIGURE 4-1. **Interaction of JWA, Nrdp1 and VAMP3 with  $\beta$ -subunits.** The cyto-Y2H system was applied to analyze interaction of AMPK- $\Delta\beta$  subunits with VAMP3, JWA and Nrdp1 respectively. AMPK  $\Delta\beta$ -subunits were expressed as fusion proteins of a plasma membrane anchor and the C-terminal end of ubiquitin, conjugated to a transcription factor (bait). VAMP3, JWA and Nrdp1 were expressed as fusions to the N-terminal end of ubiquitin (prey). Interactions were detected by activation of reporter gene transcription allowing growth on medium lacking adenine and histidine. Spots represent yeast grown for 48h at 30°C.  $\Delta\beta$ , N-terminal domain of AMPK  $\beta$ -subunit used for Y2H screening. For more details see Part V -Experimental Procedures.

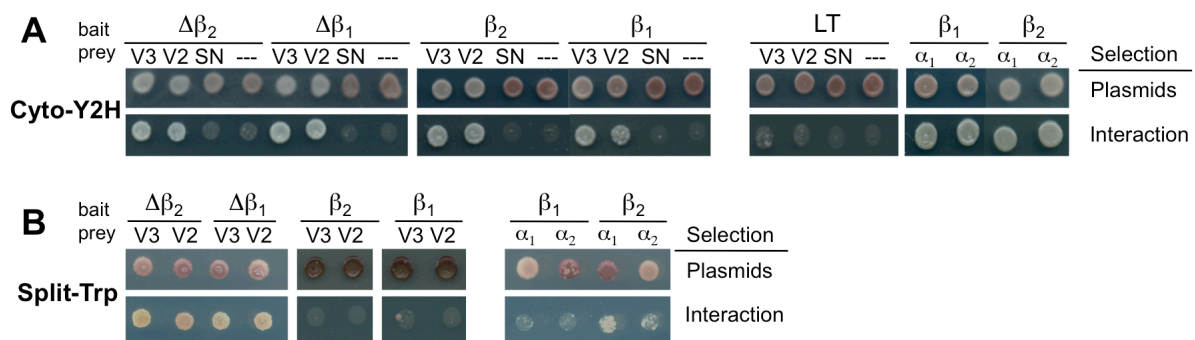
VAMP3 interacted as strongly with  $\Delta\beta_1$  as with  $\Delta\beta_2$ . JWA interacted with AMPK  $\Delta\beta_1$  but not with the  $\Delta\beta_2$ -subunit, it thus appears to be an AMPK isoform-specific interaction partner. Nrdp1 interacted with both  $\Delta\beta_1$  and  $\Delta\beta_2$  subunits, even if the interaction appeared to be stronger with  $\Delta\beta_1$  (Fig. 4-1). These data warrant further investigation into these candidates.

The list of published AMPK interaction partners and targets (Annexes 2 & 3) was analyzed for the presence of the putative AMPK interaction partners identified in this screen. Only one of the identified proteins, VAMP3, was found among the putative AMPK interactors (Annex 3). VAMP3 has been identified in a large-scale co-immunoprecipitation study using the AMPK  $\beta$ 1 subunit as bait (Ewing et al. 2007). Moreover, a functional link between AMPK and VAMP is supported by the implication of both in the translocation of glucose transporter GLUT4 (Kurth-Kraczek et al. 1999, Yamaguchi et al. 2005, Webster et al. 2010) and fatty acid transporter CD36 (van Oort et al. 2009) from intracellular

stores to the cell surface (Chapter I) Thus, the putative PPI between AMPK and VAMP3 was selected for further investigations.

### 2.3. AMPK interaction with different VAMP family members in yeast

VAMP3 (cellubrevin) and its closest homologue VAMP2 (synaptobrevin), were analyzed in paired interaction assays using two independent split-protein Y2H techniques (Fig. 4-2). Both, VAMP2 and VAMP3 interacted with  $\Delta\beta_1$  and  $\Delta\beta_2$  in the cyto-Y2H system. The interaction was also confirmed with both full-length  $\beta$ -subunits (Fig. 4-2A). The SNARE domain common to VAMPs was not sufficient to induce the interaction. In order to further confirm the AMPK-VAMP interaction, a second cytosolic Y2H system was applied. The split-Trp system, in which the two proteins are expressed as fusion to either a C-terminal part or an N-terminal part of Trp1p, PPI leads to reconstitution of Trp1p enzyme activity, thus allowing tryptophan biosynthesis. Here, both proteins are thus expressed as soluble fusions in the cytosol (See Part V - Experimental Procedures for more details). In contrast to the cyto-Y2H system, the PPI read-out will not be amplified by a transcriptional step. The split-Trp system confirmed an interaction between VAMP2 or VAMP3 and both truncated  $\beta$ -subunits, but gave a negative result for interaction with full-length  $\beta$ -subunits (Fig. 4-2B) that persisted when interaction partners were swapped between the two vectors (not shown). However, under conditions of the split-Trp system, even the dimerization of the AMPK  $\alpha$ - and  $\beta$ -subunit was also almost undetectable (Fig. 4-2B). Thus, some interactions, possibly weaker ones, escape detection in this system.



**FIGURE 4-2. Identification of the two major VAMP isoforms as AMPK interaction partners by Y2H analysis.** Two different cytosolic Y2H assays were applied to analyze interaction of AMPK with VAMP isoforms VAMP3 and VAMP2. (A) Cyto-Y2H system. AMPK subunits were expressed as fusion proteins of a plasma membrane anchor and the C-terminal end of ubiquitin conjugated to a transcription factor (bait). VAMP2 or 3 (V2, V3) and the shared SNARE domain (SN, aa 31-91) were expressed as fusions to the N-terminal end of ubiquitin (prey). Interactions were detected by growth on medium lacking adenine and histidine. Spots represent yeast grown for 72h at 30°C. (B) Split-Trp system. AMPK subunits were expressed as fusion proteins to the C-terminal (bait) and VAMP2 and 3 as fusion proteins to the N-terminal (prey) portion of Trp1p. Interactions were detected by growth on medium lacking tryptophan. Yeast was grown for 7 days at 27°C.  $\Delta\beta$ , N-terminal domain of AMPK  $\beta$ -subunit. Negative controls: LT, Large T Antigen of *Simian Virus* (aa 84-704) and empty prey vector (---). Positive controls: AMPK  $\alpha$ - $\beta$  dimerization. For more details see Part V - Experimental Procedures.

In support of this, we have observed during purification of recombinant AMPK from *Escherichia coli*, that the  $\alpha$ -subunit could be dissociated from the stronger interacting  $\beta$ - $\gamma$  dimer, also suggesting a rather weak  $\alpha/\beta$  interaction. Alternatively, AMPK- $\alpha 2$  may interact with yeast AMPK  $\beta$  homologues (e.g. Gal83) (Moreno et al. 2009) thus reducing other  $\alpha$ - $\beta$  interactions.

#### 2.4. Co-immunoprecipitation of full length AMPK and VAMP2

The interaction of VAMP with full-length functional AMPK complex was further confirmed by co-immunoprecipitation and affinity pull-down. In a first approach, recombinant expression of VAMP in *E. coli* or *in vitro* was performed. Since expression of Strep-VAMP3 in *E. coli* yielded no protein at all, different *in vitro* protein expression systems were set-up. However, *in vitro* expression of full length VAMP3-His and VAMP2-His yielded only faint quantities of protein after purification. It seems that VAMPs, as many other transmembrane proteins, are difficult to express and to purify when recombinantly expressed. In addition, co-immunoprecipitation assays with VAMP proteins recovered after purification and recombinant full length AMPK failed. We supposed that there could be additional problems with the proper folding of recombinant VAMPs, affecting the conformation required for interaction.

In an alternative approach, VAMP2 was enriched from rat brain by preparation of synaptic vesicles as described in detail in Part V. Recombinant constitutively active AMPK isoform  $\alpha_2$ T172D $\beta_2\gamma_1$  (221TD) was added to the synaptic vesicle fraction, since endogenous AMPK was low and undetectable by immunoblotting (data not shown). Immunoprecipitation or affinity pull-down of exogenous AMPK 221TD led to co-purification of endogenous VAMP2 (Fig. 4-3).

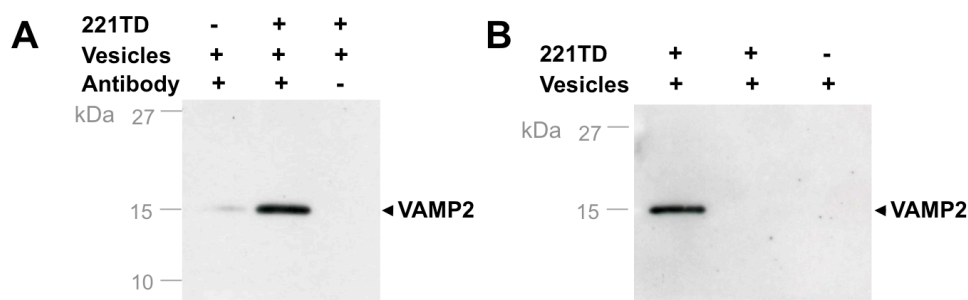


FIGURE 4-3. **AMPK interactions with endogenous VAMP2 from synaptic vesicles.** AMPK immunoprecipitation (A) or pull-down (B) were performed by using synaptic vesicles. (A) Immunoprecipitation of added constitutively active AMPK  $\alpha_2$ T172D-His $\beta_2\gamma_1$  (221TD) by anti-His-tag antibody. (B) Pull-down of VAMP2 by added 221TD using Ni-NTA Sepharose. VAMP2 in pull-down fractions or immunoprecipitates was detected by immunoblot analysis with anti-VAMP2 antibody.

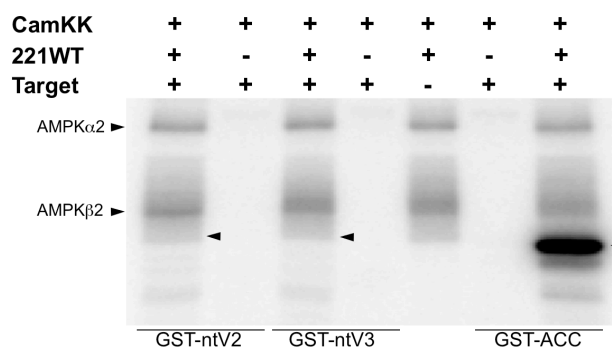
#### 2.5. VAMPs are no AMPK targets but might recruit AMPK to vesicles

Given the evidence for VAMP/AMPK interaction, we analyzed whether the different VAMPs could be AMPK targets. As a substrate we used recombinant soluble domains of VAMP fused to

GST- or Strep-tag, Strep-ntVAMP3, GST-ntVAMP3 and GST-ntVAMP2, all produced in *E. coli*. In these constructs, the C-terminal transmembrane domain was deleted, resulting in higher levels of expressed and purified protein. Addition of a GST-tag further increased protein yield, suggesting that the larger GST-tag stabilizes the different soluble VAMP constructs.

An AMPK *in vitro* phosphorylation assay was then performed using wild type AMPK isoform  $\alpha_2\beta_2\gamma_1$  (221WT) previously activated by CamKK and recombinant soluble GST-ntVAMP2 and GST-ntVAMP3. Neither of both VAMPs was phosphorylated *in vitro* (Fig. 4-4). The same negative results were obtained when Strep-ntVAMP3 and constitutive active AMPK 221TD were used (data not shown). These data suggest that VAMP is not a direct AMPK target. However, as already mentioned, the correct conformation of these VAMP constructs has not been verified so far, and phosphorylation assays should be repeated using immunoprecipitated VAMP from vesicle fractions. The fact that neither VAMP2 nor VAMP3 presented a putative AMPK phosphorylation recognition site as revealed by a Prosite Scan with the AMPK recognition motive [MLIFV]-[XRKH]-[XRKH]-X-X-[ST]-X-X-X-[MLIFV] (Dale et al. 1995, Scott et al. 2002) support however the notion that these VAMPs are no AMPK targets.

**FIGURE 4-4. VAMPs are no AMPK *in vitro* targets.** AMPK 221 (4 pmol) previously activated by CamKK (1 pmol) was incubated with GST-ntVAMP3 or GST-ntVAMP2 (200 pmol) or with GST-ACC (100 pmol) for 8 min at 37°C. *In vitro* phosphorylation assays were analyzed by SDS-PAGE and Typhoon phosphoimager; also revealing AMPK autophosphorylation.



Next we tested whether VAMP may recruit AMPK to vesicles, which then could lead to phosphorylation of other vesicle-associated proteins. The same *in vitro* phosphorylation assay, using constitutive AMPK 221TD, was applied to both, synaptic vesicles and synaptosomes. Several putative AMPK targets could be detected in these fractions (Fig. 4-6). However, these vesicle fractions were still too complex to allow direct protein identification from the gel by mass spectrometry. Further pre-fractionation or 2D gel resolution will be required to identify these putative targets.



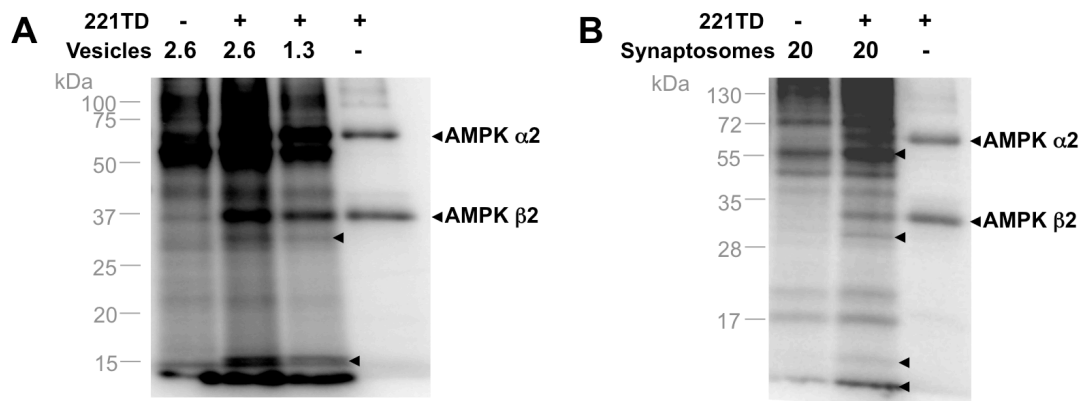


FIGURE 4-6. **Putative AMPK targets in synaptosomes and synaptic vesicles.** AMPK 221TD (30 pmol) was incubated with (A) synaptic vesicles (2.6 or 1.3  $\mu$ g) or with (B) synaptosomes (20  $\mu$ g) for 2 min at 37°C. *In vitro* phosphorylation assays were analyzed by SDS-PAGE and Typhoon phosphoimager; also revealing AMPK autophosphorylation.

Another protein associated with vesicles and investigated in the laboratory is  $\gamma$ -aminobutyric acid (GABA) receptor-associated protein-like 1 (GEC1), a potential member of the GABARAP family involved in the transport of GABA receptors. *In vitro* phosphorylation assays using AMPK 221WT previously activated by CamKK revealed specific AMPK-mediated GEC1 phosphorylation (Fig. 4-7A). Therefore, interaction of GEC1 with truncated  $\Delta\beta$  subunits was analyzed.

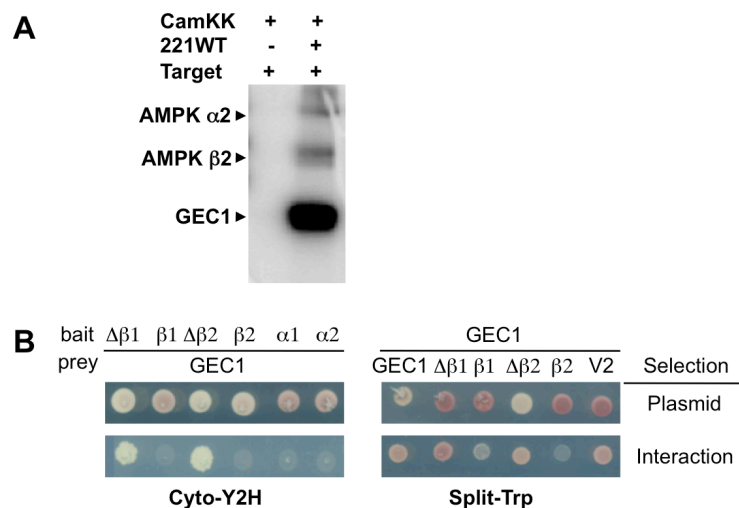
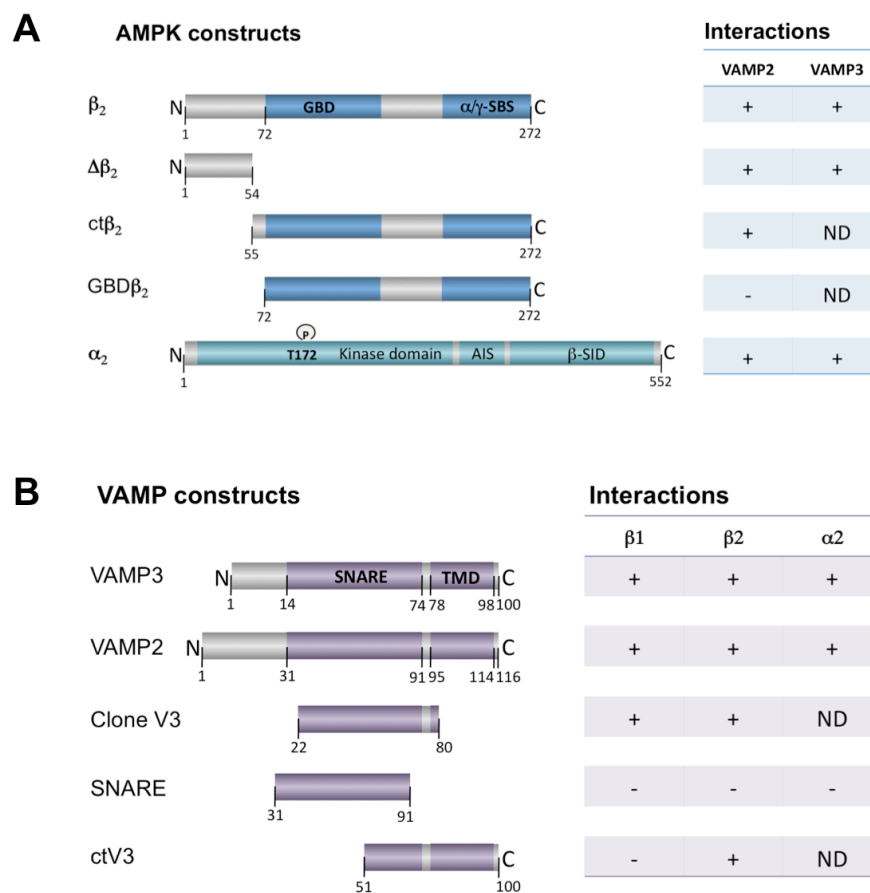


FIGURE 4-7. **AMPK phosphorylations of and interactions with vesicle-associated protein GEC1** (A) AMPK *in vitro* phosphorylation assay. AMPK 221WT (4 pmol) previously activated by CamKK (1 pmol) was incubated with GEC1 (200 pmol) for 5 min at 37°C. Phosphorylation assays were analyzed by SDS-PAGE and Typhoon phosphoimager; also revealing AMPK autophosphorylation. (B) Two different cytosolic Y2H systems were applied to analyze AMPK-GEC1 interaction. In cyto-Y2H (left), AMPK  $\alpha$  and  $\beta$  subunits and truncated  $\Delta\beta$  (aa 1-54) were expressed as bait and GEC1 was expressed as prey. Spots represent yeast grown for 72h at 30°C. (B) In SplitTrp Y2H (right) AMPK  $\beta$  subunits, VAMP2 (V2) or GEC1 (positive control) were expressed as prey and GEC1 was expressed as bait. Spots represent yeast grown for 72h at 27°C.

In both, cyto-Y2H and Split-Trp Y2H assays, an AMPK-GEC1 interaction was detected (Fig. 4-7B). The interaction was not detectable with full-length  $\beta$ -subunits in Y2H assays, but could be confirmed by surface plasmon resonance (SPR) experiments using GEC1 and full-length AMPK 221WT complex (A. Berthier, data not shown). Since GEC1 also interacted with VAMP2 in the Split-Trp Y2H assay (Fig. 4-7B) it may be speculated that all three proteins, VAMP, AMPK and GEC1 participate in higher order complexes.

Altogether, these data suggest that different AMPK targets may be associated to synaptic and other intracellular vesicles. We thus suggest that VAMP is recruiting AMPK to vesicles to phosphorylate other vesicle- or vesicle transport-associated proteins.

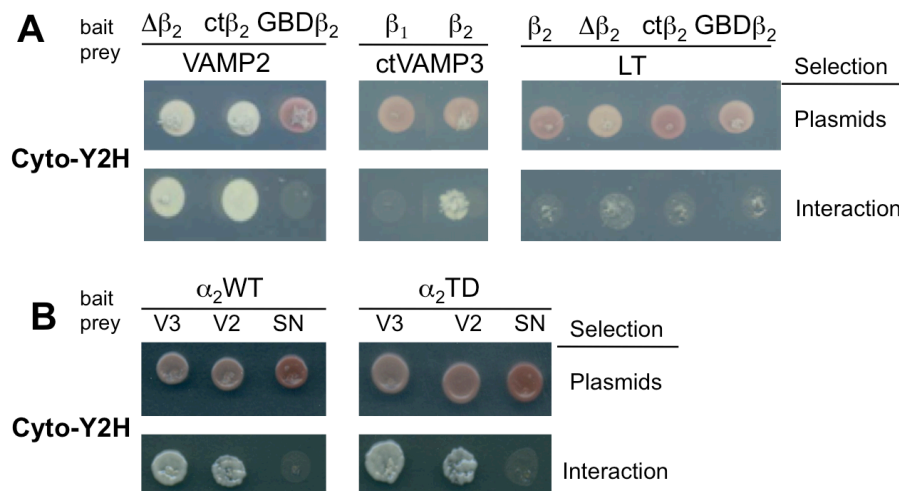


**FIGURE 4-8. AMPK and VAMP constructs used in Y2H assays to determine the interaction domain** (A) Different AMPK  $\beta$ 2-subunit constructs were used.  $\beta_2$ , full length subunit;  $\Delta\beta_2$ , N-terminal 54 aa; ct $\beta_2$ , aa 55-272; GBD $\beta_2$ , aa 72-272 bearing the glycogen binding domain (GBD) and the C-terminal  $\alpha/\gamma$ -subunit interacting sequence ( $\alpha/\gamma$ -SDS);  $\alpha_2$ , full length subunit bearing the kinase domain, an auto inhibitory sequence (AIS) and the C-terminal  $\beta$ -subunit interaction domain ( $\beta$ -SID). (B) Different VAMP constructs. Full length VAMP2/3 were used. Clone V3, clone identified in the Y2H screen (aa 22-80); VAMP2/3 common SNARE domain (aa 31-91 of VAMP2); ctV3, C-terminal 49 aa of VAMP3 bearing a part of the SNARE domain and the C-terminal transmembrane domain (TMD). ND, not determined.

## 2.6. Determination of the AMPK-VAMP interaction domain

AMPK interaction with proteins to trigger recruitment to specific cellular compartments would be a novel element in AMPK signaling. Experimental manipulation of such protein-protein interactions requires further mapping of the AMPK-VAMP interaction domain. To this end, truncation constructs of AMPK  $\beta$ -subunits or VAMPs were cloned into the vectors of the cyto-Y2H system (Fig. 4-8).

Based on the above finding that the very N-terminal domain of AMPK  $\beta$  is sufficient for interaction with VAMP, N-terminal truncations were realized to obtain a minimal N-terminal AMPK sequence necessary for interaction (Fig. 4-8A). When testing these constructs in cyto-Y2H assays with VAMP2, interaction was detected with the  $\Delta\beta_2$  (amino acids 1-54) and also with ct $\beta_2$  (aa 55-272), but not with GBD $\beta_2$  (aa 72-272) (Fig. 4-9A). These results suggest that the N-terminal domain (amino acids 1-71) of the AMPK  $\beta_2$  subunit harbours a VAMP interaction domain spanning across the fragment boundary at residue 54.



**FIGURE 4-9. Identification of the VAMP interacting AMPK domain by Y2H analysis.** Two different cytosolic Y2H systems were applied to analyze interaction of truncated AMPK subunits with VAMP2 and truncated VAMP3. (A) AMPK  $\beta_2$ -subunit or truncated  $\beta_2$  constructs were expressed as bait, VAMP2, ctVAMP3 or LT (negative control) were expressed as prey. Spots represent yeast grown for 48h at 30°C. (B) Wild type ( $\alpha_2$ WT) or constitutive active ( $\alpha_2$ TD) AMPK  $\alpha_2$ -subunits were expressed as bait, VAMP2/3 or the SNARE domain as prey. Interactions were detected by growth on medium lacking adenine and histidine. Yeast was grown for 48 h at 30°C.  $\Delta\beta$ , aa 1-54 of AMPK  $\beta$ -subunit. Ct $\beta_2$ , aa 55-272 of AMPK  $\beta_2$ -subunit. GBD $\beta_2$ , aa 72-272 of AMPK  $\beta_2$ -subunit. CtVAMP3, aa 51-100 of VAMP3. SN, SNARE domain (aa 31-91). For more details see Part V - Experimental Procedures.

For VAMPs, Y2H analysis already revealed that amino acids 22-80 present in the interacting Y2H VAMP3 clone V3 are sufficient for interaction, but not the SNARE domain alone (Fig. 4-2A). Therefore, a construct extending a truncated SNARE domain towards the C-terminus, including some additional cytosolic residues and the C-terminal transmembrane domain of VAMP3 (amino acids 51-100) was cloned (Fig. 4-8B). This construct thus includes residues present in the interacting clone V3, but missing in the isolated SNARE domain. When tested with full-length AMPK  $\beta$ -subunits, this

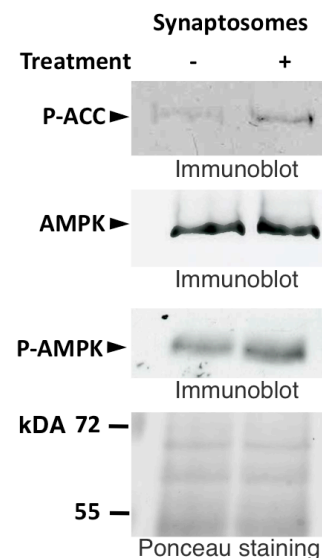
truncated VAMP3 showed weak interaction with AMPK  $\beta$ 2, albeit not with AMPK  $\beta$ 1. This result suggests some differences between  $\beta$ 1 and  $\beta$ 2 in VAMP3 interaction, with a possibly crucial role for the three amino acids separating SNARE and transmembrane domain in both VAMPs. Taken together, different interaction epitopes within the SNARE and C-terminal part of the cytosolic domain of VAMP seem to be necessary for full AMPK/VAMP interaction. However, there may be also some folding problems with the chosen constructs, so further experiments will be necessary to determine the exact AMPK- $\beta$  interaction domain within the cytosolic domain of VAMPs.

Further AMPK subunits were tested for their interaction with VAMPs in the cyto-Y2H screen. However, AMPK  $\gamma$ 2 was not correctly expressed in yeast strain NMY51, since an AMPK  $\gamma$  screen and a binary interaction assay did not lead to reproducible results. In addition, there was no immunodetectable  $\gamma$ -construct present in protein extracts from transfected yeast (not shown). With AMPK  $\alpha$ 2, either wild-type or constitutive active T172D mutant, interaction was observed with both, VAMP2 and VAMP3 (Fig. 4-9B). However, AMPK  $\alpha$ 2 is able to interact with yeast AMPK  $\beta$  homologues, and these could mediate an indirect interaction of AMPK  $\alpha$ 2 proteins with VAMPs. No interaction of AMPK  $\alpha$ -subunits with VAMP2 was observed in the SplitTrp Y2H system (not shown). In any case, it will be important to test whether disruption of VAMP interaction with N-terminal AMPK  $\beta$  domain, e.g. by using appropriate inhibitory peptides, is sufficient to inhibit interaction with native AMPK complex.

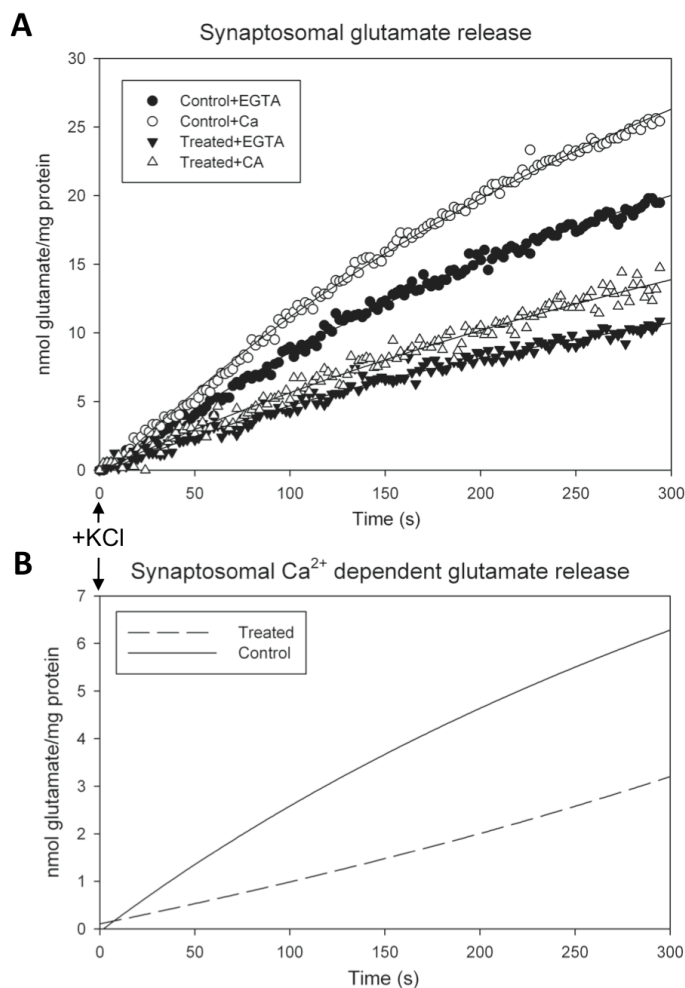
### 2.7. Putative role of AMPK in neurotransmitter release

VAMP proteins like VAMP2 have a major role in the trafficking of synaptic vesicles, leading to neurotransmitter release at the synapse. Given the observed AMPK/VAMP interaction, we examined whether the exocytotic glutamate release from synaptosomes is affected by AMPK activation. Synaptosomes were isolated from rat forebrain and endogenous AMPK was activated with A-769662 compound (Fig. 4-10).

**FIGURE 4-10. Immunoblots, showing that AMPK is localized in synaptosomes and can be activated by A-769662 compound.** Synaptosomes were treated (+) or not (-) with A-769662 compound for 30min at 37°C. Immunoblotting of 50 $\mu$ g synaptosomal proteins using (A) anti-P172 AMPK (Ponceau staining as loading control) or (B) anti-phospho-ACC and anti-AMPK antibodies. Data from different experiments.



After 30 min incubation at 37°C with the specific AMPK activator A-769662, the AMPK signaling pathway was effectively activated as shown by increases in AMPK  $\alpha$ T172 phosphorylation and phosphorylation of the AMPK downstream target ACC (Fig. 4-10).



**FIGURE 4-11. A-769662 reduces Ca<sup>2+</sup>-dependent glutamate release from synaptosomes.** (A) Time course of exocytotic glutamate release from synaptosomes, induced by depolarization with KCl. Glutamate release was measured by a fluorimetric assay detecting NADH production by glutamate dehydrogenase (GlutDH) and. Synaptosomes previously incubated for 30 min with A-769662 (triangles) or not (Control, circles) were incubated with Ca<sup>2+</sup> (white) or EGTA (controls, black). After addition of GlutDH, glutamate release was activated by depolarization with KCl (t<sub>0</sub>). At the end of each run 10 nmol glutamate were added as internal standard to quantify released glutamate. (B) Time course of net Ca<sup>2+</sup>-dependent glutamate release, calculated as the difference between the release in presence of EGTA and the release in the presence of Ca<sup>2+</sup>. One of two experiments is shown.

After treatment, the glutamate release assay was performed in medium containing glutamate dehydrogenase (GlutDH) and NADP<sup>+</sup>. The release of glutamate into the medium leads to NADP reduction and thus to an increase in fluorescence emission at 460 nm (Nicholls et al. 1986). Neurotransmitter release is activated by Ca<sup>2+</sup> and triggered by addition of KCl leading to depolarization. To account for basal, Ca<sup>2+</sup>-independent release, control measurements were done in the presence of Ca<sup>2+</sup>-chelating agent EGTA (Fig. 4-11A). Ca<sup>2+</sup>-dependent glutamate release, i.e. the difference between control measurements and measurements in presence of Ca<sup>2+</sup>, was reduced after treating synaptosomes with A-769662 by a factor of 2 to 3 (Fig. 4-11B). Although these are presently only preliminary data (two experiments) and conclusions should be drawn with caution, activation of AMPK seems to reduce the Ca<sup>2+</sup>-dependent glutamate release in isolated synaptosomes.

### 3. DISCUSSION

In this study, a new variant of the cyto-Y2H system was used to screen for novel AMPK interaction partners. Seven specifically AMPK interacting candidates were identified that were not characterized so far as AMPK targets or interaction partners. With one exception, all identified candidates are membrane proteins, indicating a certain bias of this screening system for membrane proteins.

Among the most interesting putative AMPK interactors are members of the large family of **vesicle-associated proteins**. VAMP3 has been identified in the non-biased Y2H screen, and the homologous VAMP2 was confirmed as additional AMPK interactor in a targeted follow-up study. The focus on this group of membrane proteins is justified by several reasons. First, like AMPK, VAMPs function in surface expression of nutrient transporters such as GLUT4 or CD36, and further in many other exo- and endocytotic processes such as trafficking of synaptic vesicles for neurotransmitter release. Thus, VAMPs may be involved in downstream AMPK signaling e.g. for increased nutrient uptake. Second, VAMP3 is the only of our candidates which was independently reported by a large-scale co-immunoprecipitation study using the AMPK  $\beta$ 1 subunit as bait (Ewing et al. 2007).

In a preliminary characterization of the AMPK interaction with VAMP2/3, we could confirm this interaction by different Y2H assays and Co-IP or pull-down experiments using synaptic vesicles and full length, active AMPK complex. Analysis of different truncated AMPK- $\beta$ 2 subunits provided some initial mapping of the interaction domain to the AMPK- $\beta$  N-terminus. Either amino acids 1-54 or 55-71 are sufficient for interaction. The same probably applies to AMPK- $\beta$ 1, given the high sequence homology with AMPK- $\beta$ 2. Interaction of AMPK  $\alpha$ 2 with VAMP2/3 detected in only one Y2H assay may be rather indirect, mediated by yeast homologues of the AMPK  $\beta$ -subunit. In VAMPs, different epitopes within the cytosolic domain, including residues close to the membrane and other(s) in the SNARE domain seem to be necessary for full interactions, but further experiments are required.

Many VAMP proteins have a broad tissue distribution (McMahon et al. 1993) and play a role in a large variety of exo- and endocytotic processes. VAMP3 is present in recycling endosomes and endosome-derived vesicles and has been implicated in recycling of transferrin receptors (Galli et al. 1994), secretion of  $\alpha$ -granules in platelets (Polgar et al. 2002), recycling of T-cell receptors to the immunological synapses (Das et al. 2004) and membrane trafficking during cell migration and adhesion (Luftman et al. 2009). Surprisingly, VAMP3 knockout mice have no obvious phenotype (Yang et al. 2001), which might result from compensation by other VAMP family members. VAMP2 is the family member displaying the greatest sequence homology to VAMP3 (74%). Contrary to genetic deletion of VAMP3, VAMP2 knockout mice are not viable (Schoch et al. 2001). VAMP2 is enriched on synaptic vesicles and mediates neurotransmitter release, aquaporin-2 exocytosis in renal cells (Procino et al. 2008), cell migration *via* integrin trafficking (Hasan et al. 2010).

Most relevant for the putative AMPK-VAMP interaction, both proteins share a functional link to translocation of nutrient transporters such as GLUT4 or CD36 from intracellular stores to the cell surface. VAMP2 is involved in translocation of glucose transporter GLUT4 after insulin-stimulation in adipocytes (Martin et al. 1998) and cardiomyocytes (Schwenk et al. 2010). By this latter very recent study, family member VAMP3 was linked to stimulation-induced GLUT4 and CD36 translocation in cardiomyocytes (Schwenk et al. 2010). In case of AMPK, activation by AICAR or contraction leads to translocation of glucose transporter GLUT4 and long-chain fatty acid transporter CD36 to the cell membrane, followed by increased glucose and fatty acid uptake as shown in adipocytes (Yamaguchi et al. 2005), cardiomyocytes (Luiken et al. 2003, Webster et al. 2010) and skeletal muscle (Kurth-Kraczek et al. 1999). Different vesicle populations with different combinations of VAMP proteins were involved in translocation of either GLUT4 or CD36, and also in the response to either insulin or contraction (Schwenk et al. 2010). In addition, stimulation of GLUT4 or CD36 translocation by AMPK seems to be rather specific, since translocation of other membrane proteins like EAAC1, a high affinity glutamate transporter (see also below), was reported to be inhibited by activated AMPK (Sopjani et al. 2010). In this thesis work, we show first preliminary data on a negative regulation of neurotransmitter release by pharmacological activation of AMPK in brain synaptosomes. In summary, these data support a direct link of AMPK to vesicle transport and exo- or endocytosis. As VAMPs are indispensable for these processes, the AMPK-VAMP interaction could play a crucial role.

However, the precise molecular mechanism for AMPK-stimulated translocation of nutrient transporters is still largely unsolved. Whether VAMPs are part of this mechanism remains to be established. Neither VAMP3 nor VAMP2 were phosphorylated by AMPK *in vitro*. They might thus rather function as scaffolds that recruit AMPK to vesicles for regulating vesicle transport and priming of membrane fusion, as both are ATP requiring processes. Indeed, several proteins in a fraction of purified synaptic vesicles were phosphorylated by AMPK *in vitro*, and we found GEC1, a protein associated with vesicles and cytoskeletal elements like tubulin (Mansuy et al. 2004) to be indeed phosphorylated by AMPK *in vitro*. GEC1 was found also associated to autophagic vesicles (Chakrama et al. 2010), consistent with a role of AMPK in autophagy (Hoyer-Hansen et al. 2007, Herrero-Martin et al. 2009, Poels et al. 2009), and has been proposed to play a role in intracellular transport of vesicles and proteins like GABA receptor.

Taken together, the hypothesis of AMPK being recruited to vesicles to regulate energy consuming transport and membrane fusion seems the most conclusive to date. Further studies will have to show whether disruption of the AMPK-VAMP interaction, e.g. by inhibitory peptides corresponding to the AMPK interaction domain, can affect AMPK-induced GLUT4 translocation or prevent the observed inhibition of glutamate release. Further confirmation of AMP-mediated inhibition of neurotransmitter release would also be of interest *per se* as, to the best of my knowledge, this would be the first evidence for a role of AMPK in this process.

The rodent homologues of **JWA protein** (PRA1 family protein 3) were identified as glutamate transporter EAAC1-interacting proteins. EAAC1 is involved in glutamate and cysteine uptake,

important for mature neurons. Glutamate uptake was increased in JWA knockdown cells, and reduced when JWA was over-expressed (Lin et al. 2001), but the mechanism for the EAAC1 regulation by JWA is not yet clear. It seems that JWA phosphorylation by protein kinase C (PKC) increases both, JWA and EAAC1 translocation to the membrane, followed by increased glutamate/cysteine uptake (Watabe et al. 2008). Independent of cell surface transporters, JWA reduces cancer cell migration (Chen et al. 2007) possibly by regulation of integrin translocation to the cell membrane in a similar way as for EAAC1 (Bai et al. 2010). Again, phosphorylation of JWA by PKC seems to be required for JWA-mediated inhibition of cancer cell migration (Chen et al. 2007). A very recent study now involves also AMPK in such intracellular transport processes (Sopjani et al. 2010). Coexpression of constitutive active AMPK with glutamate transporter EAAC1 in *Xenopus* oocytes led to reduced surface expression of the transporter. The authors suggest that AMPK-mediated activation of Nedd4-2, an E3 ubiquitin-ligase (see also below), is responsible for increased EAAC1 degradation. However, our interaction data suggest that JWA/EAAC1 translocation to the cell membrane could be directly regulated by AMPK. Further experiments have to show whether AMPK could be a second kinase regulating JWA activity. At least one AMPK phosphorylation consensus site is present in the JWA sequence.

The only soluble AMPK interaction candidate identified in this study is the **E3 ubiquitin-protein ligase Nrdp1**. This class of proteins is part of the cellular machinery for protein ubiquitination which targets proteins to the ATP-dependent proteolytic pathway proceeding at proteasomes. The large number of E3 ubiquitin-protein ligases, often termed simply ubiquitin ligases, catalyze the final step of ubiquitination and assume the substrate specificity of the entire process (Deshaies et al. 2009). Nrdp1 is involved in degradation of receptor tyrosine-protein kinase erbB-3, a member of the epidermal growth factor receptor (EGFR) family controlling cell proliferation and differentiation. Since dysregulated erbB-3 expression or function is involved in various cancers (reviewed in (Sithanandam et al. 2008)) it is a promising target for therapeutic intervention (Yen et al. 2006). The AMPK-Nrdp1 interaction may thus add to the multiple antitumorigenic roles of AMPK. These include beneficial effects of AMPK activators like AICAR and metformin in animal models and humans (Evans et al. 2005, Swinnen et al. 2005), and direct antiproliferative AMPK signaling via mTOR or p53 (reviewed in (Motoshima et al. 2006)). However, it cannot be excluded that it is rather Nrdp1 that acts on AMPK. In a recent study, another E3 ubiquitin-protein ligase, malin, in complex with the dual-specificity protein phosphatase laforin, was shown to promote ubiquitination of AMPK  $\beta$ -subunits (Moreno et al. 2010). In this case, ubiquitination rather promoted stability of the AMPK complex. Taken together, Nrdp1 certainly is a promising candidate for further investigations. Ongoing work in our group is analyzing whether AMPK is able to phosphorylate Nrdp1 and whether this affects ErbB3 degradation in breast cancer cell lines. Indeed, such AMPK-controlled protein degradation has been described for E3 ubiquitin-ligase Nedd4-2. AMPK mediated Nedd4-2 phosphorylation leads to increased epithelial Na<sup>+</sup> channel (ENaC) ubiquitination and degradation (Bhalla et al. 2006, Almaca et al. 2009).



Two of the identified putative AMPK interactors, **APLP2** and **ITM2B**, could link AMPK signaling to neurodegenerative diseases. APLP2 is a homologue of APP, the  $\text{a}\beta$ -peptide precursor, whose accumulation and aggregation occurs in Alzheimer disease (Crews et al. 2010). ITM2B regulates APP and  $\text{a}\beta$ -peptide secretion (Fotinopoulou et al. 2005, Soba et al. 2005), and its cleavage is involved in Familial British and Danish dementia (Vidal et al. 1999). However, a serious note of caution is mandatory in this case, since both, APLP2 and ITM2B, interact with APP. They could thus interact directly with the transmembrane  $\text{A}\beta$  anchor of APP used in the AMPK bait construct. However, a recent study showed reduced extracellular accumulation and stimulated lysosomal degradation of  $\text{a}\beta$ -peptide after pharmacological activation of AMPK by AICAR or resveratrol (Vingtdeux et al. 2010). Although this could be explained by stimulated autophagy of  $\text{a}\beta$ -peptide via the mTOR pathway (Vingtdeux et al. 2010), it may be worth to reanalyze a direct link of AMPK, APLP/ITM2B, and  $\text{a}\beta$ -peptide secretion.

In conclusion, our preliminary evidence proposes VAMP2 and VAMP3 as scaffolding proteins for AMPK and potentially other proteins, recruiting the kinase complex to different intracellular vesicle populations and thus regulating ATP-dependent transport and membrane fusion events in various tissues. JWA protein and Nrdp1 are interesting interactors with roles in intracellular protein transfer and protein degradation that could be potentially phosphorylated by AMPK. All three candidates require further characterization to affirm or discard their potential role in AMPK signaling.

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**- CHAPTER V -****A TWO-DIMENSIONAL SCREENING STRATEGY REVEALS NOVEL  
ISOFORM-SPECIFIC SUBSTRATES OF AMP-ACTIVATED PROTEIN KINASE.****Anna Brückner<sup>1#</sup>, Cécile Polge<sup>1#</sup>, Yolanda Auchli<sup>2</sup>, René Brunisholz<sup>2</sup>, and Uwe Schlattner<sup>1</sup>**

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**Abstract:** AMP-activated protein kinase is an important cellular signaling hub involved in energy homeostasis and cell proliferation, recognized as a potential drug target for treating metabolic syndrome and cancer. Drug development requires precise knowledge on the AMPK signaling network. Here we present a new *in vitro* two-dimensional screening approach combining a biophysical interaction assay with phosphorylation assays to identify AMPK-interacting substrates potentially dependent on specific AMPK isoforms. Among the identified proteins, we confirm fumarate hydratase (or fumarase) and fatty acid binding protein 1 as *in vitro* AMPK targets, preferentially interacting with and phosphorylated by the AMPK $\alpha$ 2 isoform. AMPK mediated phosphorylation of fumarate hydratase leads to significant enzymatic activation of this enzyme.

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**1. INTRODUCTION**

AMP-activated protein kinase (AMPK) is member of a Ser/Thr kinase family conserved across the eukaryotic kingdom, occurring as SNF1 complex in yeast and as SnRK1 in plants. These kinases function as heterotrimeric complexes composed of one catalytic  $\alpha$ -type subunit and two regulatory subunits, the  $\beta$ - and  $\gamma$ -type subunits (Davies et al. 1994, Mitchelhill et al. 1994, Jiang et al. 1997, Bouly et al. 1999). Subunits of mammalian AMPK occur in form of different isoforms ( $\alpha$ 1,  $\alpha$ 2,  $\beta$ 1,  $\beta$ 2,  $\gamma$ 1-3) and splice variants (of  $\gamma$ 2 and 3), potentially generating a multitude of different heterotrimeric complexes.

In mammals, AMPK functions mainly as an energy sensor, integrating signals from the cell, the cellular environment, and the whole organism (for reviews see (Neumann et al. 2003a, Hardie 2007)). The activation mechanism involves AMP-induced conformational changes and covalent activation by upstream kinases (Woods et al. 2003, Riek et al. 2008). AMPK signaling has also been involved in different human pathologies like diabetes and cancer, and is proposed as a potential target for treating these pathologies (Steinberg et al. 2009, Fogarty et al. 2010). Indeed, two of the major drugs widely used for treating diabetes type II, metformin and thiazolidinedione, activate AMPK (Zhou et al. 2001,

Fryer et al. 2002) and most of their therapeutic effects are mediated by AMPK signaling, particularly in case of metformin (Shaw et al. 2005). Moreover, the identification of a known tumor suppressor, LKB1, as an upstream kinase of AMPK has generated substantial interest for AMPK signaling in cancer development. Activated AMPK negatively regulates cell proliferation and the cell cycle, mediated by mTOR and p53, respectively (Kimura et al. 2003, Jones et al. 2005, Alessi et al. 2006). However, treatments based on a systemic activation of AMPK may not only be beneficial, given the largely pleiotropic effects of AMPK signaling linked to its growing number of targets. Different strategies are currently followed-up to circumvent this potential limitation.

Systemic activation may be avoided to some degree by targeting specific AMPK isoform combinations, either because some of them show a partial albeit not absolute tissue-specificity (Viollet et al. 2009), or because some of them may recognize specific substrates. In yeast (Vincent et al. 1999) and plants (Polge et al. 2008), the variable N-terminal region of the  $\beta$ -subunits has been proposed to mediate interaction of the kinase with its targets. In mammals, the  $\alpha$ 1 and  $\alpha$ 2 subunits were shown to exhibit subtle different substrate preferences when using variants of the SAMS peptide, suggesting that the two isoforms could phosphorylate different substrates within the cell (Woods et al. 1996).

Another strategy is to further identify downstream targets with the ultimate goal to interfere more specifically with AMPK downstream signaling instead of AMPK itself, e.g. by inhibiting protein-protein interactions of AMPK, a general prerequisite for cell signaling. Although a large number of putative AMPK-protein interactions have been reported, most derive from large-scale screening like immunoprecipitation of flag-tagged AMPK- $\beta$ 1 followed by LC-ESI-MS/MS analysis (Ewing et al. 2007), and only very few have been independently verified by more than one method ((Solaz-Fuster et al. 2006, Kuramoto et al. 2007, Vernia et al. 2009), Chapter III) or correspond to known AMPK targets. In fact, immunoprecipitation experiments do not allow discriminating between binary AMPK protein interaction and formation of higher order protein complexes.

In this study, we aim at identifying AMPK substrates *in vitro* using a screen including AMPK-target interaction. This approach is expected to reveal AMPK substrates that interact with the protein kinase rather strongly and potentially show a preference for specific AMPK isoform-combinations. For this purpose, we have set up a screening procedure involving prefractionation, a biophysical interaction screen using surface plasmon resonance (SPR), and a final *in vitro* kinase assay followed by protein identification with mass spectrometry (MALDI-MS/MS) already successfully applied in an earlier study (Tuerk et al. 2007). From the list of putative AMPK targets obtained, we could (i) confirm two new AMPK targets, fumarate hydratase (FH) and fatty-acid binding protein 1 (FABP1), by yeast two-hybrid and kinase assays, (ii) show interaction and phosphorylation in an AMPK isoform-specific manner, and (iii) reveal AMPK-dependent enzymatic activation of FH *in vitro*.

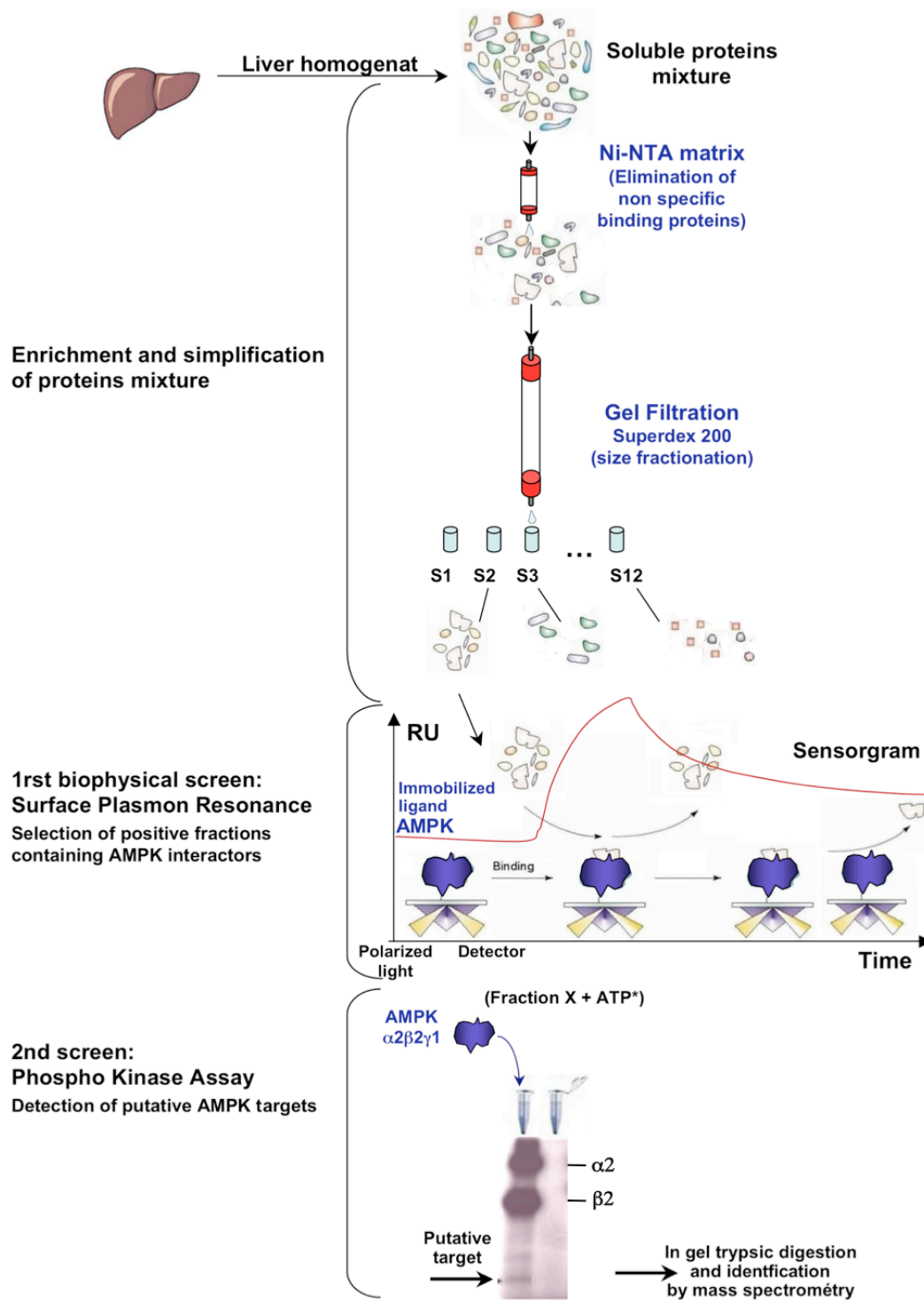


FIGURE 5-1. **Flow chart of the *in vitro* screening procedure.** A tissue-derived extract containing soluble proteins is pre-fractionated by two serially connected columns with different sorbent chemistries. Each fraction is then analyzed by SPR for the presence of AMPK221 interacting proteins. Positive fractions are subjected to *in vitro* phosphorylation assays for detection of potential downstream targets. After SDS-PAGE separation and autoradiography, positive lanes are excised and proteins identified by mass spectrometry.



## 2. RESULTS

### 2.1. Setup of an *in vitro* target screen coupling biophysical interaction to a kinase assay

With the rationale to use the affinity between the kinase and its substrates as an additional parameter in a two-dimensional screening matrix for new substrates, we set out the following strategy. Starting with soluble proteins within a cell extract of rat liver, we (i) reduced complexity via gel filtration chromatography, (ii) screened for fractions containing AMPK interactors by using Biacore SPR with His-tagged AMPK $\alpha$ 2 $\beta$ 2 $\gamma$ 1 (AMPK221) immobilized on an NTA sensor chip, and finally (iii) screened SPR-positive fractions by using *in vitro* kinase assays with constitutively active AMPK221 (Fig. 5-1). Preliminary experiments revealed a high degree of non-specific binding of proteins to the NTA surface in the SPR screening step (not shown). To remove such non-specifically binding proteins, we added Ni-NTA chromatography as first prefractionation step and only proceeded with the flow through (Fig. 5-1).

For further prefractionation, size exclusion chromatography with a Superdex 200 column was chosen, allowing the separation of molecules ranging from 10 to 600 kDa. In fact, it is important for the subsequent SPR screen to compare in a single fraction proteins of similar size. The SPR signal is directly correlated to the refractive index, i.e. density of the chip surface, and thus not only to the number but also to the size of the bound protein. Protein eluted from the column was collected in 12 fractions and individually analyzed by Biacore SPR (Fig. 5-2). Since unprocessed chromatography fractions with different refractive index as compared to running buffer already produce a SPR signal during injection phase, a reporting point was chosen during dissociation phase (80 s after dissociation start) to evaluate the results. We designated a fraction as positive when its SPR response was higher on the AMPK221 surface as compared to a blank surface. This was the case for seven fractions, S1, S2, S3, S4, S10, S11 and S12. Fraction S1 was discarded, since chromatography and SDS-PAGE suggested that it contains mainly aggregates (data not shown).

Positive fractions (S2, S3, S4, S10, S11 and S12) supposed to contain putative AMPK221 interacting proteins, as well as an interaction-negative fraction (S6) were subjected to a kinase assay with constitutively active AMPK221 to screen for putative targets (Fig. 5-1). Assays performed with and without active kinase yielded ten AMPK-specific bands in the interaction-positive fractions and three in the interaction-negative fraction S6 (Fig. 5-3).

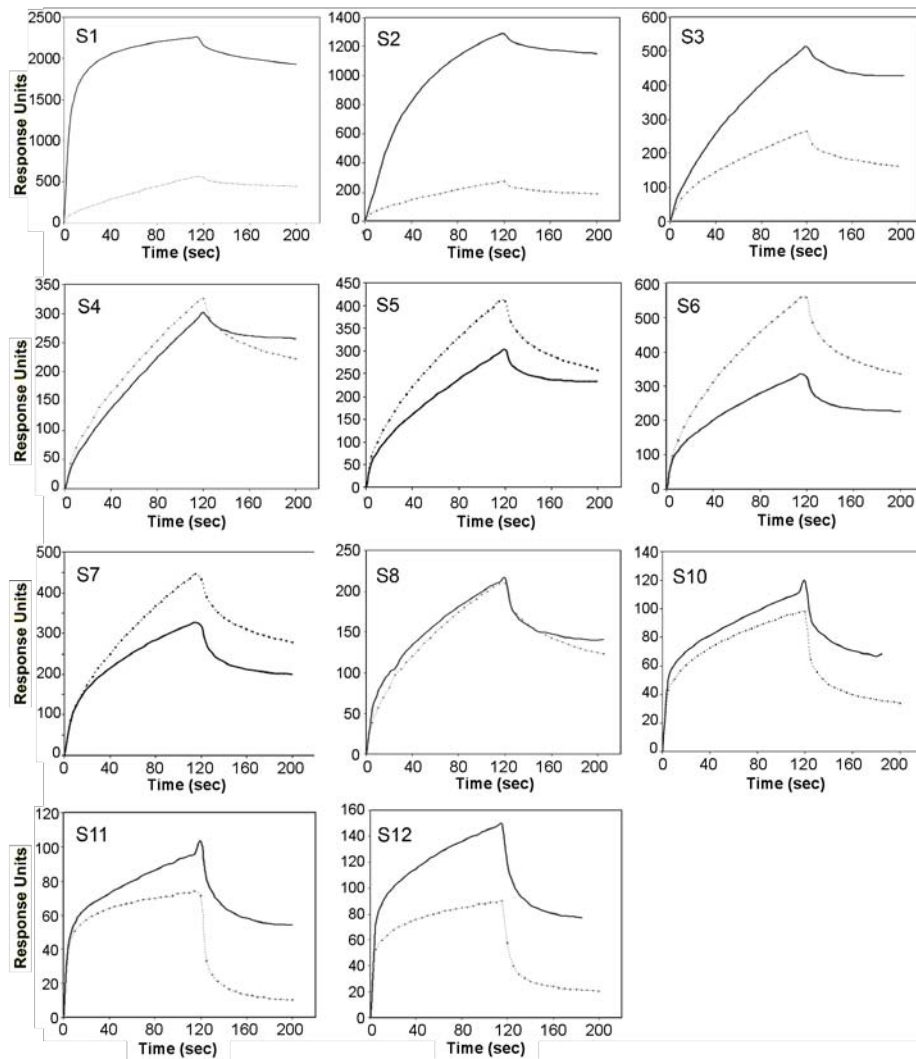


FIGURE 5-2. **SPR interaction screening.** Sensorgrams of different fractions from size exclusion chromatography injected onto immobilized AMPK221 (full lines) compared to association/dissociation on empty surfaces (control, dotted lines).

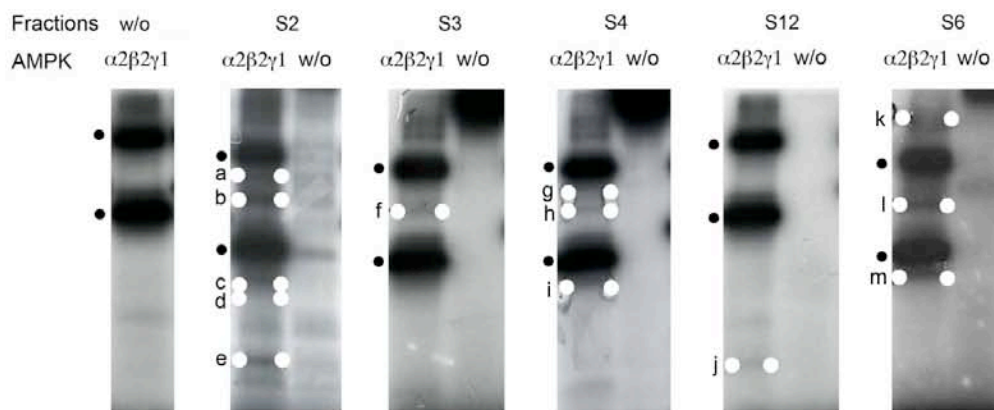


FIGURE 5-3. **Kinase assay screening.** SPR positive fractions and negative fraction S6 were subjected to *in vitro* phosphorylation assays with constitutively active  $\alpha$ T172D AMPK221 or without added AMPK (w/o, control) and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  for 2 min at  $37^\circ\text{C}$ . Assay mixtures analyzed by SDS-PAGE and Typhoon phospho-imager revealed AMPK auto-phosphorylation of  $\alpha$  and  $\beta$  subunits (•) and putative AMPK targets (○, white circles.). Fractions S10 and S11 are similar to S12 and not shown.

## 2.2. Identification of candidate targets of AMPK.

AMPK-specific bands were excised, subjected to tryptic in-gel digestion and analyzed by MALDI-MS/MS mass spectrometry. Results indicated that these samples still contained several vertebrate proteins. Table 5-1 only gives those proteins that could be identified with significant MASCOTT score (> 50). To select candidates for follow-up experiments, we therefore applied as additional filter the following conditions: (i) their presence in the cytosol, since AMPK has not been shown to have intra-mitochondrial localization, and (ii) the presence of at least one AMPK phosphorylation consensus motif  $\phi(X,\beta)XXS/TXXX\phi$  (where  $\phi$  represents a hydrophobic residue and  $\beta$  a basic residue). The resulting target candidates are, fumarate hydratase (FH; accession number P14408) and fatty-acid binding protein 1 (FABP1 or FABPL; accession number P02692). They are highlighted in grey in Table 5-1. Gamma-actin has already been identified as a putative AMPK target in our earlier *in vitro* phosphorylation screen using the AMPK111 complex (Tuerk et al. 2007), but contained only non-stringent phosphorylation sites. The FH gene product shows a dual localization in mitochondria and cytosol (Regev-Rudzki et al. 2009), with the preprotein (54 kDa) showing three putative AMPK sites, while the mature proteins lacking the mitochondrial target peptide (50 kDa) and retains only one consensus motif. FABP1 is a small protein of 14.6 kDa that carries a single AMPK consensus motif.

TABLE 5-1. Protein identification by mass spectrometry.

Fraction	Band	Identified protein	Accession no.	Score	Mass (kDa)	Peptides	Consensus site	Subcellular localization
<i>Biacore positive fractions</i>								
S2	a	ATP synthase subunit $\alpha$	P15999	281	60	5	Yes (1)	mitochondria
		Cytochrome P450 2D26	P10634	150	57	3	Yes (1)	mitochondria
	b	3-ketoacyl-CoA thiolase	P13437	210	41	6	Yes (1)	mitochondria
S4	g	Aldehyde dehydrogenase	P11884	74	54	6	Yes (1)	mitochondria
	h	$\gamma$ -actin	P63259	104	42	2	No	cytoplasm
		<b>Fumarate hydratase (FH)</b>	P14408-2	101	54	3	Yes (1)	cytoplasm
S12	j	<b>Fatty-acid binding protein 1 (FABP1)</b>	P02692	53	14,6	1	Yes (1)	cytoplasm
<i>Biacore negative fractions</i>								
S6	l	Fumarate hydratase	P14408	460	54	11	Yes (1)	cytoplasm
		Medium-chain specific acyl-CoA dehydrogenase	P08503	298	47	9	No	mitochondria
	m	Electrontransfer flavoprotein $\beta$	Q68FU3	500	28	9	Yes (1)	mitochondria
		14-3-3 protein $\beta/\alpha$	P35213	192	28	5	No	cytoplasm
		Enoyl-CoA hydratase	P14604	153	31	3	Yes (1)	mitochondria

Candidate targets are highlighted in grey. Selection criteria for candidate substrates were (i) cytosolic localization and presence of the stringent consensus phosphorylation motif  $\phi(X,\beta)XXS/TXXX\phi$  ( $\phi$  represents a hydrophobic residue,  $\beta$  a basic residue). Only vertebrate proteins identified with a significant MASCOTT score and different from AMPK subunits are shown.

## 2.3. FH and FABP1 preferentially interact with AMPK $\alpha$ 2 and (partially) $\beta$ 2 subunits.

We next wanted to confirm whether the newly identified FABP1 and FH are in fact those proteins that were directly interacting with AMPK221 in the first dimension and phosphorylated by AMPK221 in the second dimension of our screen.

To confirm direct protein-protein interactions in an *in vivo* environment, we applied a cytosolic yeast two-hybrid (Y2H) assay. This is complementary to the SPR method applied in the previous screening protocol (Bruckner et al. 2009). The applied Y2H is a variant of the split-protein Cyto-Y2H system (Mockli et al. 2007). The bait fused to a transcription factor is anchored in the plasma membrane and bait-prey interaction leads to reconstitution of ubiquitin, triggering release of transcription factor by ubiquitin-specific proteases and thus expression of reporter genes (for details see in Part V). Different AMPK subunits ( $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$  and  $\beta 2$ ), FABP1 and the mature form of FH were cloned into Cyto-Y2H vectors. The unrelated Large T antigen of *Simian virus* served as negative control. The analysis of pairwise protein-protein interactions (Fig. 5-4) revealed that FH interacted with the  $\alpha 2$  and  $\beta 2$  AMPK subunits, but not with  $\alpha 1$  and  $\beta 1$  subunits. FABP1 showed the same specificity for  $\alpha 2$ , while it interacted only very weakly with both  $\beta 1$  and  $\beta 2$ . These results confirmed that FH and FABP1 are (i) true interactors of AMPK and (ii) specifically interacting with the AMPK $\alpha 2$  catalytic subunit as well as, in case of FH, the AMPK $\beta 2$  regulatory subunit. These are the two subunits present in the AMPK complex used for the initial SPR screen. Moreover, this result suggests that, at least for the putative targets identified here, different AMPK isoforms can diverge in their substrate recognition.

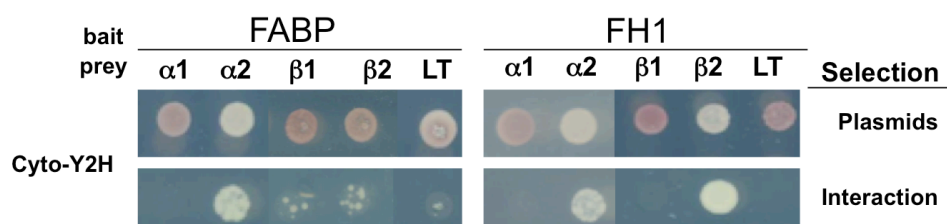
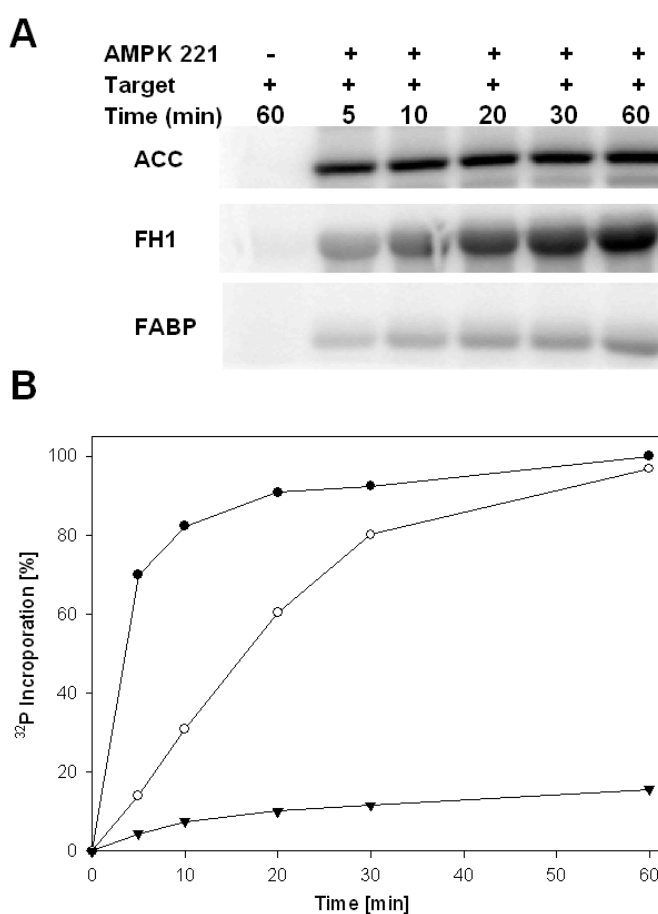


FIGURE 5-4. **FABP1 and FH directly interact with AMPK in Y2H analysis.** A cytosolic Y2H system was applied to analyze interaction of AMPK with FABP1 and FH. Both proteins were expressed as fusion proteins of a membrane anchor and the C-terminal end of ubiquitin conjugated to a transcription factor (bait) and AMPK subunits as fusions to the N-terminal end of ubiquitin (prey). Interactions were detected by activation of reporter gene transcription allowing growth on medium lacking adenine and histidine. Spots represent yeast grown for 72h at 30°C (1/10 dilution).  $\alpha 1/2$ , AMPK  $\alpha$ -subunit 1 or 2;  $\beta 1/2$ , AMPK  $\beta$ -subunit 1 or 2. Control: LT, Large T Antigen of *Simian Virus* (aa 84-704; negative control). For more details see Part V - Experimental Procedures.

#### 2.4. FH and FABP1 are directly phosphorylated by AMPK221

To verify direct phosphorylation of FH and FABP1 by AMPK, we set up kinase assays with purified candidate substrates. This is not only important because MS/MS detected the most abundant proteins in the tryptic digest which may not be identical with the phosphorylated ones, but also because candidate targets may have been phosphorylated by an upstream kinase that co-eluted in a particular fraction and that was itself activated by AMPK. Rat FH (mature form lacking signal peptide) and FABP1 were cloned, expressed and purified as Strep-tagged recombinant proteins. Both proteins and the reference AMPK target acetyl-CoA carboxylase (ACC) as positive control were

incubated *in vitro* with [ $\gamma$ - $^{32}$ P]ATP in the presence or absence of recombinant AMPK221 that was previously activated by the AMPK upstream kinase CamKK $\beta$ . Incorporation of  $^{32}$ P was detected by SDS-PAGE and subsequent autoradiography. As shown in Figure 5-5, both candidate targets, FH and FABP1, were directly phosphorylated by AMPK221 in a time-dependent manner. Both proteins are thus, at least *in vitro*, true targets of AMPK. Incorporation of  $^{32}$ P into FH reached similar levels as in ACC, albeit with a somewhat slower kinetics, arguing for a 1:1 stoichiometry of phosphorylation.  $^{32}$ P incorporation was lower in recombinant FABP1, and appeared also low relative to endogenous liver protein as observed in the previous screen (Fig. 5-3, S12). Possibly, additional factors or secondary modifications were missing in this recombinant protein.



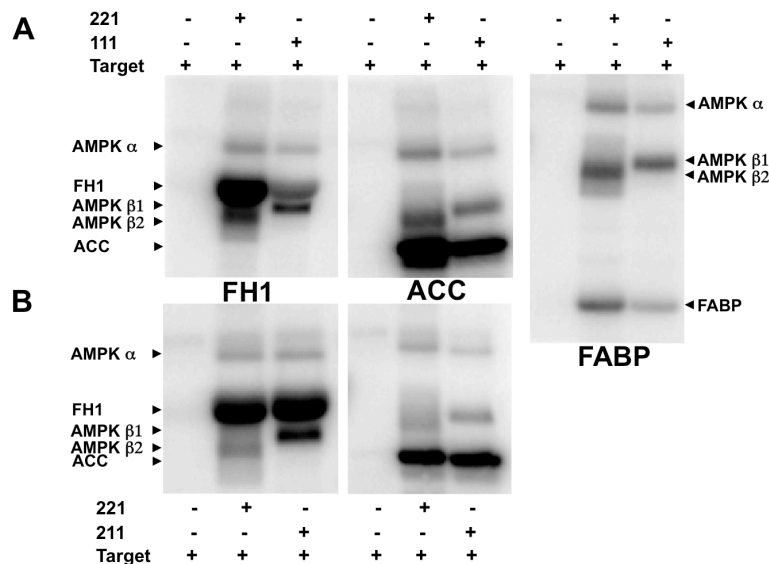
**FIGURE 5-5. FABP1 and FH are direct AMPK targets.** (A) Time course of FABP1 and FH phosphorylation by AMPK. AMPK 221WT (4 pmol) first activated by CamKK $\beta$  (1 pmol) was incubated with purified FABP1, FH (200 pmol) or ACC (positive control, 100 pmol) for 5 to 60 min at 37°C. *In vitro* phosphorylation assays were analyzed by SDS-PAGE and Typhoon phosphoimager. (B) Quantification of the phosphorylation time course using Image Quant TL. Data is normalized to maximal ACC phosphorylation. Symbols: GST-ACC (●) FH (○), FABP1 (▼).

The identification of phosphosites is beyond the scope of this study. However, a stringent consensus motif as applied for the selection of substrate candidates (see Table 5-1) is present in both proteins analyzed here, with T133 in FH (I228-G-R-T-H-T-Q-D-A-V237) and S100 in FABP1 (F95-K-G-I-K-S-V-T-E-F104). Few information is available on the molecular basis of divergent substrate recognition of  $\alpha$ 1- and  $\alpha$ 2-subunits. Sequence criteria proposed from an earlier study based on various peptide substrates (Woods et al. 1996) would only partially explain the preferred interaction of AMPK $\alpha$ 2 with FH and FABP1 at the above mentioned putative phosphosites.

### 2.5. FH and FABP1 are preferentially phosphorylated by $\alpha 2$ -containing AMPK complex

We then wanted to know whether our screening protocol aiming at AMPK221 interacting targets had possibly yielded targets that are indeed preferentially phosphorylated by AMPK complexes containing these subunit isoforms. This is at least suggested by the cyto-Y2H results (Fig. 5-4), showing preferential interaction of FH with  $\alpha 2$  and  $\beta 2$  subunits, and of FABP1 at least with  $\alpha 2$ . Like above, recombinant FH, FABP1 and ACC were incubated *in vitro* with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  in presence or absence of three different recombinant AMPK complexes: AMPK221, AMPK211, AMPK111, all previously activated by CamKK $\beta$ . ACC was again taken as a control to account for slightly different specific activities of the AMPK preparations. It is known that the SAMS peptide that is derived from the ACC1 phosphosite is an equally good substrate for  $\alpha 1$ - and  $\alpha 2$ -containing AMPK complexes (Woods et al. 1996).

**FIGURE 5-6. AMPK isoform composition affects FH and FABP1 phosphorylation.** (A) AMPK-221 phosphorylates FH and FABP1 more efficiently than -111. (B) AMPK-221 and -211 phosphorylate FH and ACC with similar efficiency. Conditions: AMPK-221, -211 or -111 (3 pmol) first activated by CamKK $\beta$  (1 pmol) were incubated with purified FH, FABP1 or ACC (200 pmol) for 8 min at 37°C. *In vitro* phosphorylation assays were analyzed by SDS-PAGE and Typhoon phosphoimager.



We first investigated the effect of different  $\alpha$ -subunits on FH phosphorylation (Fig. 5-6A). The phosphorylation of ACC by CamKK $\beta$ -activated AMPK221 and -111 was somewhat different with a ratio of 1,5 for P-ACC<sub>(221)</sub>/P-ACC<sub>(111)</sub>. However, when using FH as a substrate, the ratio P-FH<sub>(221)</sub>/P-FH<sub>(111)</sub> was increased to 5,0. Thus, FH is a much better substrate of AMPK $\alpha 2$  compared to AMPK $\alpha 1$ . To a minor degree, the same was true for FABP with a ration of 2,0 for P-FABP<sub>(221)</sub>/P-FABP<sub>(111)</sub>.

Then the effect of different  $\beta$  subunits was analyzed. Here, complexes AMPK221 and AMPK211 phosphorylated both FH and ACC with similar efficiency, with calculated ratios of 0.9 for P-FH<sub>(221)</sub>/P-FH<sub>(211)</sub> and 1,1 for P-ACC<sub>(221)</sub>/P-ACC<sub>(211)</sub> (Fig. 5-6B). FABP1 did neither show different phosphorylation with the two AMPK complexes (not shown). Thus, at least *in vitro*, AMPK  $\beta$ -subunits have no effect on FH and FABP1 phosphorylation.

In conclusion, these results strongly suggest that for some substrates, AMPK isoform composition can determine substrate specificity. FH and FABP1 interact with AMPK $\alpha 2$  and are preferentially

phosphorylated by  $\alpha$ 2-containing complexes. FH and, to a much lesser degree, FABP1 also interact with the  $\beta$ 2-subunit, but this does not affect phosphorylation efficiency.

### 2.6. FH phosphorylation by AMPK221 increases its catalytic efficiency

We have chosen FH for further experiments addressing the effect of substrate phosphorylation because of several reasons: (i) recombinant protein is obtained in large quantities, (ii) it showed the highest phosphorylation stoichiometry *in vitro*, and (iii) enzymatic activity of FH can be exactly quantified *in vitro*. FH catalyzes the reversible hydration/dehydration of fumarate to malate involved in the tricarboxylic acid (TCA) cycle and in the metabolism of fumarate produced by the urea cycle. We determined enzyme kinetics of FH before and after *in vitro* phosphorylation by CamKK $\beta$ -activated AMPK221, using malate as substrate and measuring fumarate formation by spectrophotometry. FH phosphorylation led to a 37 % increase in  $k_{cat}$  whereas the apparent  $K_m$  remained almost unaffected (Fig. 5-7; Table 5-2).

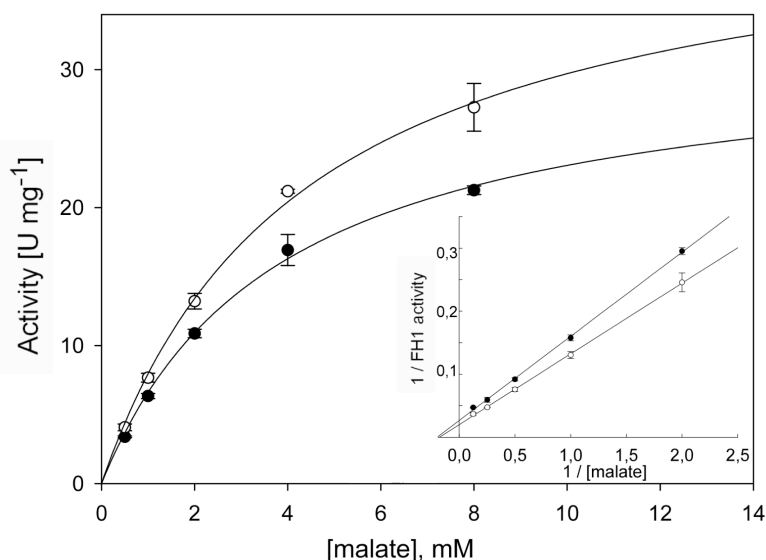


FIGURE 5-7. **AMPK-mediated phosphorylation affects enzymatic catalysis of FH.** Enzyme activity of phosphorylated ( $\circ$ ) and non-phosphorylated ( $\bullet$ ) FH at different concentrations of malate, measured as described in Experimental Section (Part V). The direct fit to original data (large graph) and Lineweaver-Burk plot (insert graph) are shown. The derived catalytic constants are given in Table 5-2.

TABLE 5-2. **Phosphorylation by AMPK activates FH.** Enzyme kinetic parameters of recombinant FH before and after phosphorylation with AMPK 221 WT activated by CamKK $\beta$ .

	$V_{max}$ ( $U\ mg^{-1}$ )	$k_{cat}$ ( $s^{-1}$ )	$K_m$ (malate) ( $mM$ )	$k_{cat}/K_m$ ( $mM^{-1}\ s^{-1}$ )
FH	$32,9 \pm 0,4$	$110,0 \pm 1,3$	$4,2 \pm 0,1$	26,2
P-FH	$45,1 \pm 1,4$	$150,8 \pm 4,7$	$4,6 \pm 0,3$	32,8

Measurements with variable concentrations of malate at 25°C. Enzyme activity is given in U, equivalent to 1  $\mu$ mol/min. Catalytic efficiency is calculated as  $k_{cat}/K_m$ . Results are given as means  $\pm$  SE (n=2) of two independent phosphorylation experiments.

### 3. DISCUSSION

We have developed an original two-dimensional *in vitro* screen combining a biophysical interaction assay based on surface plasmon resonance with a phosphorylation assay in order to identify new AMPK targets. Using rat liver extracts, we provide a proof of principle that such a screening protocol can indeed discover AMPK substrates that are novel and phosphorylated in an AMPK isoform-specific manner. We show at the example of FH and FABP1 that mainly the  $\alpha$ -subunit of AMPK is involved in substrate interaction and recognition, as well as in substrate-specific phosphorylation. In case of FH, the phosphorylation increases enzymatic turnover.

SPR technology has so far not been used to explore kinase/substrate interaction for screening of novel kinase substrates. It has only been applied as high-throughput readout device to measure interactions between phosphorylated kinase substrates and anti-phospho antibodies (Takeda et al. 2006, Takeda et al. 2010). We show here that SPR can detect AMPK interactors in complex mixtures of soluble proteins when different conditions are satisfied: (i) need of highly pure, native kinase, as we have established by a polycistronic bacterial expression (Neumann et al. 2003b); (ii) the use of a readily recoverable chip surface as e.g. with Ni-NTA, allowing repeated immobilization of fresh kinase; (iii) prior removal of proteins with non-specific affinity to the chip surface, particularly relevant for Ni-NTA; (iv) the use of prefractionated extracts as source of soluble protein. We have assembled these conditions in our protocol (Fig. 5-1), providing exploitable data. However, the resolving power of the screen may be improved by using multidimensional prefractionation (e.g. combining ion exchange with size exclusion chromatography) or detection approaches (e.g. 2D-PAGE).

Importantly, fractions positive in SPR for AMPK221 interacting proteins contained several AMPK target candidates, two of them novel candidates first described in this report, namely FH (or fumarase) and FABP1. Another member of the FABP family, FABP5, has been already found associated to AMPK $\beta$ 1 in an anti-bait co-immunoprecipitation study (Ewing et al. 2007). We found that FH and FABP1 were not only interacting exclusively with the  $\alpha$ 2- and (in case of FH)  $\beta$ 2-subunits used in the SPR screening, and not with  $\alpha$ 1 and  $\beta$ 1, but they were also preferentially phosphorylated by AMPK complexes containing the  $\alpha$ 2 subunit as compared to those containing  $\alpha$ 1. This is one of the first reports showing substrate specificity of mammalian AMPK complexes *in vitro*. Such substrate specificity may contribute to tissue- and compartment-specific AMPK signaling whose mechanisms are so far largely unsolved. SPR may also prove to be useful for identification of further elements in AMPK signaling specificity, such as scaffolding proteins or regulatory interactors.

FH or fumarase is encoded by a single gene, but dual-targeted to mitochondria and cytosol by a mitochondria-dependent and metabolically regulated mechanism that has been studied in detail in yeast (Regev-Rudzki et al. 2009). After cleavage of the signal peptide in mitochondria, identical mature FH variants relocate to the mitochondrial matrix or the cytosol. We show AMPK-dependent



phosphorylation and activation of such mature FH enzyme, which can be supposed to happen outside mitochondria given the known cellular distribution of AMPK. However, while the function of FH in the TCA cycle is known since decades, its function in the cytosol has only been discovered most recently. Here, the enzyme does not only scavenge fumarate produced in the urea cycle or from degradation of amino acids, but also functions as regulator of HIF stress signaling (O'Flaherty et al. 2010) and most importantly as a DNA damage response protein (Yogev et al. 2010). Upon cell stress leading to DNA double-strand breaks, FH translocates to the nucleus, where its enzymatic activity is required to activate the double-strand break response and DNA repair machinery. Several lines of evidence point to AMPK-dependent phosphorylation of FH as an important role in this sequence of events: (i) Fumarase, like the LKB1-AMPK pathway, has been identified as tumor suppressor (Tomlinson et al. 2002). (ii) Different pathways link sensors of DNA damage and genotoxic stress to activation of AMPK to preserve genomic integrity, including p53 or ataxia-telangiectasia mutated protein (ATM), which activate AMPK via the p53 targets Sestrin1 and Sestrin2 (Budanov et al. 2008) or AMPK upstream kinase LKB1 (Alexander et al. 2010), respectively. (iii) Only AMPK complexes containing the  $\alpha 2$  isoform show nuclear localization (Salt et al. 1998), in agreement with our finding that the activating phosphorylation of FH is largely dependent on  $\alpha 2$  subunits. Therefore, we propose that the tumor suppressor activity of AMPK includes a DNA repair response via phosphorylation of FH occurring under genotoxic stress. This would occur either in the nucleus, further activating FH to trigger the DNA repair response, or in the cytosol, possibly interfering with nuclear targeting of FH.

FABP1 is member of a family of small intracellular lipid binding proteins, which function as intracellular carriers of hydrophobic components such as retinoids, long chain fatty acid (FA) and bile acids with high affinity (for reviews see (Wolfrum 2007, Storch et al. 2009)). Liver FABP1 is abundantly found in hepatocytes, but is also expressed in some other tissues like stomach, pancreas, intestine and kidney. Recombinant FABP1 was phosphorylated by AMPK albeit only at low level when compared to ACC. This could be due to specific properties of FABP. First, FABP1 is associated *in vivo* with fatty acids and other intracellular proteins which directly impact on FABP structure (Storch et al. 2009). Such cofactors are possibly still present in the initial phosphorylation screen but lacking in recombinant protein. Second, FABP1 interacted in our study only with the  $\alpha 2$  subunit, not with  $\beta 2$  as it was the case with FH, possibly leading to lower affinity to the kinase. In any case, FABP1 represents an attractive substrate candidate within AMPK signaling under energy stress. A key effect of activated AMPK is stimulation of FA import and FA oxidation via translocation of FA transporter FAT/CD36 (van Oort et al. 2009) and regulation of ACC (Hardie et al. 2002), respectively. In liver, both FA uptake and  $\beta$ -oxidation are regulated in correlation with intracellular FABP1 levels as e.g. in FABP1 knock out mice (Wolfrum 2007). Thus, all three processes are closely linked. We propose that AMPK could affect intracellular fatty acid transport via interaction and phosphorylation.

In summary, we provide proof of concept that combining classical *in vitro* AMPK phosphorylation assays with SPR-based AMPK-protein interaction screening can not only identify novel AMPK substrates but also enrich for AMPK isoform-specific targets. FH and FABP are exclusively interacting with and preferentially phosphorylated by  $\alpha 2$ -containing AMPK complexes. Activation of FH by AMPK-dependent phosphorylation, together with recent findings on FH functions in the DNA damage response, open new avenues for research linking AMPK to the maintenance of genetic stability and tumor suppressor function.

#### 4. ACKNOWLEDGEMENTS

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**- CHAPTER VI -****HIGH AFFINITY INTERACTION OF AMP-ACTIVATED PROTEIN KINASE WITH GLUTATHIONE TRANSFERASES MU AND PI LEADS TO THEIR PHOSPHORYLATION AND PI-1 ENZYMATIC ACTIVATION****Anna Brückner<sup>1</sup>, Alexandre Berthier<sup>1</sup>, Cécile Polge<sup>1</sup>, Sacniete Ramirez<sup>1</sup>, Nicolas Lentze<sup>2</sup>, Daniel Auerbach<sup>2</sup>, and Uwe Schlattner<sup>1</sup>**<sup>1</sup>Inserm, U884, Grenoble, France; Laboratory of Fundamental and Applied Bioenergetics, University Joseph Fourier, Grenoble, France, <sup>2</sup>Dualsystems Biotech AG, Schlieren, Switzerland.*Revised version to be resubmitted to Journal of Biochemistry*

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**Abstract:** AMP-activated protein kinase (AMPK) is a cellular and whole body energy sensor with manifold functions in regulating energy homeostasis, cell morphology and proliferation in health and disease. Here we implicate AMPK in the cellular anti-oxidative and detoxification machinery by characterizing AMPK interactions with isoforms of glutathione S-transferase (GST), using different cytosolic yeast two-hybrid (Y2H) techniques, classical pull-down or immunoprecipitation, and quantitative SPR. AMPK interacted *in vitro* and *in vivo* (Y2H and rat liver) with GSTs of the Mu-family, including mammalian GSTM1 and GST of the parasite *Schistosoma japonicum* used for tagging protein. The latter imposes a note of caution for the use of GST-fusion proteins in AMPK research. Other mammalian GST isoforms like Pi-family member P1 also interacted with AMPK, but less so members of the Alpha family. AMPK revealed high affinity for GST ( $K_D$  for M1 at about 5 nM). The N-terminal domain of AMPK beta was sufficient to induce this interaction. Activated AMPK phosphorylated mammalian GSTM1 and -P1 in a glutathione-independent manner, which in turn reduced interaction with the transferase. Importantly, this led to a strong increase in catalytic efficiency of GSTP1, but not GSTM1. Interaction with and phosphorylation of GST did not alter AMPK activation or AMPK target phosphorylation. GST interaction, phosphorylation and activation by AMPK is proposed to contribute to oxidative stress defense, but may also interfere with further of the pleiotropic GST functions.

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**1. INTRODUCTION**

AMP-activated protein kinase (AMPK) is an evolutionary conserved serine/threonine kinase playing a central role in sensing and regulating energy homeostasis at the cellular and whole-body level (reviewed in (Kahn et al. 2005, Hardie 2007, Neumann et al. 2007, Viollet et al.)). In response to diverse external and internal stimuli signaling impaired cellular energy state in diverse physiological and pathological situations (hormones, nutrients, AMP), the heterotrimeric AMPK is activated covalently by phosphorylation on the  $\alpha$ -subunit and allosterically by AMP binding to the  $\gamma$ -subunit, involving a conformational change (Riek et al. 2008). Increasing evidence suggests that AMPK is also activated by reactive oxygen or nitrogen species (ROS, RNS), either via their inhibitory effect on mitochondrial ATP generation that increases AMP/ATP ratios (Hawley et al.), or putative effects on AMPK upstream signaling as we have shown for peroxynitrite (Zou et al. 2004, Xie et al. 2008). Activated AMPK then inhibits ATP-consuming (anabolic) processes (e.g. fatty acid and glycogen

synthesis) and stimulates ATP-generating (catabolic) processes (e.g. fatty acid oxidation, glucose uptake, glycolysis) (Kahn et al. 2005, Hardie 2007, Neumann et al. 2007, Viollet et al.).

AMPK thus integrates various input signals on the energy and nutritional state of the cell and the entire organism to finally trigger a large variety of cellular responses to relieve energy stress. Further roles of AMPK beyond the pure regulation of cellular energy homeostasis became apparent more recently, such as in the central nervous system e.g. for hypothalamic appetite regulation, or in controlling cell shape or proliferation (Minokoshi et al. 2004, Lee et al. 2007). Due to its links to metabolic and proliferation signaling, AMPK became a prime pharmacological target for treating type II diabetes, the metabolic syndrome or cancer (Zhang et al. 2009, Fogarty et al.).

Despite this widespread potential application of AMPK-targeted pharmaceutical intervention, the AMPK signaling network is far from being fully understood, thus potentially limiting the development of save AMPK-targeted drugs. The multitude of targets and cell-type specific responses of AMPK suggest the involvement of specific protein/protein interactions in addition to target phosphorylation. *In vitro* phosphorylation screens (Tuerk et al. 2007) and *in vivo* yeast two-hybrid techniques (Solaz-Fuster et al. 2006, Pan et al. 2008, Bruckner et al. 2009, Fu et al. 2009, Moreno et al. 2009, Vernia et al. 2009, Song et al.) have been successfully applied by us and others to characterize the AMPK signaling network.

Glutathione S-transferases (GSTs) form a superfamily of ubiquitous proteins with multiple enzymatic and non-enzymatic functions. A common feature of GSTs is their ability to catalyze the binding of electrophilic compounds to the thiol group of glutathione, rendering the products more water-soluble and thus facilitating their elimination from the cell (reviewed in (Hayes et al. 2005, Frova 2006, Lo et al. 2007)). This reaction is very important for eliminating secondary, highly reactive metabolites formed during oxidative stress, and also for detoxification of xenobiotics, elimination of chemotherapeutic drugs and some biosynthetic pathways. GSTs can also serve as peroxidases, isomerases, thiol transferases and dehydroascorbate reductases, many of these activities being also linked to antioxidative defense (Hayes et al. 2005, Frova 2006). Additional, non-enzymatic functions have been described for some GSTs as ligands or signal transduction modulators of JNK, ASK1, PKC, PKA or EGFR signaling, during which either the interacting protein partner undergoes functional alteration or the GST protein itself is posttranslationally modified and/or functionally altered or relocated within the cell (Adler et al. 1999, Yin et al. 2000, Cho et al. 2001, Gilot et al. 2002, Lo et al. 2007). In particular GSTP1 was proposed to initiate a coordinated regulation of stress kinases under oxidative stress to reduce cell death (Adler et al. 1999, Yin et al. 2000).

In mammals, 15-20 different GST genes have been identified that give rise to seven classes of cytosolic GSTs, including the Alpha, Mu, and Pi families (Hayes et al. 2005, Frova 2006). Lower eukaryotes also contain GST orthologs of these mammalian GSTs. A GST isoform of the unicellular parasite *Schistosoma japonicum* (*S. japonicum*) used in the popular GST-tag (Smith 2000) also belongs to the Mu-family. In such protein fusions, GST favors good solubility during expression e.g. in *E. coli* and provides a high binding capacity to glutathione facilitating protein purification.

Here we reveal a high affinity interaction between AMPK and GST Mu and Pi isoforms by four independent, complementary *in vivo* and *in vitro* interaction assays, including yeast two-hybrid, pull-down, immunoprecipitation and surface plasmon resonance (SPR). We show that GST/AMPK complexes involve the AMPK  $\beta$ - subunit N-terminus, are modulated by AMPK activation, lead to GST phosphorylation and enzymatic activation of GSTP1. Caution is indicated when working with GST-engineered constructs together with AMPK.

## 2. RESULTS

### 2.1. *Schistosoma* GST interacts directly with AMPK via its $\beta$ -subunit N-terminal domain

Tagging proteins with GST has become a widely used method to improve and facilitate their recombinant expression and purification, as well as to enrich interaction partners of the fused protein by pull-down assays with immobilized glutathione. By applying such interactomic approaches, we observed pull-down of AMPK with GST from *Schistosoma japonica* (GST-Sj). To verify a potential direct interaction between GST-Sj and AMPK, we applied two different cytosolic Y2H assays (Bruckner et al. 2009).

Cyto-Y2H is a cytosolic, split-protein-based assay where bait/prey interaction leads to reconstitution of ubiquitin, subsequent release of transcription factor by ubiquitin-specific proteases, and transcription of reporter genes that allow growth on nutrient-deficient medium. Such transcriptional read-out is very sensitive and can detect also weak or transient interactions. Cyto-Y2H clearly revealed GST-Sj interaction with AMPK  $\beta$ 1 and  $\beta$ 2, but not with AMPK  $\alpha$ 1- or  $\alpha$ 2-subunits (Fig. 6-1A, left). The significance of this result is confirmed by (i) control interactions yielding the expected results, i.e. confirming GST homodimerization and AMPK  $\alpha/\beta$ -subunit interaction, while showing no interaction with unrelated protein Large T (LT; Fig. 6-1A, right), and (ii) GST-Sj in both orientations, i.e. as bait or as prey construct, yielding similar results (not shown). We further used truncated AMPK  $\beta$  constructs to identify the interaction domain. As shown in Fig. 6-1, an N-terminal domain comprising only amino acids 1-54 ( $\Delta\beta$ 1 or  $\Delta\beta$ 2) was sufficient to induce GST/AMPK interaction in the yeast cytosol (Fig. 6-1A, left).

The cyto-Y2H system set up and applied here differs from the published version (Mockli et al. 2007) in using a plasma membrane-anchored bait. Aware of potential problems with false positives in Y2H assays, in particular if a corresponding data base is still lacking, we used a second, complementary Y2H assay. Split-Trp-Y2H is another cytosolic split-protein-based assay where C- and the N-terminal domain of an enzyme in tryptophan biosynthesis are fused to bait and prey. Their interaction leads to reconstitution of enzymatically active protein and growth on Trp-deficient medium. (Tafelmeyer et al. 2004). This direct read-out lacks intermediate transcriptional amplification, thus lowering the rate of false positives and increasing proportionality between interaction strength and read-out signal. The assay entirely confirmed results obtained with Cyto-Y2H for the GST-Sj/AMPK interaction or positive (GST/GST) and negative (LT) controls (Fig. 6-1B). It is



to note, however, that in this assay the interaction between AMPK  $\alpha$ - and  $\beta$ -subunits appeared as rather weak opposed to what was observed in case of cyto-Y2H.

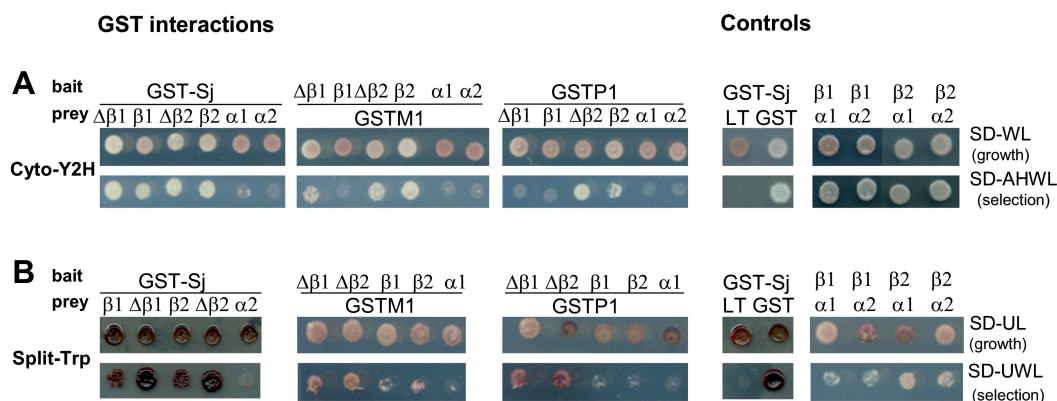
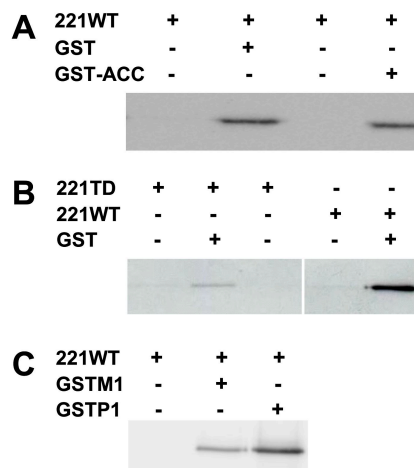


FIGURE 6-1. **Identification of GST isoforms as AMPK interaction partners and of the interacting AMPK domain by Y2H analysis.** Two different cytosolic Y2H systems were applied to analyze interaction of AMPK with three GST isoforms: *Schistosoma japonicum* GST (GST-Sj) and mammalian (rat) GSTM1 and GSTP1. (A) Cyto-Y2H. GST and AMPK subunits were expressed as fusion proteins of a membrane anchor and the C-terminal end of ubiquitin conjugated to a transcription factor (bait) or as fusions to the N-terminal end of ubiquitin (prey). Interactions were detected by activation of reporter gene transcription allowing growth on medium lacking adenine and histidine (SD-AHWL). Spots represent yeast grown for 72h at 30°C. (B) Split-Trp system. GST and AMPK subunits were expressed as fusion proteins to the C-terminal (bait) or the N-terminal (prey) portion of Trp1p. Interactions were detected by growth on medium lacking tryptophan (SD-UWL). Yeast was grown for 8-9 days at 27°C.  $\Delta\beta$ , N-terminal domain of AMPK  $\beta$ -subunit. Controls: LT, Large T Antigen of *Simian Virus* (aa 84-704; negative control); GST, GST-Sj (positive control). For more details see Experimental Procedures.

We further confirmed GST-Sj interaction with full-length, functional AMPK complex using classical GST pull-down assays. When recombinant pure GST-Sj and AMPK 221WT were incubated together, AMPK was present after GST pull-down and washing as detected by immunoblotting (Fig. 6-2A). Since GST-Sj has gained enormous popularity for its use in GST fusion proteins, we also tested as example a fusion of GST-Sj with the catalytic domain of acetyl-CoA carboxylase (ACC) (Scott et al. 2002). ACC does not build stable complexes with AMPK (Aranda et al. 2010, Lee 2010). However, similar quantities of AMPK were pulled down with GST-ACC as with GST-Sj alone (Fig. 6-2A). Finally, GST-Sj pulled down AMPK WT more efficiently than 221TD, indicating more stable complexes with inactive AMPK WT (Fig. 6-2B).

FIGURE 6-2. **GST isoforms and GST-tag interact with full-length AMPK in pull-down assays.** Pure recombinant AMPK  $\alpha2\beta2\gamma1$  wild type (221WT) was pulled down with following proteins: (A) GST-Sj (*S. japonicum*) alone or in fusion with acetyl CoA carboxylase (GST-ACC), (B) GST-Sj with 221WT or constitutively active AMPK mutant (221TD), (C) GSTM1 and -P1 (*R. norvegicus*). In all assays, AMPK (0.075 mg/ml) was incubated with or without (negative control) GST (0.075 mg/ml) and Glutathione Sepharose 4B. Pull-down samples were subjected to immunoblot analysis using anti- $\alpha$  AMPK antibody.



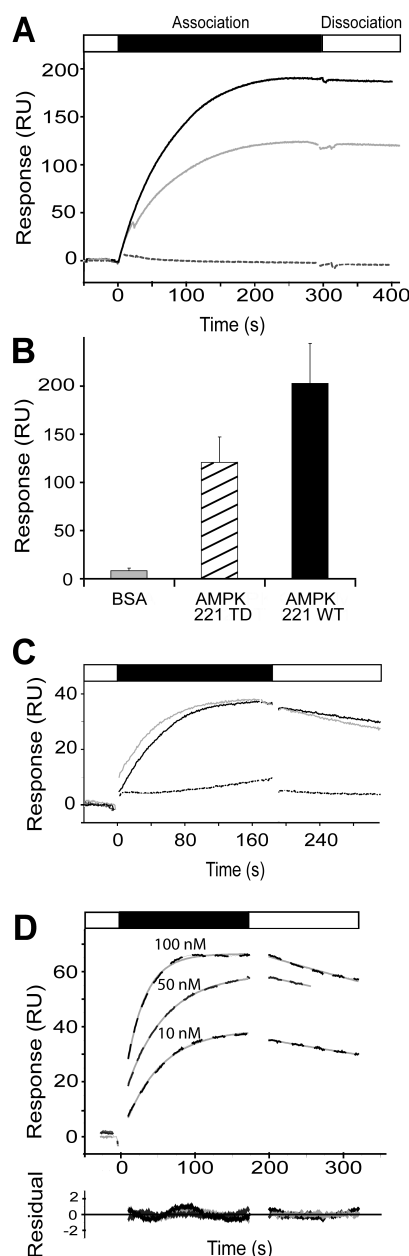
## 2.2. Abundant mammalian GSTM1 and P1 show similar interaction with AMPK

To analyze whether the detected interaction occurs with different eukaryotic GSTs, we cloned, expressed and purified two of the most abundant mammalian cytosolic GSTs, GSTM1 and GSTP1 from a rat cDNA library. GSTM1 is the rat homologue of GST-Sj (44% sequence identity), while GSTP1 is less related to GST-Sj (30% sequence identity). Both mammalian GSTs were assayed with the same AMPK constructs and the same two Y2H systems as GST-Sj (Fig. 6-1A,B). Again, both GSTs did not interact with AMPK  $\alpha$ -subunits, but in almost all examined configurations with the N-terminal domain of  $\beta$ -subunits ( $\Delta\beta$ 1 and  $\Delta\beta$ 2). When growth was prolonged, also interaction with AMPK  $\beta$ -subunits became more evident with the Split-Trp system. In addition, GST pull-down experiments confirmed interaction of GSTM1 and P1 with full-length AMPK (Fig. 6-2C). These results suggest that the GST/AMPK interaction is not specific to one GST family member but involves at least the two different GST classes Mu and Pi.

## 2.3. GST/AMPK interaction is of high affinity

To obtain quantitative data on the GST/AMPK interaction *in vitro* in respect to kinetics and affinity, we performed a series of experiments with surface plasmon resonance spectroscopy (SPR). We first used a sensor chip where the gold surface had been functionalized with a self assembled monolayer that reduces non-specific adsorption to the surface and allows immobilization of low ligand densities. GST-Sj, which turned out to be the more stable interaction partner, was covalently immobilized and 10 nM AMPK 221WT or BSA (negative control) were injected onto this surface (Fig. 6-3A). The resulting sensorgrams showed a direct and specific AMPK/GST interaction with rather fast association and very slow dissociation, indicating high affinity. The equilibrium response was  $203 \pm 41.5$  RU for AMPK 221WT, which was reduced to  $121 \pm 27$  RU for AMPK 221TD, as compared to  $8 \pm 3.7$  RU for BSA as a negative control (Fig. 6-3B).

Conventional CM5 sensor chips were used to confirm the specific interaction of native AMPK with mammalian GST (Fig. 6-3C,D). AMPK 221WT (10 nM) interacted with both GSTM1 and GSTP1. A concentration series of AMPK 221WT was then injected onto GSTM1 to extract further quantitative parameters. The resulting kinetics could be very well fitted to a simple Langmuir 1:1 model (Fig. 6-3D) as seen by the very low residuals of the fit ( $< 1$  RU). The quantitative results confirm a fast association rate ( $k_a = 3,1 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ) and a very slow dissociation rate ( $k_d = 1,6 \cdot 10^{-3} \text{ s}^{-1}$ ), with a resulting  $K_D$  of about 5 nM. The  $K_D$  of the AMPK/GSTP1 interaction was in the same nM range (data not shown).



**FIGURE 6-3. Surface plasmon resonance identifies high affinity interactions between GSTs and AMPK.** Freshly diluted, recombinant full-length AMPK  $\alpha 2\beta 2\gamma 1$  was injected onto immobilized GST. (A) GST-Sj binding of 10 nM AMPK 221WT (black full line), constitutive active AMPK 221TD (grey full line) or BSA (grey dotted line) at a flow rate of 20  $\mu\text{l}/\text{min}$  (surface: self-assembled monolayer). (B) Equilibrium response from (A), mean  $\pm$  SD of 12 (221TD), 6 (221WT) or 3 (BSA) independent experiments. (C) Comparison of GSTM1 (black) or GSTP1 (grey) association and dissociation kinetics of 10 nM AMPK 221WT (full lines) or 100 nM of BSA (dotted lines) and a flow rate of 30  $\mu\text{l}/\text{min}$  (surface: CM5). (D) GSTM1 association and dissociation kinetics of AMPK 221WT at different concentrations (dashed black lines) and a flow rate of 30  $\mu\text{l}/\text{min}$  (surface: CM5), single exponential fit of experimental data (grey lines) and corresponding residuals (to assess the quality of the fit, lower panel). Representative sensorgrams of at least two repetitions are shown. Bars on the top of sensorgrams indicate protein injection (association, black) or injection of running buffer (white).

#### 2.4. AMPK/GST interaction occurs in rat liver

We then investigated the interaction of endogenous GST isoforms and AMPK in liver. First we used specific antibodies for GSTM1/2 and GSTP1 for immunoprecipitation experiments from liver extracts (Fig. 6-4A). Endogenous AMPK co-immunoprecipitated with both GSTs, confirming an *in vivo* interaction between AMPK and these GSTs. Since GSTM1 is more abundant in liver than P1 (Rowe et al. 1997), the prominent co-IP of AMPK with GSTP1 may indicate an even stronger interaction with this isoform *in vivo*.

We then added recombinant AMPK 221WT or 221TD to liver extracts, and detected co-purification of both with endogenous GST by pull-down assays with Glutathione Sepharose beads (Fig. 6-4B). Finally, endogenous AMPK was pulled down together with endogenous GST from the liver extracts. The three major GST isoforms were identified by MALDI mass spectrometry to be GSTA1, GSTA3 and GSTM1 (Fig. 6-4C). Similar amounts of AMPK were pulled down under more

stringent conditions in presence of glutathione, where mainly GSTM1 was detectable, suggesting that GSTM1, but not GSTA1 or A3 are main AMPK interaction partners in liver.

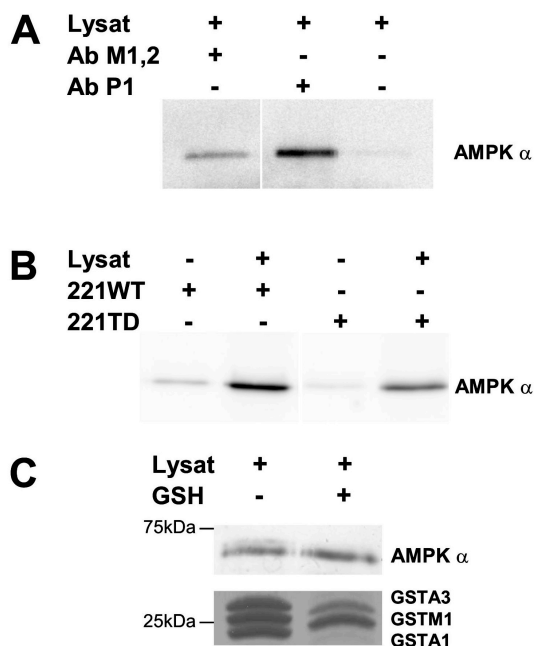


FIGURE 6-4. **AMPK interacts with endogenous GST isoforms in rat liver.** GST immunoprecipitation (A) or pull-downs (B,C) were performed with rat liver extracts. AMPK in pull-down fractions or immunoprecipitates was detected by immunoblot analysis with anti- $\alpha$  AMPK antibody. (A) Immunoprecipitation of endogenous AMPK by anti-GSTM1/2 or anti-GSTP1 antibodies. (B) Pull-down of added constitutively active AMPK  $\alpha 2\beta 2\gamma 1$  (221TD) or wild type (221WT) by endogenous GSTs. (C) Pull-down of endogenous AMPK by different endogenous GST isoforms in absence or presence of glutathione. Note: Addition of glutathione reduces pull-down of GSTA isoforms without affecting pull-down of AMPK.

### 2.5. AMPK activation reduces GST/AMPK interaction

The GST/AMPK interaction could be regulated by glutathione-binding to GST or activation of AMPK. While the presence of glutathione did not affect GST/AMPK interaction in pull-down and SPR experiments (not shown), AMPK activation, i.e. the use of constitutively active AMPK 221TD mutant, reduced GST/AMPK complex formation as compared to 221WT. As observed already with GST-Sj in pull-down assays (Fig. 6-2C) and SPR (Fig. 6-3A,B), this was confirmed in the pull-down assay with endogenous GST (Fig. 6-4B).

### 2.6. GST/AMPK complex formation does not affect AMPK signaling

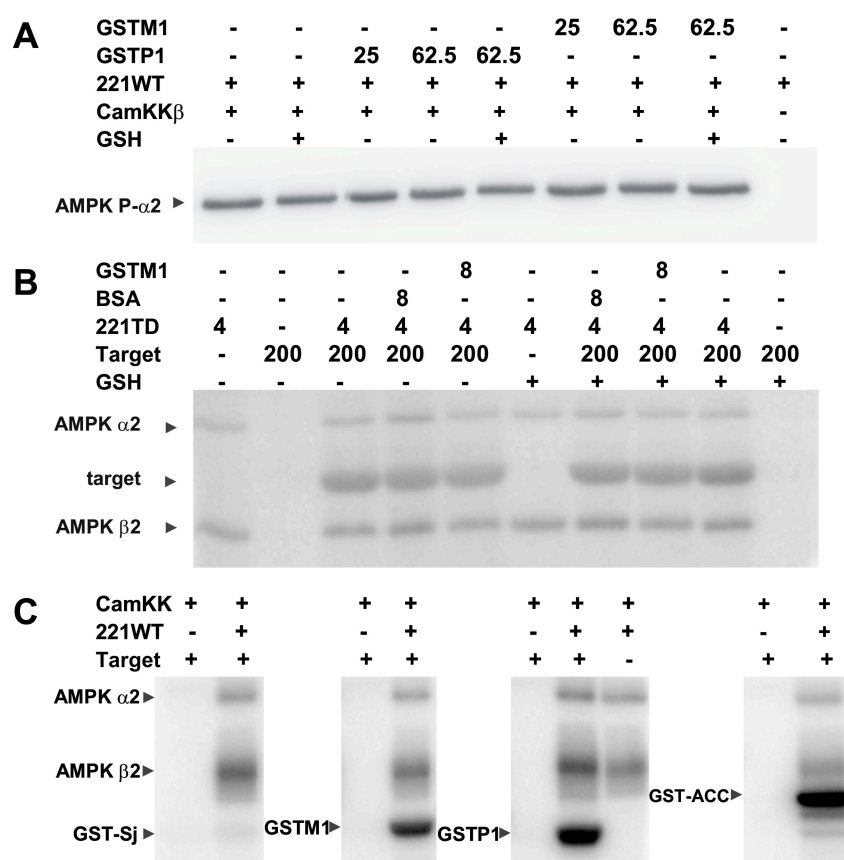
GSTP1 and GSTM1 were shown to interact with stress-related protein kinases like JNK and ASK1 to coordinatively regulate their anti-oxidative and anti-apoptotic signaling pathways (reviewed in (Lo et al. 2007)). We therefore analyzed whether GST also affects energy-stress signaling by AMPK, in particular AMPK activation or phosphorylation of AMPK downstream targets. *In vitro* phosphorylation of AMPK 221WT at  $\alpha T172$  by its upstream kinase CamKK $\beta$  was not modified by GSTM1 or GSTP1 (Fig. 6-5A). GSTM1 did neither affect the capability of AMPK 221TD to phosphorylate an AMPK downstream target currently under investigation in our laboratory (Ramirez-Rios & Schlattner, unpublished data, Fig. 6-5B). Similar results were obtained with GSTP1, even when used at higher concentrations (200 pmol; not shown). Both, AMPK phosphorylation and AMPK target phosphorylation in presence of GST were independent of glutathione (Fig. 6-5A,B).

GST could in theory also modify AMPK properties by direct glutathionylation. However, we could not detect GST-dependent incorporation of glutathione into AMPK 221WT (not shown). Taken

together, these results suggest that, at least under the conditions examined here, there is no regulatory effect of GST in AMPK signaling.

### 2.7. AMPK activation leads to phosphorylation of mammalian GST

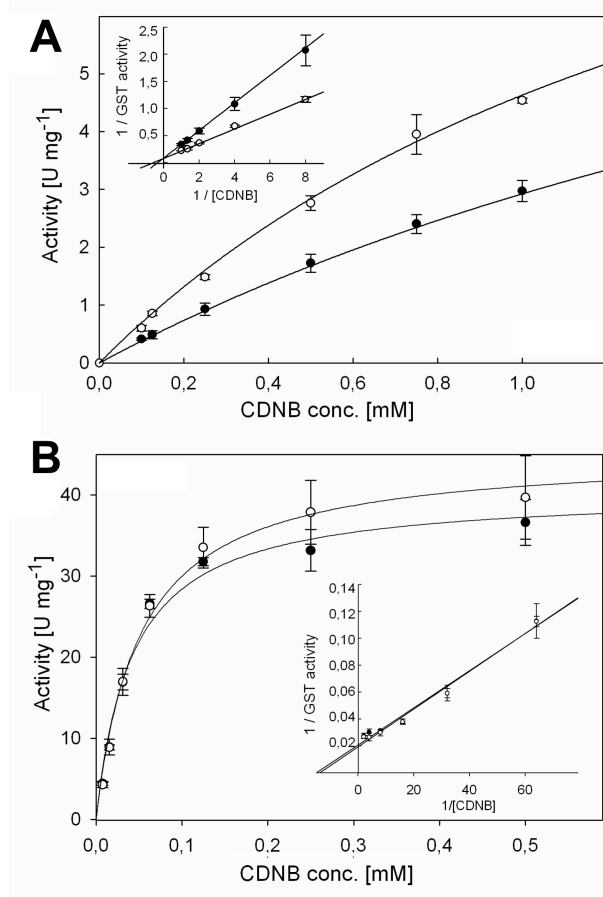
Given the evidence for a strong GST/AMPK interaction, we analyzed whether the different GSTs could be AMPK targets. An *in vitro* phosphorylation assay was set up using purified constitutive active or CamKK $\beta$ -activated AMPK and a 60-fold excess of purified GST. Both, GSTM1 and GSTP1 were phosphorylated by 221TD (not shown) and even more efficiently by CamKK $\beta$ -activated 221WT (Fig. 6-5C). The presence of glutathione did not further increase GST phosphorylation (not shown) as reported for PKA and PKC (Lo et al. 2004). Under the conditions used here, GST-Sj was not phosphorylated (Fig. 6-5C).



**FIGURE 6-5. GST/AMPK complexes affect GST phosphorylation but not AMPK signaling.** (A) GSTM1 and -P1 do not affect AMPK phosphorylation by CamKK $\beta$ . GSTs were preincubated with  $\alpha$ 2 $\beta$ 2 $\gamma$ 1 (221WT, 12.5 pmol), with or without 10 mM glutathione, in presence of ATP prior to addition of CamKK $\beta$  (0.63 pmol). Phosphorylation assays were subjected to immunoblot analysis using anti-P-T172- $\alpha$  AMPK antibody. (B) GSTM1 does not affect AMPK target phosphorylation. AMPK 221TD was preincubated with GSTM1 or BSA (negative control) before incubation with target and [ $\gamma$ - $^{32}$ P]ATP in absence (left) or presence (right) of 10 mM glutathione. Protein quantities are given in pmol. (C) GSTM1 and -P1, but not GST-Sj, are phosphorylated by AMPK. AMPK 221WT (3.5 pmol) first activated by CamKK $\beta$  (1 pmol) was incubated with purified GST-Sj, GSTM1, GSTP1, or GST-ACC (200 pmol). *In vitro* phosphorylation assays (B,C) were analyzed by SDS-PAGE and Typhoon phosphoimager; also revealing AMPK autophosphorylation.

## 2.8. GSTP1 phosphorylation increases catalytic efficiency

In addition to AMPK, other Ser/Thr protein kinases like PKA and PKC were reported to phosphorylate GSTP1, leading to GST enzyme activation (Lo et al. 2004). We therefore analyzed enzyme kinetics of GSTP1 and -M1 in presence or absence of CaMKK $\beta$ -activated AMPK 221WT, using the model substrate 1-chloro-2,4-dinitrobenzene (CDNB). In case of GSTP1, phosphorylation led to a strong decrease in apparent  $K_m$  and a slight increase in  $v_{max}$  (Fig. 6-6A), resulting in a doubling of the catalytic efficiency  $k_{cat}/K_m$  (Table 6-1). In contrast, phosphorylation of GSTM1 did not show any effect on apparent  $K_m$  and  $v_{max}$  values for CDNB (Fig. 6-6B, Table 6-1).



**FIGURE 6-6. AMPK-mediated phosphorylation affects enzymatic catalysis of GSTP1, but not GSTM1.** Enzyme activity of phosphorylated and non-phosphorylated GSTP1 (A) and GSTM1 (B) at different concentrations of the model substrate CDNB and saturating glutathione concentrations, measured as described in Experimental Procedures. The direct fit to original data (large graph) and the inverse Lineweaver-Burk plot (insert graph) are shown. The derived catalytic constants are given in Table 6-1.

**TABLE 6-1. Phosphorylation by AMPK activates GSTP1.** Enzyme kinetic parameters of recombinant GSTP1 and -M1 before and after phosphorylation with AMPK 221WT activated by CamKK $\beta$ .

	$V_{max}$ ( $U\ mg^{-1}$ )	$k_{cat}$ ( $s^{-1}$ )	$K_m$ (CDNB) (mM)	Catalytic efficiency $k_{cat}/K_m$ ( $mM^{-1}\ s^{-1}$ ) change (%)	
GSTP1	$11,4 \pm 1,9$	$8,9 \pm 1,4$	$2,9 \pm 0,5$	3,0	
P-GSTP1	$12,5 \pm 1,7$	$9,8 \pm 1,3$	$1,7 \pm 0,3$	5,8	+ 97%
GSTM1	$39,7 \pm 1,3$	$31,1 \pm 1,0$	$0,039 \pm 0,005$	790	
P-GSTM1	$42,2 \pm 1,5$	$33,0 \pm 1,2$	$0,043 \pm 0,006$	764	- 3%

Measurements with variable concentrations of model substrate CDNB (1-chloro-2,4-dinitrobenzene) at saturating glutathione concentration (2mM for GSTP1 and 10mM for GSTM1) and 25°C. Enzyme activity is given in U, equivalent to 1  $\mu$ mol/min. Results are given as means  $\pm$  SE (n=3-4) of a phosphorylation experiment that has been independently repeated four times.

### 3. DISCUSSION

This study provides a detailed characterization of a novel AMPK interaction partner and target that is glutathione S-transferase (GST). This finding has two important implications. First, it links the energy-stress sensor AMPK via GST phosphorylation to the cellular anti-oxidative defense and detoxification machinery. Second, it imposes a note of caution for working with GST fusion proteins in AMPK research.

Although a rapidly growing body of evidence suggests that AMPK is one of the central hubs in the cellular signaling network, few efforts have been made to identify interaction partners that may include but are not limited to AMPK targets (Gimeno-Alcaniz et al. 2003, Solaz-Fuster et al. 2006, Pan et al. 2008, Fu et al. 2009, Moreno et al. 2009). Here we present qualitative and quantitative data using different, complementary approaches to show that GSTM and GSTP isoforms are such AMPK interaction partners, and that one consequence of this interaction is GSTP1 phosphorylation and activation. The physiological relevance of these events is supported by the findings that the interaction (i) occurs *in vitro* and also *in vivo* in yeast cells or liver extracts, (ii) is of high, nanomolar affinity, and (iii) regulates GSTP1 activity via GST phosphorylation. Although we could show high affinity interaction with GST isoforms of the Mu and Pi family, this seems not be the case with all GSTs, since e.g. GST alpha and omega isoforms do not pull-down AMPK (not shown).

An important feature of AMPK/GST complex formation is the role of the N-terminal domain of the AMPK  $\beta$ -subunit. No specific function has been described so far for this domain in mammals, although it is fairly well conserved across the AMPK protein family. The core structure of mammalian AMPK and its yeast ortholog have been solved at molecular resolution, but these structures lack the N-terminal  $\beta$  domain (reviewed in (Sanz 2008)). This may indicate a high degree of flexibility in this region. Taken together with our data, we suggest that the N-terminal  $\beta$  domain represents a docking domain for protein/protein interactions. Many putative AMPK interactors were found associated with the  $\beta$ -subunit (IntAct database, (Aranda et al.)). However, in contrast to binding of glycogen and other effectors to the more C-terminal glycogen binding domain of AMPK  $\beta$  (Polekhina et al. 2005, McBride et al. 2009a, McBride et al. 2009b), interactions of the N-terminal domain do not seem to affect AMPK activity, at least in case of bound GST, and with or without its substrate glutathione. However, GST binding to AMPK could make it a good substrate for phosphorylation by the activated kinase.

The GST/AMPK complex formation could affect properties of both partners. Since GSTM1 and -P1 were identified as signal transduction modulators, we verified whether GST binding to AMPK could modify activation or enzymatic properties of the kinase. First, GSTs could participate in glutathionylation of AMPK, but this seems not to be the case. However, for the signaling of JNK and other stress-activated kinases, it has been well described that tight complex formation with GSTs *per se* can regulate stress and apoptosis signaling (Adler et al. 1999, Yin et al. 2000). In case of AMPK, although the affinity of the interaction is similar, we could not detect so far such a non-catalytic,

ligand binding regulatory activity of GST, neither on AMPK activation by upstream kinases nor on AMPK target phosphorylation *in vitro*. However, there may be further functions that are worth to be analyzed, such as substrate specificity or subcellular localization of AMPK.

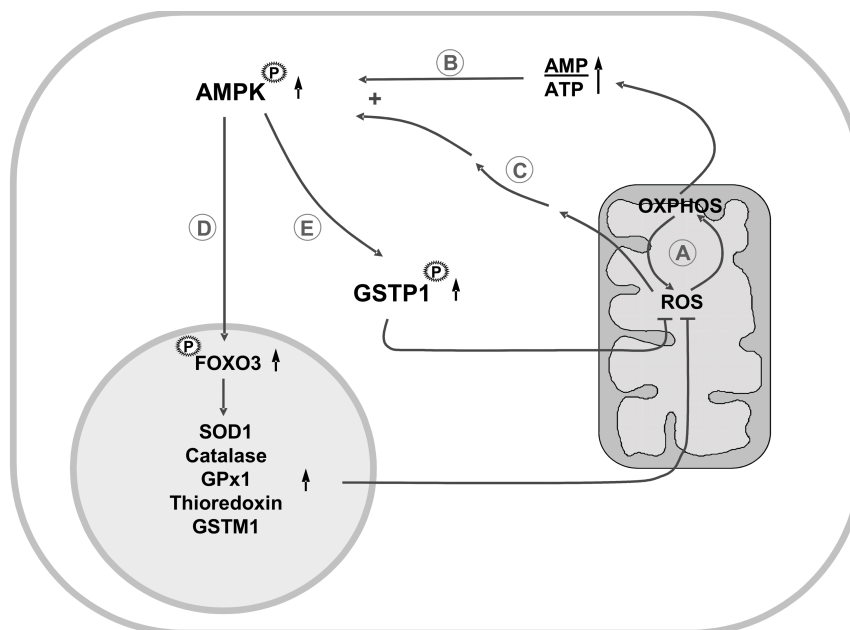
In contrast to AMPK, the AMPK/GST complex formation has a clear effect on the bound mammalian GST. AMPK is able to phosphorylate GSTM1 and even more so GSTP1. Also, once AMPK is activated, the binding to GST is reduced, suggesting that recruitment of GST prior to AMPK activation is important. GSTM1 and -P1 contain several less stringent putative AMPK sites, as e.g. present in mammalian target of rapamycin or eukaryotic elongation factor 2 kinase (Neumann et al. 2007). None of them is conserved between the Pi and Mu sub-families, possibly explaining the divergent effect of phosphorylation on GST enzyme activity. Further studies will have to show whether a single or multiple sites of these GSTs are phosphorylated. Interestingly, there is no significant phosphorylation of the *Schistosoma* GST-Sj enzyme, which is an ortholog of GSTM1. However, since the AMPK used throughout the study is of mammalian origin, there could be a problem with substrate recognition and *Schistosoma* AMPK may be required. Alternatively, the function linked to this phosphorylation step has only developed later during evolution.

An important effect of AMPK/GST interaction and GST phosphorylation is the doubling of GSTP1 catalytic efficiency. Similar activation of GSTP1 has been shown for phosphorylation by PKA and PKC (Lo et al. 2004) or epidermal growth factor receptor (Okamura et al. 2009), and interaction with the Franco anemia group C protein (Cumming et al. 2001). Increased GSTP1 activity will reinforce its functional role in antioxidative defence and detoxification. GSTs, together with some other enzymes, are in the second line of defense against ROS and RNS damage. While antioxidant enzymes aim at scavenging ROS/RNS species before they inflict damage on membrane lipid, DNA, protein and other cell constituents, GSTs protect against the reactive by-products of oxidative stress like lipid hydroperoxides. Our findings on GSTP1 activation fit very well into the emerging picture of a role of AMPK in oxidative stress and radical signaling (summarized in Fig.6-7). There is increasing, most recent evidence that AMPK is not only activated by reactive oxygen and nitrogen species, possibly via different mechanisms (Zou et al. 2004, Xie et al. 2008, Hawley et al.), but is also in turn up-regulating the cellular antioxidative defense machinery (Hou et al. , Greer et al. 2007, Colombo et al. 2009, Wang et al.). Various elements of the glutathione system are stimulated by AMPK activation at the gene expression level, including GSTM1 (Greer et al. 2007), but not GSTP1 (Colombo et al. 2009). Our data propose an inverse regulation of these GSTs at the enzyme activity level, with GSTP1 but not GSTM1 protein being activated.

An important point to be aware of is the double edged function of GST-mediated detoxification in cancer. While GSTs are beneficial by inactivating carcinogens, they also participate in elimination of anti-cancer drugs. In particular GSTP1 is frequently up-regulated in neoplastic cells, and this is a key element in drug-induced resistance to chemotherapy (19, (Lo et al. 2007). In fact, GSTP1 activation by tyrosin phosphorylation decreased drug sensitivity (Okamura et al. 2009). Although prodrugs that are



activated but not eliminated by GST are now developed to circumvent the problem, this puts a note of caution on the use of AMPK activators as anti-proliferative anti-cancer drugs.



**FIGURE 6-7. GSTP1 activation in the context of AMPK signaling in oxidative stress.** (A) Reactive oxygen and nitrogen species (ROS, RNS) generated by oxidative phosphorylation (OXPHOS) and other sources during oxidative stress will induce molecular damage finally reducing oxidative ATP-generation. **Dual activation of AMPK by ROS/RNS:** (B) Reduced mitochondrial ATP generation will translate into increased cytosolic AMP/ATP ratios that activate AMPK (Hawley et al. 2010). (C) Alternatively, ROS/RNS may have direct effects via unknown mediators and upstream kinases on AMPK activation (Zou et al. 2004, Xie et al. 2008). **Dual action of AMPK in antioxidative defense:** (D) AMPK activates transcription factor FOXO3 known to promote resistance to oxidative stress and longevity (Greer et al. 2007). AMPK-activated FOXO3 is involved in expression of antioxidative genes like manganese superoxide dismutase (Kukidome et al. 2006, Colombo et al. 2009, Wang et al. 2010), catalase (Colombo et al. 2009, Wang et al. 2010), thioredoxin (Hou et al. , Colombo et al. 2009), metallothioneins (Greer et al. 2007), or uncoupling protein 2 (Xie et al. 2008). Among AMPK-FOXO3-induced genes are also  $\gamma$ -glutamylcysteine synthase, the first enzyme in glutathione biosynthesis (Colombo et al. 2009), glutathione peroxidase (GPx) that uses glutathione to reduce lipid and hydrogen peroxides (Wang et al. 2010), as well as GSTM1 (Greer et al. 2007), but not GSTP1 (Colombo et al. 2009). (E) In the present study, we show that GSTP1 is by contrast activated via AMPK interaction and phosphorylation at the protein level.

In contrast to GSTP1, phosphorylation of GSTM1 did not change its catalytic properties. Another known function of GST phosphorylation is mitochondrial targeting (Robin et al. 2003). Various cytosolic GST isoforms were found in mitochondria, and mitochondrial GST content increases during oxidative stress (Raza et al. 2002, Gallagher et al. 2006, Goto et al. 2009). However, cytosolic GSTs lack a cleavable, N-terminal targeting sequence, and it has been convincingly shown for GST-A4 that phosphorylation by PKA or PKC is a prerequisite for its mitochondrial targeting (Raza et al. 2002). Like GSTA4, the P1 and M1 isoforms are major cytosolic GSTs also found in mitochondria (Gallagher et al. 2006, Goto et al. 2009). GSTP1 distributes between cytosol and mitochondria, and directly protects mitochondria against oxidative stress (Goto et al. 2009). Recently, we could identify

GSTM1 as a putative AMPK target in mitochondrial fractions (Ramirez-Rios & Schlattner, unpublished data) by using an *in vitro* phosphorylation screen developed earlier (Tuerk et al. 2007). We therefore propose that AMPK interaction and phosphorylation of GSTM1 and possibly also of GSTP1 could be linked to their mitochondrial targeting. Further experiments will be necessary to test this hypothesis.

Since GST-Sj is used in commercially available vectors for the expression of GST fusion proteins, results obtained with such constructs should be considered with care when working on AMPK signaling. At least GST pull-down should not be used to identify AMPK interaction partners. The approach may still be compatible with the identification of target phosphorylation, but one should consider that phosphorylation of such target constructs may be favoured by the strong AMPK interaction.

In summary, we have described a novel interaction between AMPK and GST, and emphasized the need for different, complementary interactomics approaches to ascertain accuracy of a given protein-protein interaction and to avoid false positives (Bruckner et al. 2009). We used different *in vitro* (GST pull-down, immunoprecipitation and SPR assays) and *in vivo* (Y2H) systems to show a strong and specific interaction between some Mu and Pi class GSTs and AMPK. These results suggest that GST is an *in vivo* interaction partner of AMPK. Moreover, the Y2H data imply that this interaction involves the N-terminal domain of both AMPK  $\beta$ 1- and  $\beta$ 2-subunits. The binding of mammalian GSTM1 and -P1 to AMPK led to their phosphorylation, which may have different physiological functions. We show here that one function in case of GSTP1 is enzyme activation with putative functions in oxidative stress response. Further research will be necessary to fully elucidate the AMPK-GST crosstalk. In any case, our data put a note of caution on working with GST-tagged proteins when analyzing AMPK function.

#### 4. FOOTNOTES

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## CORRIGENDUM

After submission of this thesis, MS-analysis of phosphorylated GST-constructs revealed significant phosphorylation within the Strep-tag that was present in the construct. When kinase assays were then repeated with GST protein lacking Strep-tag, the observed phosphorylation level was very low as compared to physiological AMPK substrates like acetyl-CoA carboxylase. Thus, GST phosphorylation is probably not physiologically relevant. Further activity measurements showed that the GST-activating effect of AMPK does neither involve GST phosphorylation but is rather due to the AMPK-GST interaction itself. The functional role of AMPK-GST interaction is currently further investigated in the laboratory. It seems to involve a secondary modification of AMPK catalyzed by GST. These data are currently submitted for publication (Klaus et al (2013) Glutathione S-transferases interact with AMP-activated protein kinase: evidence for S-glutathionylation and activation *in vitro*).



## - CHAPTER VII -

### DISCUSSION

AMPK acts as an energy sensor regulating whole body energy providing and consuming processes at the cellular and whole body level. Thus taking center stage in metabolic regulation, AMPK has gained enormous interest. Major efforts are directed towards the identification of AMPK upstream and downstream signaling pathways, in particular towards the identification of AMPK downstream target (Tuerk et al. 2007, Gwinn et al. 2008, Thali et al. 2010). In contrast, available information on the AMPK interactome is scarce, despite the fact that protein interaction networks define specificity of protein function within the context of cellular complexity and may facilitate the identification of novel drug targets (Strong et al. 2007, Chautard et al. 2009). In this work, different *in vivo* and *in vitro* methods were combined to reliably identify new AMPK interacting proteins and putative downstream targets (see Chapter 2). In the following, implication of AMPK in three cellular processes involving these AMPK partners will be discussed: vesicle transport, alternative functions of a metabolic enzyme, and cellular detoxification.

#### 1. AMPK IS ASSOCIATED WITH INTRACELLULAR VESICLES

A specific class of transmembrane proteins, the soluble NSF attachment protein receptors (SNARE), mediate fusion of vesicles with target membranes by interaction of vesicle-located v-SNAREs with target membrane-located t-SNAREs. SNAREs thus drive the targeting of intracellular vesicle pools to the surface (and the inverse) as key players of exo- and endocytotic processes. A Y2H screen of a human brain cDNA library revealed binary interaction between AMPK and v-SNARE family members VAMP2 and VAMP3 (Chapter IV). This interaction was confirmed by coimmunoprecipitation of synaptic vesicle VAMP2 with constitutive active  $\alpha 2T172D\beta 2\gamma 1$  AMPK. Neither VAMP2 nor VAMP3 were phosphorylated *in vitro* by AMPK, suggesting that they are not direct AMPK targets *in vivo*. However, different vesicle-associated proteins were shown to be phosphorylated *in vitro* by AMPK, suggesting that VAMP proteins recruit AMPK to intracellular vesicles for regulation of vesicle transport and/or vesicle endo- and exocytosis.

AMPK has been previously implicated in vesicle transport mainly for GLUT4 translocation. However the AMPK-mediated increase of GLUT4 translocation from intracellular vesicle pools to the plasma membrane was suggested to involve indirect regulation via AS160 and TBC1D1 phosphorylation. The AMPK-mediated phosphorylation increases AS160/TBC1D1 binding to 14-3-3-protein and thus inhibits its interaction with Rab proteins (Geraghty et al. 2007, Chen et al. 2008). The insulin sensitive AKT pathway also regulates AS160 (Chen et al. 2008). However, very recently,



contradicting data showed that AMPK mediated AS160 phosphorylation inhibits basal and insulin-stimulated glucose uptake in adipocytes (Gaidhu et al. 2010). In addition, AMPK activation by AICAR but not by metformin or phenformin induced AS160 phosphorylation (Geraghty et al. 2007), suggesting that additional mechanisms should be involved in the regulation of tissue-dependent glucose uptake.

AMPK- and insulin-activated GLUT4 translocation seem to involve different vesicles pools. Indeed, it has been shown recently that insulin induced GLUT4 translocation involves VAMP2, 5 and 7, whereas AMPK-activated translocation requires VAMP3, 5 and 7 (Schwenk et al. 2010). In addition, AMPK and insulin also stimulated translocation of FA transporter CD36. Even if the mechanism by which AMPK regulates CD36 translocation remains unclear, it was shown to involve VAMP3, 4 and 5 whereas insulin stimulated translocation required the presence of VAMP2, 4 and 5 (Schwenk et al. 2010). These data correlate the presence of specific VAMPs with AMPK-mediated intracellular vesicle mobilization. An AMPK-mediated regulation of plasma membrane protein levels was also suggested for endothelial Na<sup>+</sup>-channel (eNAC) and Na<sup>+</sup>-coupled glutamate transporter EAAT3 (Bhalla et al. 2006, Sopjani et al. 2010). In the latter two cases, AMPK is supposed to regulate ubiquitin-ligase Nedd4-2 mediated internalization and degradation. However internalization of eNAC and EAAT3 again relies on the v-SNARE protein family VAMP (Grefen et al. 2008).

Among the putative AMPK targets and interacting proteins published (Annex 2 & 3), further proteins involved in vesicle transport and endo- and exocytosis can be found. These include not only cytoskeletal proteins (e.g. actin and tubulin) but also dynamin-2 and synapsin-1 (Ewing et al. 2007, Tuerk et al. 2007). Dynamins are in principle involved in the scission of newly formed vesicles from the membrane of a cellular compartment and their targeting to, and fusion with, another compartment, involving e.g. the cell surface and the Golgi apparatus (Durieux et al. 2010). Synapsins bind synaptic vesicles to components of the cytoskeleton, which prevents them from migrating to the presynaptic membranes and releasing transmitters. Synapsin phosphorylation, mainly by CamKII, releases the vesicles from the cytoskeletal associated storage pool to the plasma membrane associated readily releasable pool. As this release is activated by increasing intracellular Ca<sup>2+</sup> concentration, which would also activate AMPK, the latter kinase could have an alternative control of this process in order to inhibit the energy demanding transport and exocytosis of synaptic vesicles. Indeed, synapsin-1 was phosphorylated by AMPK *in vitro* (Tuerk et al. 2007) and our preliminary data on synaptosomal glutamate release suggest that this process is inhibited by AMPK. Altogether, these data warrant further investigations on the role of AMPK in vesicular transport. It might e.g. be of interest to analyze the effect of AMPK activation/inhibition on intracellular distribution of synaptic or GLUT4 vesicles to the different intracellular vesicle pools.

## 2. ALTERNATIVE METABOLIC REGULATION

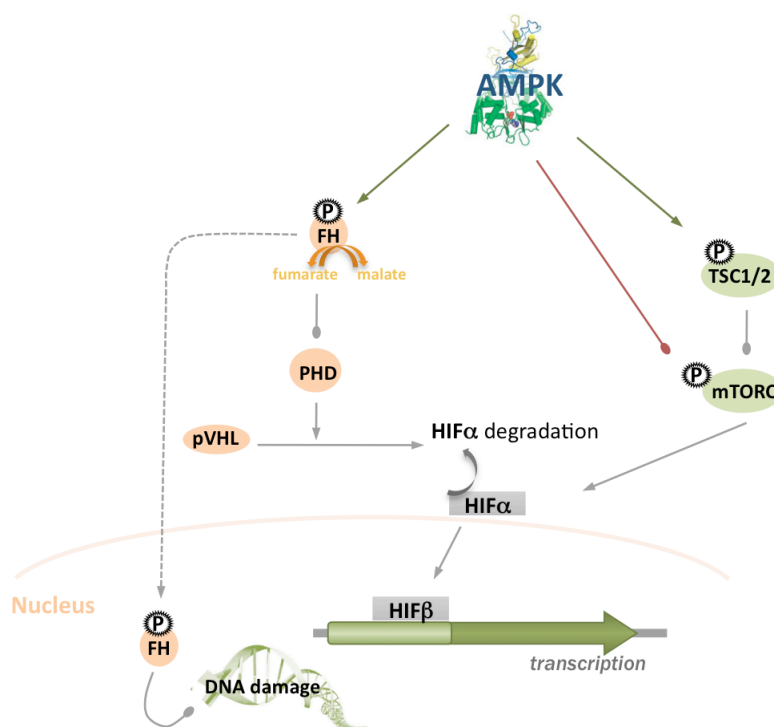
In a combined approach of biophysical interaction screening and *in vitro* AMPK phosphorylation we identified FABP and FH as direct AMPK interacting downstream targets *in vitro* (Chapter V).

### 2.1. Pleiotropic functions of fumarate hydratase regulated by phosphorylation?

Human tissue fumarate hydratase (FH) is almost equally distributed between the mitochondria, where the enzyme catalyzes the reversible hydration of fumarate to malate as a part of the TCA cycle, and the cytosol, where it is involved in the metabolism of the fumarate released by the urea cycle (Bowes et al. 2007). The malate formed in the cytosol can then enter the mitochondria and the TCA cycle. Activity of the urea cycle is important in particular for carnivores directly after meal, when up to 90 % of their energy requirements are covered by the oxidation of amino acids. Following protein degradation, amino acids are transformed into  $\alpha$ -keto acids (e.g. pyruvic and oxalacetic acid) which can then enter the tricarboxylic acid (TCA) cycle. This reaction involves the liberation of an amino group which is used either for amino acid biosynthesis or eliminated through the urea cycle.

Here we report, that AMPK interacts with, phosphorylates and activates cytosolic FH. Under stress conditions that activate AMPK, increased FH activity would have several beneficial effects (Fig. 7-2). First it would increase ATP generation through TCA substrate supply. Second, it would avoid accumulation of cytosolic fumarate that otherwise would inhibit hypoxia-inducible factor (HIF) $\alpha$  prolyl hydroxylase (PHD) which is triggering degradation of HIF $\alpha$  mediated by von Hippel-Lindau disease tumor suppressor (pVHL). HIF $\alpha$  upregulates the expression of genes involved in glucose metabolism and angiogenesis (O'Flaherty et al. 2010). Third, it has recently been reported that cytosolic FH can translocate to the nucleus where its enzymatic activity is required to activate the DNA damage repair machinery (Yogev et al. 2010). The latter two “non-metabolic” roles of FH may be responsible for its tumor suppressor function, since mutations in the enzyme have been involved in the etiology of different forms of cancers (Alam et al. 2005, King et al. 2006).

In addition to the phosphorylation of cytosolic FH, it might also be possible that AMPK plays a role in regulation of FH import into mitochondria. Mitochondrial FH differs from cytosolic FH only by the additional N-terminal mitochondrial targeting sequence (Regev-Rudzki et al. 2008), which contains two additional putative AMPK recognition sites (Ser19, Ser 43). Their phosphorylation was not directly tested, since the full-length protein could not be purified from *E. coli*. A regulation of mitochondrial import by AMPK has also been proposed for other putative mitochondrial targets of AMPK like citrate synthase or glutamate dehydrogenase identified in earlier screens (Tuerk et al. 2007). However, this hypothesis has not been tested so far, e.g. by studying mitochondrial translocation in response to AMPK activation or inhibition by immunofluorescence.



**FIGURE 7-2. Role of AMPK-activated fumarate hydratase in triggering DNA damage repair and HIF-mediated transcription.** AMPK phosphorylates and activates FH. Translocation of FH into the nucleus activates the DNA damage repair machinery (left). FH reduces cytosolic fumarate levels which indirectly activates HIF $\alpha$ -mediated transcription (middle, for details see text). Independently, HIF $\alpha$ -mediated transcription is activated by the AMPK-TSC1/2-mTORC pathway. Phosphorylations are indicated by (P). Activating and inhibiting processes are indicated in green and red respectively. Indirectly inhibited processes are indicated in grey. Abbreviations: PHD, HIF $\alpha$  prolyl hydroxylase; pVHL, von Hippel-Lindau disease tumor suppressor; HIF hypoxia inducible factor; TSC, tuberous sclerosis protein; mTORC, mammalian target of rapamycin complex.

## 2.2. Putative regulation of FABP mediated FA transport

The family of cytoplasmic FA-binding protein (FABPs) allows intracellular long-chain FA transport through binding. Nine different FABPs are expressed in mammals in a tissue specific manner (Storch et al. 2008). Despite modest amino acid sequence homologies, FABPs exhibit very similar tertiary structures. It has been suggested that subtle three-dimensional changes occur upon ligand binding and that these changes may promote specific protein-protein or protein-membrane interactions (Storch et al. 2009). Mice lacking FABP4 and FABP5 showed significant alterations in FA transport, availability, and composition, which resulted in a marked protection against diet-induced obesity, insulin resistance, type 2 diabetes, and fatty liver disease as well as increased AMPK activity (Maeda et al. 2005). Since FABP links cellular FA uptake to mitochondrial FA oxidation, two processes very well known to be controlled by AMPK, additional control of FABP is conceivable.

AMPK does not only interact with liver FABP (FABP1), as shown in our study, but also with FABP5, as detected by coimmunoprecipitation (Ewing et al. 2007). We show in addition that FABP1 is phosphorylated by AMPK *in vitro*, although the phosphorylation level appeared rather weak as compared to ACC or FH. This may be explained by a lack of necessary cofactors such as FA.

FABPs are known to bind to other proteins like hormone-sensitive lipase (HSL) and peroxisome proliferator activated receptors (PPAR), leading to their activation. In case of HSL, FABP binding was shown to depend on FA and HSL phosphorylation (Smith et al. 2007). Interestingly, AMPK seems to regulate directly or indirectly both FABP-interacting proteins, HSL and PPARs. AMPK phosphorylates HSL and thus blocks its activation by PKA. AMPK interaction with and phosphorylation of FABP may have an additional inhibitory effect on HSL. AMPK was also proposed to phosphorylate PPAR coactivators, thus repressing the ligand-dependent transactivating function of the receptor (Leff 2003). Here, if FABP functions as PPAR coactivator, its phosphorylation could be involved in short-term regulation of PPAR activity.

### 3. REGULATION OF CELLULAR REDOX HOMEOSTASIS AND GLUTATHIONYLATION

The mammalian glutathione transferases (GST) catalyze the conjugation of reduced glutathione (GSH), via a sulfhydryl group, to electrophilic centers on a wide variety of substrates. This activity detoxifies electrophilic xenobiotics, such as chemical carcinogens, environmental pollutants, and antitumor agents. The GST family comprises cytosolic, mitochondrial, and microsomal subfamilies (Hayes et al. 2005). In Chapter VI we report on a strong interaction of AMPK with mammalian cytosolic GST family members P1 and M1. Both GSTs were phosphorylated *in vitro* by AMPK. In the case of GSTP1 this led to increased catalytic enzyme activity. In contrast to the reported GSTP1 phosphorylation by PKC (Lo et al. 2004) and EGFR (Okamura et al. 2009), phosphorylation by AMPK was not enhanced in the presence of GSH. However, our results suggest a new role of AMPK in cellular detoxification.

Besides detoxification, GSTs inactivate endogenous reactive oxygen species (ROS) (e.g. unsaturated aldehydes, quinones, epoxides, and hydroperoxides) (Hayes et al. 2005). ROS are formed during cellular respiration when approximately 1–3% of electrons in the electron transport chain are prematurely donated to oxygen, producing the free radical superoxide (Valko et al. 2007). Superoxide is considered the “primary” ROS that can further interact with other molecules to generate “secondary” ROS, either directly or prevalently through enzyme- or metal-catalyzed processes. Overproduction of ROS results in oxidative stress (Valko et al. 2007). Their effect on DNA, protein, and/or lipid oxidation can cause irreversible cellular damage that has been associated with pathogenesis of a wide variety of diseases and also with degenerative processes associated with aging (Droge 2002, Balaban et al. 2005). However at lower concentrations, ROS have been shown to mediate the activation of signaling cascades that are needed to regulate growth, differentiation, proliferation, and apoptosis (Droge 2002). Indeed, ROS were reported to directly activate AMPK (Horie et al. 2008, Zmijewski et al. 2010) and AMPK activity has been associated to redox homeostasis mainly through long-term transcriptional regulation (Schulz et al. 2008, Colombo et al. 2009). AMPK mediated GSTP1 phosphorylation and activation might thus constitute a short-term

response to increased ROS production. In contrast to GSTP1, phosphorylation of GSTM1 did not affect its enzymatic activity. However, GST phosphorylation could also regulate its translocation to the mitochondria in response to increased mitochondrial ROS production as it has been reported for isoenzyme GSTA4 (Robin et al. 2003).

Although different roles can be attributed to the AMPK-mediated GST phosphorylation, the role of the strong interaction between both enzymes remains unclear. Even if the interaction might favor rapid GST phosphorylation, generally an interaction of “kiss-and-run” type is sufficient for kinase target phosphorylation (S. Ramirez, unpublished data). A similar strong interaction has been reported between GSTP1 and stress-activated c-Jun N-terminal kinase (JNK) where GST association alone inhibits JNK activation (Adler et al. 1999). Such an inhibitory effect was not observed with AMPK bound to GSTP1 or -M1, neither with AMPK activation by CamKK, nor with AMPK target phosphorylation *in vitro*. However, it has recently been reported that AMPK can be glutathionylated in response to oxidation (Zmijewski et al. 2010). It has also been shown that GST can potentiate S-glutathionylation reactions following oxidative and nitrosative stress (Townsend et al. 2009). It may be hypothesized that the GST-AMPK complex could constitute an “oxidative stress sensor” allowing rapid AMPK activation via glutathionylation and/or rapid detoxification of toxic carbonyl-, peroxide-, and epoxide-containing metabolites by GST activation. This hypothesis could be verified by studying the effect of the GST-AMPK interaction e.g. on ROS-mediated AMPK activation or GST functions.

#### 4. CONCLUSION

A combination of multiple interactomics approaches was applied to reliably identify novel AMPK interacting proteins and AMPK targets. The application of a cytosolic Y2H system led to the identification of new AMPK interaction partners, VAMP family members 2 and 3, which are not AMPK targets but might be scaffolding proteins as part of a new AMPK signaling complex. A combination of a biophysical SPR-based interaction screen and *in vitro* phosphorylation assays led to the identification of two new AMPK interacting targets that are preferentially phosphorylated by AMPK221 isoform. One target, FABP, suggests a role of AMPK in intracellular FA transport. The second target, FH, is strongly phosphorylated and activated by AMPK, which may favor HIF-mediated gene transcription and/or DNA damage repair. Finally a combination of different methods (i.e. Y2H, SPR, phosphorylation assays) provided reliable data for GSTP1 and -M1 being AMPK interacting targets. Both, the strong interaction with these GSTs and their phosphorylation suggest a new role of AMPK in oxidative stress defense.

The role of the identified interactions has now to be analyzed in more detail *in vivo*. The various models proposed above could be tested by inhibiting a given interaction, using specific peptides or overexpression of AMPK interaction domains. Analysis of AMPK-mediated phosphorylation will require identification of target phosphorylation sites, thus allowing target mutagenesis and generation of phospho-specific antibodies for *in vivo* studies.

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# PART III



## ANALYSIS OF BCK COMPARTMENTATION IN BRAIN

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### CHAPTER VIII :

*BCK compartmentation and interactomics*

### CHAPTER IX :

*BCK association to subcellular structures*

### CHAPTER X :

*Analysis of the BCK interactome by a cytosolic Y2H approach*

### CHAPTER XI :

*Discussion: Cellular compartmentation of BCK*

## AIM OF PART III

Creatine kinase (CK) isoforms play a major role in the acute response to increasing energy demands. High intracellular concentrations and fast diffusibility of phosphocreatine (PCr) allow the CK/PCr system to function as energy buffer and energy shuttle or -circuit. Since the solution of the molecular structures of all CK isoenzymes by the end of the 1990s, the CK/PCr system has been studied in molecular detail. The functional coupling of mitochondrial CKs to mitochondrial ATP synthesis and the role of cytosolic muscle MCK for muscle contraction, in particular its structural and functional association with myofibrils and SR calcium pumping, are well accepted now. Much less is known about the structural and functional organization of the CK/PCr system in the brain. Some older studies give little molecular insight, and more recent studies are limited to the association of BCK to the ATP-dependent rearrangement of actin cytoskeleton.

As a first aim, the available knowledge on brain BCK in respect to subcellular localization and interaction partners had to be summarized (Chapter VIII).

The aim of the experimental studies described in the following chapters was then to elucidate in more detail the intracellular “BCK network” in brain cells. Two different approaches were chosen:

(i) BCK association to different purified cellular membrane compartments was studied by applying cell fractionation methods and an initial analysis of membrane interaction. This preliminary approach should reveal subcellular distribution of membrane-bound BCK (Chapter IX).

(ii) A novel cytosolic Y2H screen was expected to identify novel BCK interaction partners. In contrast to a classical Y2H screen applied already in the past to BCK, this screen allows interactions to proceed in the cytosol, their “natural” environment, thus including potential interactions with membrane proteins (Chapter X).

The results obtained on the subcellular compartmentation of BCK are discussed in Chapter XI.

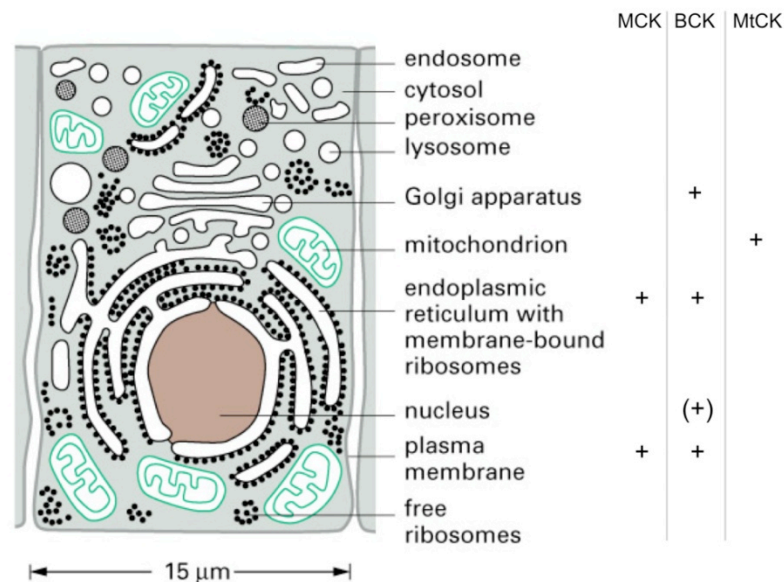


## - CHAPTER VIII -

### BCK COMPARTMENTATION AND INTERACTOMICS

#### 1. CELLULAR COMPARTMENTATION

Eucaryotic cells are divided into several compartments enclosed by unit membranes. Each compartment is characterized by specific morphology and function (Fig. 8-1). The nucleus houses the genetic information together with the replication and transcription machinery and is closely linked to the endoplasmic reticulum (ER), an interconnected network of tubules and vesicles that is involved, among others, in synthesis of proteins and lipids, metabolization of carbohydrates and other macromolecules, as well as calcium homeostasis. In continuation of the ER, the Golgi complex compartment is a combination of membrane-bound sacs and vesicles involved in synthesis and modification of different macromolecules, mainly proteins, and their sorting and packaging into different types of vesicles. Such vesicles assure the traffic of proteins e.g. to the cell surface, the breakdown of organelles and ingested particles (lysosomes), and the degradation of peroxides and fatty acids (peroxisomes).



**FIGURE 8-1. Schematic representation of cellular compartments.** The most important organelles of an eukaryotic cell are schematically represented with an emphasis on membrane systems. Association of different CK isoforms (cytosolic BCK and MCK and mitochondrial MtCK) with these different compartments as reported in literature is indicated (+). (Friedhoff et al. 1977, Rossi et al. 1990, Chen et al. 1995, Burklen et al. 2007, Schlattner et al. 2009, Ramirez-Rios et al. 2010, Yang et al. 2010) (Figure modified after (Alberts et al. 2002))

Mitochondria occur as a heterogeneous population within the cell and are subdivided by two membranes into the compartments matrix space and intermembrane/cristae space. Organization and functions of this cellular machinery require energy mainly delivered in form of ATP by either (an)aerobic glycolysis or mitochondria, with relative contributions depending whether metabolism is rather glycolytic or oxidative. Brain cells can use both types of ATP-generation, but in any case glucose is the obligatory energy substrate. Although the brain represents only 2% of the body weight, it receives 15% of the cardiac output, 20% of total body oxygen consumption, and 25% of total body glucose utilization (Ames 2000, Raichle et al. 2002, Squire et al. 2002). A metabolic differentiation exists between two major brain cell types, astrocytes and neurons. Astrocytes are likely the cells where glucose uptake occurs during activation. Lactate and pyruvate are then delivered to neurons, where energy precursors are mainly used at synaptic sites, not in perinuclear regions.

## 2. ENERGY AVAILABILITY AND DISTRIBUTION IN THE BRAIN

The generated “high energy” ATP diffuses to sites of energy demand, although this diffusion is probably not entirely free due to the crowded and highly structured cytosolic cellular space. In the brain, ATP is mainly consumed by the activities of  $\text{Ca}^{2+}$ - and  $\text{Na}^+/\text{K}^+$ -ATPases, glutamine/glutamate neurotransmitter cycling, cell signaling, and molecular synthesis and axonal transport of cell constituents (Ames 2000). Different adaptations of brain cells to organize their intracellular energy metabolism and to overcome ATP diffusion limitations have been reported. For example, localization of several glycolytic enzymes at the plasma membrane and at the  $\text{Na}^+\text{K}^+$  pump in synaptosomal membranes has been described (Knull 1978, Lim et al. 1983). Another adaptation is the concentration of mitochondria at the glutamatergic synapses, in the inner segments of retinal photoreceptors and in unmyelinated axons (Wong-Riley 1989, Nie et al. 1996, Wong-Riley et al. 1998). An alternative to such spatial organization of ATP producing and consuming processes is energy distribution *via* the creatine kinase/phosphocreatine (CK/PCr) shuttle (Hemmer et al. 1993).

An important role of the CK/PCr circuit has been confirmed by transgenic mice also for the brain. Knock-out of cytosolic brain-type CK (BCK) affects spatial memory acquisition and behaviour, development of the hippocampus, correct functioning of hair bundle cells in the auditory system, (Jost et al. 2002, Shin et al. 2007) and energy distribution within photoreceptor cells (Linton et al. 2010). These and other data support a role of the CK system as intracellular energy buffering and shuttle system in the brain, in particular in polarized large cells like neurons or hair bundle and photoreceptor cells. Here, BCK is co-expressed with ubiquitous mitochondrial CK (uMtCK) (Wallimann et al. 1994, Streijger et al. 2004). Octameric uMtCK generates phosphocreatine (PCr) in the peripheral mitochondrial intermembrane space (Brdiczka 1994, Speer et al. 2004), which is then used by the dimeric cytosolic BCK to generate ATP at sub-cellular sites of high ATP turnover (Wallimann et al. 1992, Friedman et al. 1994). To further validate the importance of this model for brain energetics, accurate knowledge of BCK

intracellular localization and its interaction with other cellular components is required. This would not only give insights into cellular energy shuttling but also define regions of high energy demand.

### 3. BCK INTERACTING PROTEINS

Only few studies have addressed BCK subcellular localization in brain cells. Functional association of BCK with cytosolic ATP-requiring processes has been postulated and described to some extent in the past, but only very few BCK/membrane or BCK/ATPase associations have been described to date. Not more than 15 proteins interacting directly or indirectly with mammalian BCK are reported in publicly accessible literature and interaction databases like IntAct and MINT (Aranda et al. 2010, Ceol et al. 2010). However, the vast majority was obtained in large-scale screenings using two-hybrid pooling or co-immunoprecipitation approaches, and only 5 were confirmed by different techniques, thus increasing confidence in these interactions (Table 8-1). These latter binding partners are SOCS box-containing protein Asb-9 (ASB9; (Debrincat et al. 2007)), metallothionein-3 (MT-3; (Lahti et al. 2005)), Golgi matrix protein 130 (GM130; (Burklen et al. 2007)), thrombin receptor (Proteinase-activated receptor 1, PAR-1; (Mahajan et al. 2000)) and the K<sup>+</sup>-Cl<sup>-</sup> co-transporter KCC2 (solute carrier family 12 member 5, SLC12A5; (Inoue et al. 2004)).

An interaction between BCK and Asb-9 was first identified during a high-throughput yeast two-hybrid approach (Rual et al. 2005). Two years later both proteins were shown to co-immunoprecipitate from different cell types and Asb-9 was shown to increase BCK polyubiquitination and to reduce total BCK levels (Debrincat et al. 2007). Asb-9 seems to be predominantly expressed in testes and kidney and, less pronounced, in heart and liver. However, it was undetectable in brain, lung, muscle, skin, spleen and thymus. Thus, the Asb-9 induced BCK degradation seems to be rather specific to testes and kidney. An interaction of BCK with other members of the SOCS box-containing family was not observed (Debrincat et al. 2007).

Immunoaffinity isolation assays identified BCK in complex with heat-shock protein 84 as an interaction partner of MT-3. The interaction was confirmed by co-immunoprecipitation of purified proteins. MT-3 may inhibit neuronal cell growth and seems to be down-regulated in Alzheimer's patients (Lahti et al. 2005).

Burklen *et al.* identified GM130 as an interaction partner of BCK in a yeast two-hybrid (Y2H) screen of a human brain cDNA library and confirmed this interaction by co-immunoprecipitation. However this interaction seems to occur only transiently during early prophase of mitosis. This transient interaction might recruit BCK to the Golgi apparatus in order to provide the energy required to induce Golgi fragmentation during mitosis (Burklen et al. 2007).

Another Y2H screen of a rat brain cDNA library, confirmed by pull-down and co-immunoprecipitation experiments, identified BCK as an interaction partner of the protein G-coupled thrombin receptor PAR-1, a seven-transmembrane protein (Mahajan et al. 2000). The authors showed that pharmacological inhibition and expression of dominant negative BCK reduced thrombin-induced

morphological changes in astrocytes and suggested that PAR-1-bound BCK provides high-energy phosphates necessary for thrombin receptor signal transduction during cytoskeleton reorganization.

Similar to the PAR-1-BCK interaction, the interaction of BCK with neuron-specific KCC2 was detected in an Y2H screen and confirmed by co-immunoprecipitation. Using an electrophysiological approach to measure the KCC2 function, the authors found that BCK enhances KCC2 activity, indicating an ATP/ADP handling role of BCK. However, the precise regulatory mechanism is, as for PAR-1, unknown (Inoue et al. 2006). KCC2 plays an important role in chloride homeostasis and in neuronal responses mediated by ionotropic GABA and glycine receptors. The expression levels of KCC2 in neurons determine whether neurotransmitter responses are inhibitory or excitatory.

**TABLE 8-1. BCK interaction partners identified by different proteomic approaches.**

<i>Bait</i>	<i>Protein name</i>	<i>Abbr.</i>	<i>Distribution</i>	<i>SwissProt</i>	<i>Method</i>	<i>Pubmed ID</i>
hBCK	Ankyrin repeat and SOCS box protein 9	ASB9	Soluble	Q96DX5	Y2H/CoIP	16189514/ 17148442
rBCK	Solute carrier family 12 member 5	SLC12A5	Membrane	Q63633	Y2H/CoIP	15094054
rBCK	Proteinase-activated receptor 1	PAR1	Membrane	P26824	Y2H/CoIP	11050237
mBCK	Metallothionein-3	MT-3	Soluble	P28184	CoIP	15802640
hBCK	Golgi matrix protein 130	GM130	Membrane	Q5PXD5	Y2H/CoIP	17036164
mBCK	14-3-3 protein zeta/delta	14-3-3 $\xi / \delta$	Soluble	P63101	CoSed	19562802
hBCK	Ubiquitin-specific peptidase-like protein 1		Soluble	Q5W0Q7	CoIP	19615732
hBCK	Ubiquitin carboxyl-terminal hydrolase 15		Soluble	Q9Y4E8	CoIP	19615732
hBCK	TRAF3-interacting protein 1	MIP-T3	Soluble	Q8TDR0	CoIP	20391533
hBCK	Mitogen-activated protein kinase 13	MAPK 13	Soluble	O15264	CoIP	19135240
mBCK	Calcium-activated potassium channel subunit alpha-1		Membrane	Q08460	CoIP	19423573
hBCK	Stress-associated endoplasmic reticulum protein 2		Membrane	Q8N6R1	Y2H	16189514
rBCK	Phosphoglycerate mutase 2	PGM-2	Soluble	P16290	Pulldown	19427860
hBCK	Nuclear factor kappa-B p105	NF $\kappa$ B p150	Soluble	P19838	CoIP	14743216
hBCK	Nuclear factor kappa-B p100	NF $\kappa$ B p100	Soluble	Q00653	CoIP	14743216

Putative BCK interaction partners were extracted either from publication databases (Pubmed, listed on top) or from protein-protein interaction databases (MINT and Intact, listed below). The latter result from large-scale screening approaches which are not confirmed by an independent method and are therefore of limited confidence. References are given as PubmedID. Abbreviations: CoIP, co-immunoprecipitation, Pulldown, GST pulldown; Y2H, yeast two-hybrid; CoSed, co-sedimentation.



#### 4. FURTHER BCK SUBCELLULAR LOCALIZATIONS

Different immunohisto- and cytochemical studies over the last two decades have localized BCK to subcellular structures like nuclei and plasma membrane (Chen et al. 1995, Shin et al. 2007). More recently the group of Bé Wieringa showed that, in macrophages, BCK co-accumulates transiently with F-actin at the nascent phagosome where it seems to play a role in active and ATP-dependent actin polymerization and particle adhesion (Kuiper et al. 2008). In astrocytes and fibroblasts, BCK facilitates actin-driven cell spreading and migration (Kuiper et al. 2009). Despite the evidences for the functional coupling of BCK with actin remodelling and the observed colocalization with F-actin, they failed to provide evidences for a structural association during pull-down, Y2H or FRAP experiments (Kuiper 2009). They thus suggested an indirect binding *via* another so far unknown actin-associated protein. One candidate could be microtubule-interacting protein associated with TRAF3 (MIP-T3), the only known BCK-interacting protein related to actin cytoskeleton (Table 1). MIP-3T is evolutionarily conserved from worms to humans, but its function remains still unknown. BCK was found to co-immunoprecipitate with MIP-T3, as did 34 other proteins in this study (Guo et al. 2010), but a direct interaction between BCK and MIP-T3 was not confirmed by other methods.

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## - CHAPTER IX -

### BCK ASSOCIATION TO SUBCELLULAR STRUCTURES

*Manuscript in preparation*

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**Abstract:** Cytosolic and mitochondrial isoforms of creatine kinase (CK) constitute an energy shuttling and buffering system. While this is structurally and functionally well characterized in muscle, much less is known in brain cells, in particular for the cytosolic BCK isoform and its coupling to ATP producing and consuming processes. This chapter aims at further analyzing the subcellular organization of BCK in brain. Synaptic vesicles and brain mitochondria were isolated from rat brain and BCK association studied by immunoblots and high pH or high salt treatments to characterize the type of interaction. Results suggest that BCK strongly associates with both, synaptic vesicles and brain mitochondria. Whereas the interaction with mitochondria appeared to be purely electrostatic, interaction with synaptic vesicles was stronger and involved additional interactions of probably hydrophobic character. Since bacterially expressed BCK did not rebind to liver or BCK-depleted brain mitochondria, it is suggested that secondary modifications of BCK are involved.

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#### 1. INTRODUCTION

Isoenzymes of creatine kinase (CK) are key elements for the management of high and fluctuating energy demands and cellular energy homeostasis in higher eukaryotes. CK isoenzymes catalyze the reversible phosphoryl transfer from ATP to creatine (Cr) yielding phosphocreatine (PCr) and ADP:  $ATP + Cr \leftrightarrow ADP + PCr$ . While these enzymes have long been thought to be entirely soluble, solid evidence has accumulated showing that they associate partially with cellular structures and proteins at sites of ATP delivery and consumption and thus maintain a CK/PCr shuttle. For example, localization and function of mitochondrial CK (MtCK) has been described in detail during recent years (Schlattner et al. 2009). These enzymes are located in the innermembrane space of mitochondria and are functionally coupled to the voltage dependent anion channel (VDAC) of the mitochondrial outer membrane and to the adenine nucleotide transporter (ANT) of the mitochondrial inner membrane (see Chapter I). Cytosolic CK isoform, BCK and MCK, use PCr for ATP regeneration. This also occurs locally, as has been shown in great detail for muscle MCK in the myofibrillar M-band (Turner et al. 1973, Hornemann et al. 2003), regenerating ATP for the myosin ATPase. Similar association of MCK with the sarcoplasmic reticulum (SR) (Rossi et al. 1990) supports the function of the highly energy-demanding SERCA calcium pump (Rossi et al. 1990). Other permanent or transient cellular

associations have been much less well characterized so far, including those with the plasma membrane (Wallimann et al. 1985) supporting the  $\text{Na}^+\text{K}^+$  pump (Guerrero et al. 1997) or the Golgi apparatus (Burklen et al. 2007). Many of these functional associations occur at membrane surfaces and thus involve interaction of CK isoenzymes with membrane proteins and/or lipids. As the well studied examples of MtCK (Schlattner et al. 2009) or myofibrillar MCK (Hornemann et al. 2003) clearly show, direct interaction of CK with the ATP delivering or consuming enzyme may not be essential.

In brain, processes linked to neuronal function consume high amounts of energy. A main ATP-requiring process besides the maintenance of ion gradients across the plasma membrane is neurotransmitter cycling involving vesicle trafficking and mitochondria located at the presynaptic membrane (Squire et al. 2002, Gazzaniga et al. 2004). Here we studied association of the brain isoform BCK to synaptic vesicles and outer membrane of brain mitochondria, membranes that have not been analyzed so far. We show strong association of BCK with both membranes, which apparently depends on different mechanisms.

## 2. RESULTS

### 2.1. Association of BCK to synaptic vesicles

Synaptic vesicles were isolated from rat forebrains by separation from nerve-ending particles (synaptosomes) as adapted from an established protocol for Guinea-pigs (Whittaker et al. 1964). First, synaptosomes were enriched by differential centrifugation, then disrupted by suspension in hypoosmotic medium, and the liberated vesicles were finally purified by density-gradient centrifugation (Fig. 9-1A). The isolation process was monitored in every step by immunoblotting with VAMP2, a vesicle-specific trans-membrane protein, as a marker for enrichment, and with exclusively mitochondrial uMtCK as a marker for purity (Fig. 9-1B). The strong increase in VAMP2 clearly shows enrichment of synaptic vesicles that are free of contaminating mitochondria after density gradient centrifugation (Fig. 9-1B).

These preparations of pure synaptic vesicles contained considerable amounts of BCK as revealed by immunoblotting (Fig. 9-2). To further examine the nature of this co-purifying BCK, an additional step was added to the purification protocol, consisting in an incubation of lysed synaptosomes with 100 mM  $\text{Na}_2\text{CO}_3$  pH 11.5 prior to density-gradient centrifugation. This treatment is known to remove peripheral membrane proteins that are only associated to the membrane surface. Typically, uMtCK was removed from the microsomal pellet containing *inter alia* broken mitochondria, while the transmembrane VAMP2 protein remained within the synaptic vesicle fraction (Fig. 9-2). uMtCK is known to associate by electrostatic interaction to cardiolipins of the inner and outer membranes of mitochondria, and such electrostatic interactions are clearly disrupted by the high pH  $\text{Na}_2\text{CO}_3$  treatment. As compared to these proteins, BCK remained bound to synaptic vesicles after high pH treatment. Thus BCK is not binding via electrostatic interactions, but is either at least partially

integrating into synaptic vesicle membranes via hydrophobic interactions or is simply localized inside the vesicles.

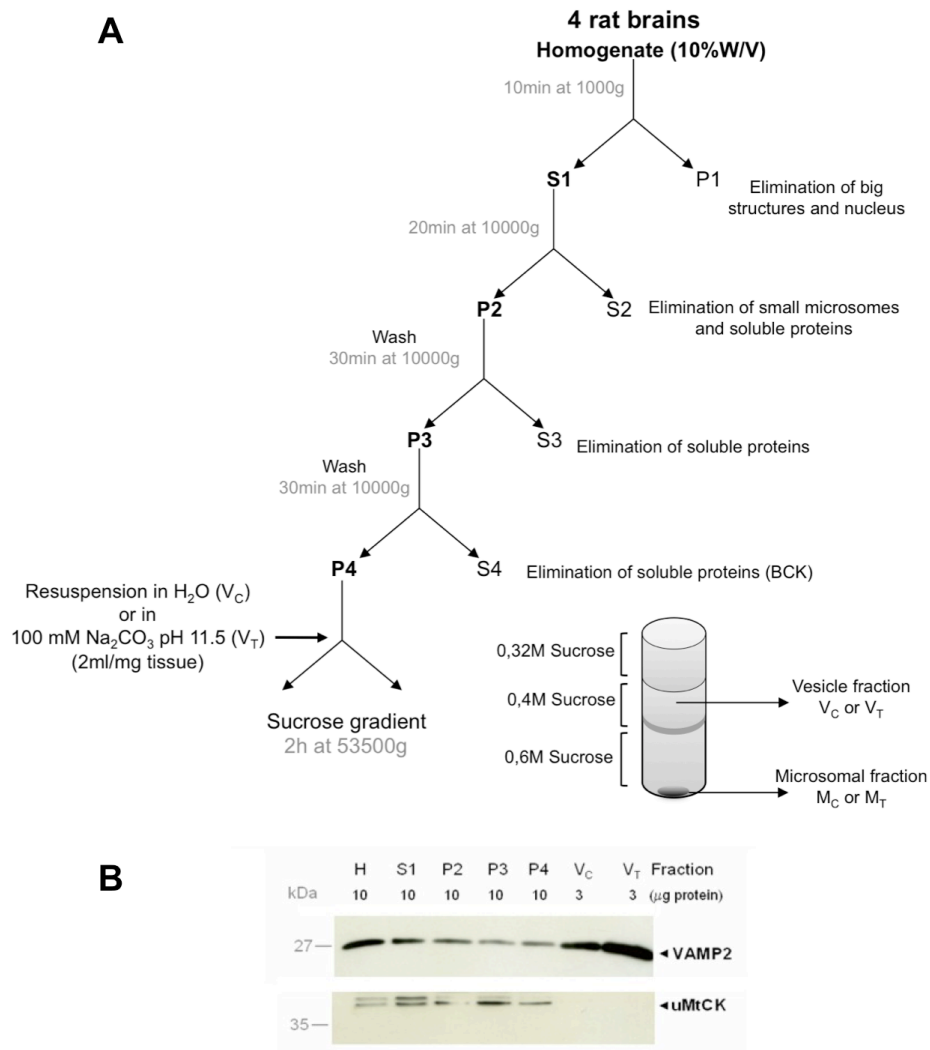


FIGURE 9-1. **Purification of synaptic vesicles from rat forebrain.** (A) Flow diagram of vesicle preparation involving differential centrifugation steps, synaptosome lysis and a sucrose gradient. Synaptosomes were disrupted in hypoosmolaric medium, either water ( $V_C$ ) or 100 mM  $\text{Na}_2\text{CO}_3$  pH 11.5 ( $V_T$ ) to remove surface-associated protein. (B) Monitoring vesicle enrichment and purity by anti-VAMP2 and anti-uMtCK immunoblotting.

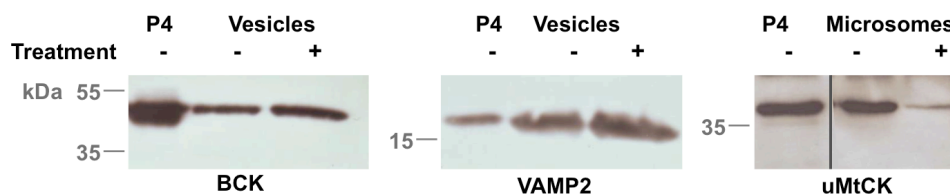


FIGURE 9-2. **BCK is co-purifying with synaptic vesicles and resistant to high pH treatment.** Immunoblot detection of BCK, VAMP2 and uMtCK in fractions of synaptic vesicles (left) or microsomes (right) isolated from lysed synaptosomes (P4) treated (+) or not (-) with  $\text{Na}_2\text{CO}_3$  at high pH.

To distinguish between these two possibilities, BCK enzymatic activity was measured either with freshly isolated intact vesicles, or vesicles that were permeabilized either by saponine or by repeated freezing/thawing cycles. Since BCK activity remained unchanged after permeabilization of synaptic vesicles (Table 9-1), it can be concluded that the isolated BCK is firmly anchored in synaptic vesicle membranes.

**TABLE 9-1. Effect of permeabilization on BCK activities co-isolated with synaptic vesicles.**

	Specific BCK activity (U.mg <sup>-1</sup> )			
	Vesicles w/o pretreatment		Vesicles with pretreatment	
	saponine	freeze/thaw	saponine	freeze/thaw
Control	1.39	1.51	1.07	1.15
Permeabilized	1.39	1.45	1.07	1.07

BCK specific activity was determined photometrically in a coupled enzyme assay measuring ATP production in the presence of 2 mM ADP and 20 mM PCr.

## ***2.2. Association of BCK to mitochondria***

Although neurons as other brain cells are mainly glycolytic, mitochondria still play an important role. For example, mitochondria close to the presynaptic membrane provide ATP for neurotransmitter release and vesicle recycling (Mironov 2009). Since in addition to MtCK, brain mitochondria seem to contain also BCK (Tokarska-Schlattner et al. 2007), association of this BCK fraction with these organelles has been analyzed. Mitochondria isolated from rat forebrain by differential centrifugation and Ficoll gradient (Fig. 9-3A) were enriched as shown by immunoblotting for ANT and uMtCK, proteins associated with the mitochondrial inner membrane (Fig. 9-3B). In these fractions, a considerable amount of BCK was detectable that had persisted throughout the entire purification procedure (Fig. 9-3C).



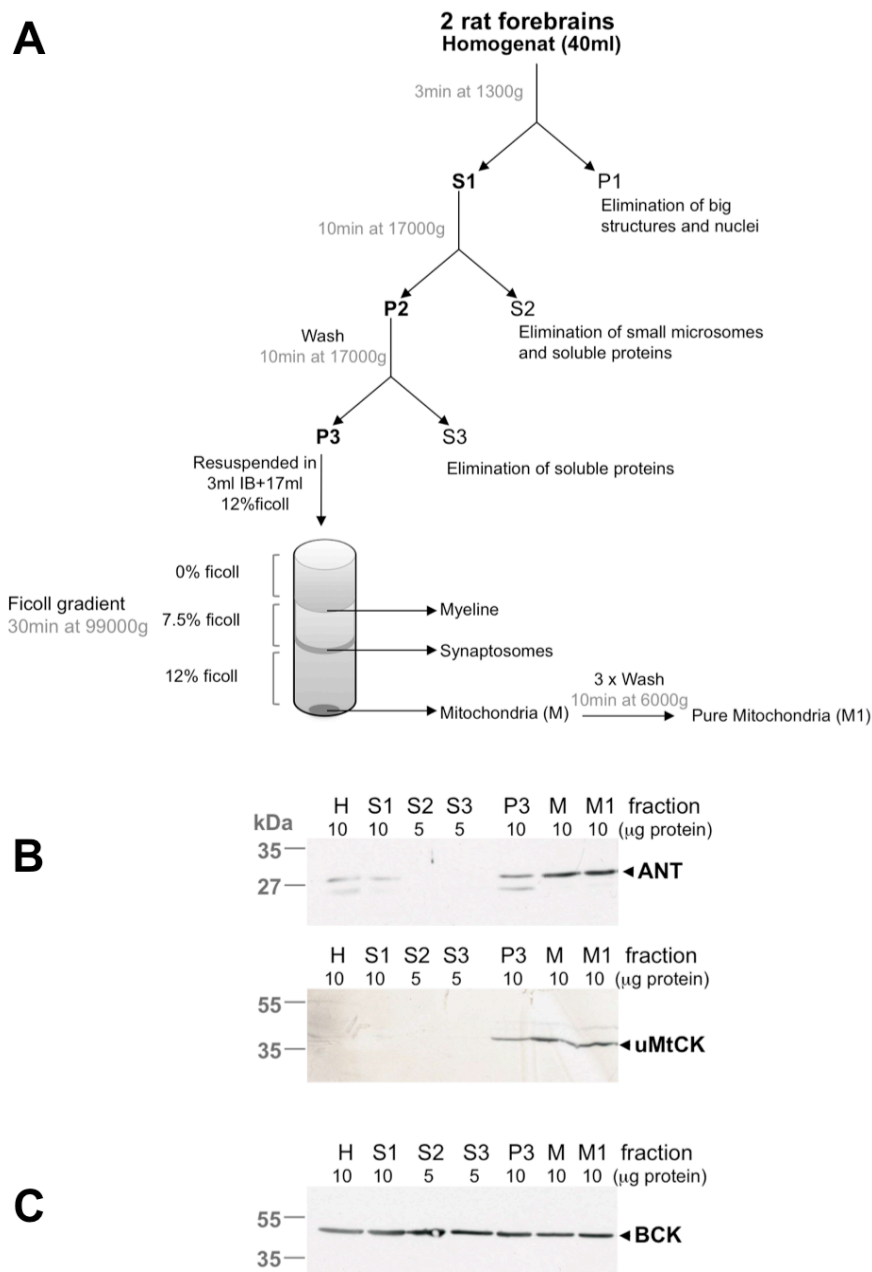
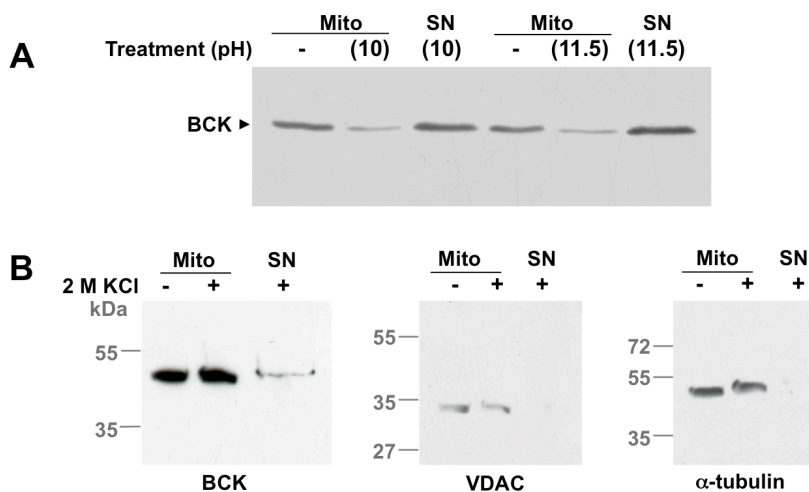


FIGURE 9-3. **Purification of brain mitochondria from rat forebrain.** (A) Flow diagram for purification of mitochondria involving differential centrifugation and a Ficoll gradient and washed 3 times (M1). (B) Monitoring enrichment of mitochondria by anti-ANT and anti-uMtCK immunoblotting. (C) Immunodetection of BCK throughout purification of brain mitochondria including the finale washing steps (M1).

Like for synaptic vesicles, mitochondria were treated with  $\text{Na}_2\text{CO}_3$  at high pH to analyze the nature of the observed BCK association. Since this isoform is strictly cytosolic and does not exhibit a mitochondrial targeting sequence, it may only attach to the outer mitochondrial membrane by electrostatic interactions, similar as MtCK in the intermembrane space, or anchor into the membrane by unknown hydrophobic interactions as seen in case of synaptic vesicles. In addition to the very stringent treatment at pH 11.5, a second slightly milder treatment with 125 mM KCl at pH 10 was applied (Fig. 9-4A). In both cases, a large fraction of membrane-associated BCK was removed from

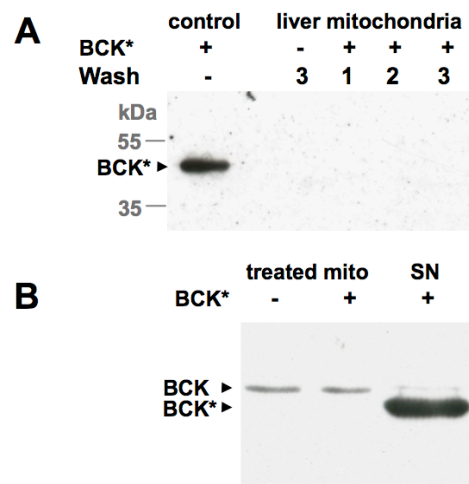
mitochondria, suggesting that BCK is associated by electrostatic interactions. The interaction strength of BCK was further compared to another cytosolic mitochondria-binding protein, tubulin. When mitochondria were incubated under conditions used earlier for tubulin (Carre et al. 2002), i.e. for 2 h at 37°C in 2 M KCl, some BCK was detected in the supernatant, while tubulin remained attached to the mitochondrial pellet (Fig. 9-4B). This indicates that the interaction of BCK with the mitochondrial membrane is weaker than that of tubulin.



**FIGURE 9-4. BCK is associated to brain mitochondria by electrostatic interactions.** Immunoblots of BCK (A,B),  $\alpha$ -tubulin and VDAC (B) of mitochondrial pellets (Mito) and corresponding supernatants (SN) from Ficoll-purified mitochondria treated to remove surface-bound protein. (A) Mitochondria incubated without (-) or with 100 mM Na<sub>2</sub>CO<sub>3</sub> pH 10, 125 mM KCl (10) or 100 mM Na<sub>2</sub>CO<sub>3</sub> pH 11.5 (11.5) in sucrose buffer for 30 min at 37°C. The pellet was washed additional three times and subjected to immunoblotting as well as the supernatant (SN) from the first centrifugation (B) Mitochondria incubated in sucrose buffer or 2 M KCl at 37 °C for 2 h. Immunoblotting of pellets (Mito) and supernatant (SN).

We further analyzed whether recombinant BCK produced and purified from *Escherichia coli* was able to associate to mitochondria. For this purpose, mitochondria were prepared from rat liver that does not express BCK. Liver mitochondria were then incubated with recombinant BCK for 1 h at 37°C and washed once with buffer. Under these conditions, no BCK was detected by immunoblotting in the washed mitochondrial pellet (Fig. 9-5A) Since mitochondria can differ between different organs, the same experiment was repeated using brain mitochondria whose BCK has been mostly removed by high pH treatment as before. Again, as detected by immunoblotting, washed mitochondrial pellets did not contain additionally associated BCK while recombinant BCK remained in the supernatant (Fig. 9-5B).

**FIGURE 9-5. Recombinant bacterially expressed BCK is unable to associate to mitochondria.** BCK immunoblots of mitochondrial pellets purified from BCK-free liver (A) or brain (B), with the latter mitochondria having most endogenous BCK removed by 100 mM Na<sub>2</sub>CO<sub>3</sub> pH 10, 125 mM KCl. (A) Purified recombinant BCK (control) and liver mitochondria incubated without (-) or with (+) recombinant BCK for 1h at 37°C followed by 1 – 3 washing steps. (B) Isolated brain mitochondria incubated without (-) or with (+) recombinant BCK and washed 3 times, together with the first supernatant (SN) BCK and BCK\* indicate migration of the endogenous and recombinant BCK, respectively.

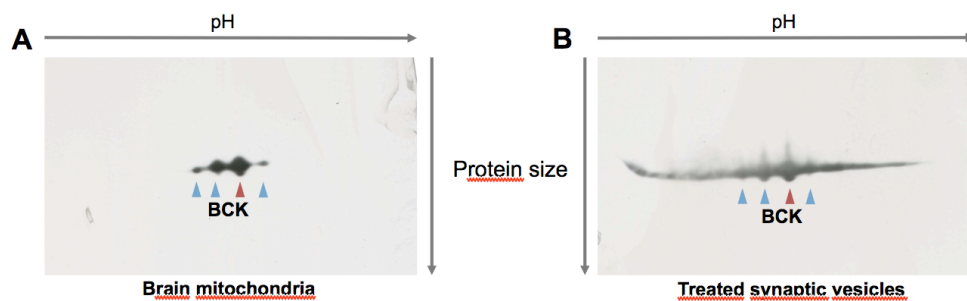


### 3. DISCUSSION

This study reveals that appreciable amounts of cytosolic BCK are bound to synaptic vesicles and mitochondrial membranes, and that these interactions are governed by different mechanisms. The association of synaptic vesicles with BCK survived the treatment with high pH that detaches peripheral membrane proteins that mostly interact electrostatically with the lipid headgroups. This observation is consistent with earlier observations that synaptic vesicle-bound CK is resistant to high salt and trypsin extraction (Friedhoff et al. 1977, Lerner et al. 1980, Lim et al. 1983). In addition BCK has also been detected in vesicles treated with 100 mM Na<sub>2</sub>CO<sub>3</sub> pH 11 by a recent study of the synaptic vesicle proteome (Takamori et al. 2006). Collectively, these data indicate that additional hydrophobic interactions are involved in the interaction of BCK with the vesicle membranes, either involving lipids or other membrane proteins.

The situation is entirely different with BCK associated with mitochondria. Such mitochondrial BCK has also been observed already earlier (Tokarska-Schlattner et al. 2007). This BCK interaction seems to be of electrostatic character, as a large part was removed by high pH and salt treatment. However, externally added recombinant BCK did neither bind to liver nor to brain mitochondria depleted of endogenous BCK. These data can be explained by two different mechanisms that either involve the membrane or the BCK binding partner: (i) A specific mitochondrial receptor is required, which is absent in liver and removed by the high pH treatment in brain, or (ii) BCK requires posttranslational modifications which are missing on the recombinant enzyme. The latter hypothesis is supported by results of 2D electrophoresis, applying isoelectric focusing in the first and SDS/PAGE in the second dimension, with both, synaptic vesicles and mitochondria. Immunoblotting of these 2D-gels revealed 4 spots, corresponding to BCK with same molecular weight but different isoelectric points (Suppl. Fig. 9-1) in both, the vesicle and the mitochondrial fraction. Unfortunately, we were

unable to identify a posttranslational modification by MS-MS analysis, which confirmed however that all 4 spots corresponded to BCK (S. Ramirez, personal communication).



SUPPLEMENTARY FIGURE 9-1. **Presence of different BCK species in brain mitochondria and synaptic vesicles.** Immunoblotting of IEF-SDS/PAGE-2D gel separations reveals at least 4 species of BCK with same protein size but different isoelectric points in mitochondria (A) and synaptosomes (B) from brain.

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## - CHAPTER X -

### ANALYSIS OF THE BCK INTERACTOME BY A CYTOSOLIC Y2H APPROACH

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**Abstract:** A cytosolic split-ubiquitin yeast two-hybrid (Y2H) technique was applied to screen for interaction partners of cytosolic brain-type creatine kinase (BCK) within the brain proteome. Full length and dimerization-incompetent BCK were used as ER membrane-anchored baits. All six identified putative BCK interaction partners are integral membrane proteins localized in the plasma membrane or cellular vesicle systems. Two candidate proteins, JWA protein (or Pra1 family protein 3) and vesicle-associated membrane proteins (VAMP2 and VAMP3) were confirmed by at least one further Y2H assay, the plasma membrane-anchored cytosolic split ubiquitin Y2H and the Split-Trp Y2H. JWA is a plasma membrane protein with roles ascribed in glutamate transport and cytoskeleton rearrangement. VAMP2 and VAMP3 are vesicle-associated proteins involved in intracellular vesicular trafficking, and endo-/exocytosis. Further candidates with less well defined roles include an organic cation transporter and RTN4/Nogo-A involved in neural outgrowth. Although not directly ATP-dependent, these proteins are close to and participate in processes that are strongly energy consuming. They may thus provide scaffolds to recruit BCK to energy-consuming microcompartments, a hypothesis that has to be verified by future research.

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#### 1. INTRODUCTION

Specific subcellular localization of CK isoforms as discussed in Chapters VIII and IX is essential for a CK/PCr shuttle to function properly. This has been shown in detail for the muscle isoforms MCK and sMtCK using *in vitro* and *in vivo* models, including single and double knock out mice (Hornemann et al. 2000, Hornemann et al. 2003, Dzeja et al. 2004). The molecular description of the CK/PCr shuttle in brain, although also an organ with high and fluctuating energy turnover, is lagging behind. In particular the elucidation of transient and/or permanent subcellular associations of the cytosolic brain isoform BCK remains an important issue. Only the identification of binding partners will allow a full molecular description of CK function in brain, including the complex phenotype of the brain CK knock out mice.

The aim of this chapter was to identify direct BCK interaction partners in brain. A cytosolic, ER membrane-anchored Y2H system was chosen for this purpose, based on our results on a rather strong association of BCK with subcellular membranes (Chapter IX), and also based on the fact that many highly ATP-consuming reactions in brain are located at or near membranes. The applied Y2H approach revealed several integral membrane proteins as putative BCK interactors. Although not directly involved in ATP-dependent reactions, they define ATP-consuming microcompartments and may therefore recruit BCK to processes such as vesicle trafficking or cytoskeleton rearrangements.

## 2. RESULTS

### 2.1. Identification of putative BCK interaction partners by a cytosolic Y2H approach

The cytosolic, membrane-associated Y2H system has already been described in detail in Chapter II. Briefly, the bait BCK was expressed as ER membrane-anchored fusion protein associated to the reporter cassette consisting of Cub and cleavable transcription factor, while as prey a human brain cDNA library was expressed as NubG-fusion proteins. In addition to full length human BCK, a truncated human BCK ( $\Delta$ BCK) was constructed and used as bait. The latter lacked the putative N-terminal CK dimerization domain, thus avoiding the potential detection of a large number of clones representing BCK. Details of both screenings are summarized in Table 10-1.

TABLE 10-1. Cyto-Y2H screen of a human brain cDNA library with ER membrane-anchored BCK.

	<i>BCK</i>	<i><math>\Delta</math>BCK</i> <sup>1</sup>
Library	human brain cDNA Library	human brain cDNA Library
3AT-concentration (mM) <sup>2</sup>	0	0
Transformation efficiency	~1x10 <sup>5</sup> clones/ug Library	~1x10 <sup>5</sup> clones/ug Library
Total number of clones	~2.8x10 <sup>6</sup>	~2.8x10 <sup>6</sup>
Clones selected on SD-AHTL	15	99
Clones after plasmid isolation	14	91
Bait dependent clones <sup>3</sup>	3	18
Clones discarded <sup>4</sup>	1(no cds), 1(false positive)	1(no cds), 5(not in frame), 3(false positive)
Selected clones	1	5 different (5xJWA)

<sup>1</sup> $\Delta$ BCK: Dimerization-incompetent truncation mutant (aa 103-318), lacking the N-terminal domain. <sup>2</sup>3-AT: 3-aminotriazole concentration necessary to suppress autoactivation was determined by a pilot screen using an empty library vector. <sup>3</sup>Clones interacting with BCK but not with Large T antigen were judged as bait dependent. <sup>4</sup>After sequencing and Blast analysis, clones lacking CDS (coding DNA sequence) or CDS in the wrong reading frame as well as supposed false positives were eliminated.

About  $2 \times 10^6$  clones were screened, yielding 15 and 99 primary interacting clones for BCK and  $\Delta$ BCK, respectively, which were able to grow on medium lacking adenine and histidine and gave a positive signal during  $\beta$ -galactosidase assays (Table 10-2). These clones were analyzed in more detail by isolating prey plasmid and performing bait-dependency assays using Large T antigen (LT) as a negative control.

Only 21 clones interacted reproducibly with  $\Delta$ BCK or BCK but not with LT. All these clones were sequenced, leading to the elimination of further 9 clones containing CDS in the wrong reading frame. From the remaining CDS, a list of putative new BCK interaction partners could be established (Table 10-2).



TABLE 10-2. New putative BCK interaction partners identified by a cytosolic Y2H screen of a human brain cDNA library.

<i>Bait</i> <sup>1</sup>	<i>Number of clones</i> <sup>2</sup>	<i>Gene name</i> <sup>3</sup>	<i>Encoded protein</i> <sup>3</sup>	<i>SwissProt</i> <sup>3</sup>	<i>Isolated peptide (amino acid no)</i>
BCK	1	VAMP3	Vesicle-associated membrane protein 3	Q15836	30-80
ΔBCK	5	ARL6IP5	Pra1 family protein 3 or JWA	O75915	1-188 (*4) 85-188 (*1)
ΔBCK	1	TMEM 144	Transmembrane protein 144	Q7Z5S9	183-345
ΔBCK	1	SLC22A17	Solute carrier family 22 member 17	Q8WUG5	266-446
ΔBCK	1	RTN4	Reticulon 4 or Nogo-A	Q9NQC3	1040-1192
ΔBCK	1	PCDHB10	Protocadherin beta-10	Q9UN67	716-800

<sup>1</sup>)Bait used to identify the interaction partner. <sup>2</sup>)Number of identified clones harboring the interaction partner. <sup>3</sup>)Gene name, protein name and SwissProt reference number for each interaction partner are given. <sup>4</sup>)Peptide encoded in the interacting clone (in parenthesis the number of clones, if applicable).

Almost all positive clones were found in the ΔBCK screen. Clones containing BCK itself as interaction partner for the formation of BCK dimers were not found in both screens. This is surprising for the full-length BCK screen. Possibly, two BCK bait proteins can already dimerize prior to interaction with prey constructs because of the high affinity dimerization interface (Eder et al. 1999). Such dimerization could also lead to some sterical hindrance affecting the bait reporter cassette, thus explaining the low number of interacting yeast clones found in the BCK screen. Interestingly all identified clones encoded membrane proteins, consistent with a partial association of BCK with cellular membranes *in vivo* as described above.

## 2.2. Evaluation of putative BCK interaction partners

From the list of putative interaction partners, some correspond to proteins whose function is very poorly described so far. These include the transmembrane protein 144 (THEM144), the solute carrier family 22 member 17 (SLC22A17, corresponding to the brain-type organic cation transporter) and protocadherin beta 10 (PCDHB10). One of the clones encoded RTN4, better known as Nogo-A that has been shown to be a critical inhibitory molecule controlling axonal growth and regeneration (reviewed in (Strittmatter 2002, Schwab 2004)). RTN4 would thus be an interesting candidate for its neurite outgrowth inhibitory functions. However, the clone identified during the Y2H screen encoded only the Nogo-66 domain, which is supposed to be extracellular (Teng et al. 2008), shedding some doubt on the physiological relevance of its interaction with BCK.

Two further clones encoded proteins that were also identified during Y2H screening with AMPK β-subunits (Chapter IV), the Pra1 family protein 3 (also called ARL6IP5 or JWA protein) and VAMP3. However, both can be considered as specific interactors. JWA interacted only with AMPKβ1

but not with AMPK $\beta$ 2, and VAMP3, although interacting with both AMPK  $\beta$ -subunits, has been verified by independent methods as true AMPK interactor (see Chapter IV). Such an interaction of both, BCK and AMPK, with JWA and VAMP3 may even indicate a physical link between BCK and AMPK which could arise from their similar but complementary functions in cellular energy homeostasis (see Chapter I and discussion in Part IV). For this reason, JWA and VAMP interactions with BCK were further analyzed.

### 2.3. BCK interacts with JWA only at the plasma membrane

To confirm a direct interaction between BCK and JWA, directed binary Y2H analysis was performed with three different systems, First, both cytosolic, membrane-associated Y2H assays were used. BCK and  $\Delta$ BCK were expressed as bait fusion proteins anchored to the ER or the plasma membrane and JWA was expressed as bait fusion protein. Under both conditions, *viz.* near the ER or the plasma membrane, JWA interacted with  $\Delta$ BCK. Interaction with full length BCK was only detectable when using the plasma membrane anchor (Fig. 10-1).

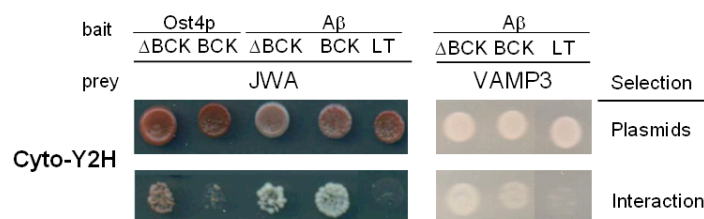


FIGURE 10-1. **Interaction of JWA and VAMP3 with BCK and  $\Delta$ BCK by Y2H analysis.** Two variants of the cyto-Y2H system were applied to analyze interaction of BCK and  $\Delta$ BCK with JWA. BCKs were expressed as fusion proteins of a ER membrane (Ost4p) or a plasma membrane anchor (A $\beta$ ) and the C-terminal end of ubiquitin conjugated to a transcription factor (bait) and JWA was expressed as fusions to the N-terminal end of ubiquitin (prey). Interactions were detected by activation of reporter gene transcription allowing growth on medium lacking adenine and histidine. Negative controls LT, Large T Antigen of *Simian Virus* (aa 84-704). For more details see Part V - Experimental Procedures.

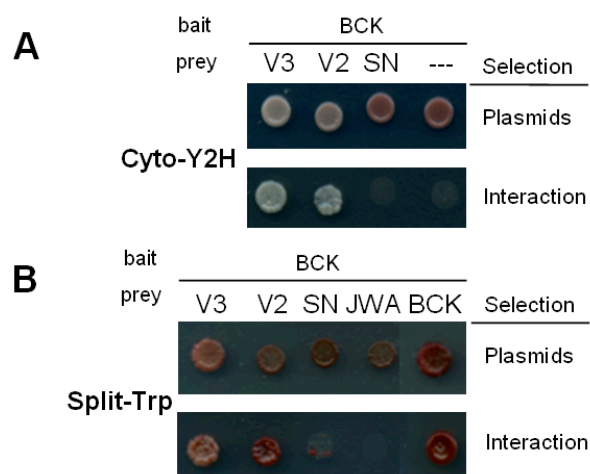
As an independent third cytosolic split-protein-based Y2H assay, the SplitTrp system was used, Here, C- and the N-terminal domain of an enzyme in tryptophan biosynthesis are fused to bait and prey. Their interaction in the cytosol leads to reconstitution of enzymatically active protein and growth on Trp-deficient medium. This assay did not reveal the BCK/JWA interaction (not shown) This may be due to the decreased sensitivity of the Split-Trp system, whose direct read-out lacks intermediate transcriptional amplification. Finally, BCK-JWA interaction could neither be detected by *in vitro* co-immunoprecipitation assays (not shown).

Taken together, these results suggest a rather weak affinity interaction between BCK and JWA, often described as “kiss-and-run”-type interaction, which would occur preferentially rather at the plasma membrane than at the ER.

### 2.4. BCK interacts with different VAMP family members

As for JWA, the interaction between  $\Delta$ BCK, BCK and VAMP3 was confirmed by the cyto-Y2H system targeting BCK fusion proteins either to the ER membrane or to the plasma membrane. VAMP3 interacted with both,  $\Delta$ BCK and BCK, at the ER (not shown) and the plasma membrane (Fig. 10-2A). We further analyzed interaction of BCK with VAMP2, which displays 74 % homology with VAMP3 and is the major VAMP isoform localized in synaptic vesicles (v-SNARE proteins). This is relevant, since we earlier found BCK associated with synaptic vesicles (Chapter IX). The major differences between VAMP2 and 3 are in the N-terminal part, which was missing in the clone identified in the Y2H screen (Table 10-2). Like VAMP3, VAMP2 interacted with BCK in the cytoY2H-assay (Fig.10-2A). The common and characteristic SNARE domain of VAMPs (amino acids 14-74 in VAMP3) was not sufficient for interaction. The interaction of BCK with both VAMP isoforms was in addition confirmed using the Split-Trp-Y2H assay (Fig.10-2B).

**FIGURE 10-2. Identification of the two major VAMP isoforms as AMPK interaction partners by Y2H analysis.** (A) Cyto-Y2H system. BCKs expressed as fusion proteins of the A $\beta$  membrane anchor and the C-terminal end of ubiquitin, conjugated to a transcription factor (bait), VAMP2 or 3 (V2, V3) and the shared SNARE domain (SN), were expressed as fusions to the N-terminal end of ubiquitin (prey). Interactions were detected by activation of reporter gene transcription allowing growth on medium lacking adenine and histidine. Negative controls: empty prey vector (---) (B) Split-Trp system. BCKs expressed as fusion proteins of a C-terminal domain of Trp1 (bait). V2, V3, SN, JWA were expressed as fusions to a N-terminal end of Trp1 (prey). Interactions were detected by reconstitution of Trp1 enzymatic activity allowing growth on medium lacking tryptophan. Positive control: BCK dimerization. For more details see Part V - Experimental Procedures.



Although these results with different Y2H systems clearly confirm an interaction of BCK with v-SNARE family members VAMP2 and VAMP3, this interaction could neither be confirmed by co-immunoprecipitation of recombinant proteins or proteins from rat synaptic vesicle fraction, nor by surface plasmon resonance (SPR). However, as seen in the studies on endogenous BCK bound to synaptic vesicles (Chapter IX), some non-electrostatic binding component is present that may require membrane lipids, and posttranslational modifications in BCK may be necessary that are missing in bacterially expressed protein. Furthermore, VAMPs are membrane proteins, and their native conformation may not be preserved when produced recombinantly in *Escherichia coli* and under the conditions used in our assays.

### 3. DISCUSSION

Novel putative BCK interacting proteins in brain were identified in a cytosolic split-protein Y2H screen using BCK and a truncated  $\Delta$ BCK variant, lacking the BCK dimerization interface, as baits. Although the latter is an N-terminally truncated version, it proved to be rather an advantage in identifying more putative interaction partners, possibly by avoiding steric hindrance occurring with dimerization of large fusion proteins. However, the mutant was not necessary to suppress BCK itself as interaction partner, possibly because full-length bait constructs dimerize before prey interaction can occur.

All interaction candidates are transmembrane proteins localized either at the plasma membrane or at cellular vesicle membranes. This emphasizes an important role for membrane-associated BCK that was already pointed out in different other studies (Wallimann et al. 1985, Inoue et al. 2004, Shin et al. 2007), including ours presented in Chapter IX. Two different mechanisms for such membrane-located CK can be envisaged. First, it can provide ATP into the undisturbed surface layer of a membrane that is rapidly depleted of ATP because of manifold energy-consuming reactions. In this case, any binding partner, protein or lipid, would just function as a scaffold for the recruitment of BCK. A good example for such a mechanism are myomesin and M-protein in the muscle that provide a scaffold for the recruitment of muscle MCK into myofibrils, in close vicinity of the myosin ATPases. Second, BCK could be associated directly to ATP-consuming membrane proteins. Two of the putative BCK interactors that we could confirm in different Y2H assays, JWA and VAMPs, seem to belong rather to the first category.

JWA was first independently described in different mammals. In humans, JWA was shown to regulate cancer cell migration through activation of MAPK cascades and rearrangement of F-actin cytoskeleton (Chen et al. 2007). More recently it has been reported that JWA might also have an inhibitory role in cancer metastasis through integrin  $\alpha_v\beta_3$  signaling (Bai et al. 2010). In rat, this protein was first identified as an interaction partner of the excitatory amino-acid carrier 1 (EAAC1), the primary glutamate transporter in neurons, using an Y2H screen of an adult rat brain cDNA library. It was thus called glutamate transporter EAAC1-associated protein (GTRAP3-18) (Lin et al. 2001). Treatment of rats with antisense oligomers resulting in decreased GTRAP3-18 showed reduced cortical glutamate uptake, suggesting that GTRAP3-18 is a putative negative regulator of EAAC1 (Lin et al. 2001). In mouse, this protein was first identified as an upregulated gene in rat amygdala nuclei after chronic morphine treatment and was called ‘addicisin’ (Ikemoto et al. 2002). All three JWA present more than 90% homology and contain at least two transmembrane domains. Presenting a cytoplasmic distribution, they are localized to the ER, Golgi and vesicular structures supposed to play a role as transport-associated proteins. Indeed it has been reported that JWA delays the ER exit of EAAC1 and might be a regulator of ER protein trafficking (Ruggiero et al. 2008). A functional role of

JWA in vesicular plasma membrane protein transport (e.g. of EAAC1 and integrin  $\alpha_v\beta_3$ ) might explain the very divergent physiological effects of JWA in different organisms (i.e. glutamate uptake and cancer cell migration). These transport processes are energy consuming. In addition, BCK has already been associated to plasma membrane ion channels as well as to cytoskeleton rearrangements (Inoue et al. 2006, Kuiper et al. 2008). JWA interacted with BCK with rather low affinity, thus possibly requiring the presence of further factors for full binding. It may be speculated that these involve also AMPK, found to interact with JWA in yeast (Chapter IV), whereas AMPK interacts only weakly and transiently with BCK (Ramirez-Rios et al. 2010). Since each of these proteins individually is involved in cytoskeleton rearrangement (Chen et al. 2007, Kuiper et al. 2008, Lee et al. 2008), it is proposed that they may be part of the same multiprotein complex.

VAMP2 and VAMP3 are exclusively vesicle-associated proteins (v-SNARE proteins), which as JWA are not directly consuming ATP. However, intracellular vesicular transport and SNARE-mediated endo- and exocytosis are very energy demanding processes (Bandorowicz-Pikula et al. 1998, Zheng et al. 1998). VAMPs could then be involved in attaching BCK to synaptic vesicles as observed in Chapter IX, where BCK would supply the required ATP while eliminating the formed ADP, which would inhibit vesicular ATPases, thus ensuring functional vesicle cycling. Although the BCK-VAMP2/3 interaction was confirmed in different, independent Y2H assays, it escaped detection by co-IP or SPR. This may have different reasons, as mentioned above, but an attractive one already discussed in Chapter IX is that secondary modifications of BCK as occurring within a eukaryotic cell are necessary for higher affinity interaction. Future experiments will have to solve these questions, in particular using BCK purified from eukaryotic cells for binding experiments and possibly for MS identification of secondary modifications (see also Chapter XI for further discussion of this issue).

Analysis of several further interaction candidates has not been pursued because of the limited data available on these proteins and their multipass transmembrane structures. THEM144 as well as SLC22A17 are such multipass membrane proteins of unknown function. SLC22A17 is an organic cation transporter (OCT), the brain isoform, of the SLC22 family (Koepsell et al. 2004). The  $\Delta$ BCK interacting clone contained amino acids 266-446 of SLC22A17, which represents the hypothetical transmembrane domains 6-9 and the rather small cytoplasmic or extracellular loops (as predicted by the UniProt KB (UniProt Consortium 2010)) (Table 10-2). OCTs are supposed to play a role in the elimination of cationic toxins and to facilitate the diffusion of different organic cations, including monoamine-neurotransmitters and pharmaceuticals. BCK was already found associated with other plasma transmembrane ion transporters like  $K^+Cl^-$  co-transporter KCC2 (Inoue et al. 2004), and shown to be functionally coupled to  $Na^+K^+$  ATPases (Blum et al. 1991). However, a direct interaction between BCK and the sodium pump or MCK and the sarcoplasmic calcium ATPase has not been detected, suggesting that CK is rather associated indirectly to these proteins, e.g. by interaction with scaffolding proteins or membrane lipid patches which themselves then bind to the ATPases.

PCDHB10 is one of the about 80 members of the protocadherin family. Protocadherins are cell-adhesion proteins, predominantly expressed in the nervous system where they are supposed to play a

role in the establishment and maintenance of specific neuronal connections and synaptic development (Frank et al. 2002, Morishita et al. 2007). PCDHB10, which shows an interaction with  $\Delta$ BCK in the Y2H screen, was detected in a clone containing amino acids 716-800 (Table 10-2). This part is exactly referred to as a putative cytosolic domain with unknown function, encouraging further experiments to confirm this interaction. However very little is known about this protein, which in addition to its transmembrane domain also contains a large extracellular portion (amino acids 27-692).

RTN4/Nogo-A, a neurite outgrowth inhibitor is a very interesting candidate that could constitute a link to the abnormal mossy fiber field size in BCK  $-/-$  knock out mice (Streijger et al. 2005). However, the clone which shows an interaction with  $\Delta$ BCK in the Y2H screen contained amino acids 1040-1192, thus including the region between the two hydrophobic transmembrane domains which also bear the Nogo-66 loop involved in extracellular Nogo-66 receptor (NgR) binding (Teng et al. 2008). The most popular topology places the Nogo-66 loop in the extracellular space (or in the lumen of the ER), which makes the possibility of a physiological interaction of BCK with this domain very unlikely. For this reason, BCK-RTN4 interaction was not further investigated.

Taken together, JWA and VAMP proteins interact with BCK in different Y2H systems, making them promising candidates for further investigation. They might link BCK to energy requiring intracellular vesicle transport.

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**- CHAPTER XI -****DISCUSSION:****CELLULAR COMPARTMENTATION OF BCK**

There has been a long-standing debate on whether the CK system works only as a global energy buffer or whether it also provides energy transfer functions and buffering of local ATP pools in so-called microcompartments. In muscle, the description of defined subcellular localization of CK isoforms and their function in association with ATP generating and consuming processes has largely contributed to the wide acceptance of a functional CK/PCr shuttle in this tissue. In brain, however, where the CK/PCr system seems to be also involved in crucial functions such as spatial memory acquisition and behaviour, development of the hippocampus and functioning of hair bundle cells in the auditory system (Jost et al. 2002, Shin et al. 2007), few is known about the underlying molecular mechanisms. Here we have started to analyze the spatial organization of the CK/PCr shuttle in brain, in particular the association of BCK to subcellular components in general as well as to specific, interacting proteins in particular. These complementary data provide evidence for a function of BCK at the membrane surface, where the kinase is anchored to proteins providing a type of scaffolding function. These proteins may attach BCK to different membranes in proximity to energy-demanding processes, but without direct interaction with ATPases. Such functions have been shown for myomesin and M-protein in muscular myofibrils (Stolz et al. 1998, Hornemann et al. 2000, Hornemann et al. 2003), and were suggested more recently for the interaction of BCK with Golgi protein GM130, occurring transiently during ATP-dependent mitotic defragmentation of Golgi (Burklen et al. 2007). Here, three specific BCK localizations are discussed in more detail: vesicle membranes, mitochondria, and plasma membrane.

**1. SYNAPTIC VESICLES**

BCK was found to be strongly bound to the surface of synaptic vesicles from rat brain. This is consistent with an earlier study by Wallimann and colleagues on *Torpedo marmorata* electrocytes, where they identified BCK associated to the postsynaptic membrane and synaptic vesicles at the presynaptic membrane (Wallimann et al. 1985). We could characterize this interaction as being of high affinity and non-electrostatic, since treatment with high pH did not dissociate BCK from this fraction. An association of BCK to synaptic vesicles is supported by the identification of the vesicle-associated membrane protein 2 (VAMP2), a transmembrane protein at the surface of these vesicles, as interaction partner of BCK by Y2H approaches.

As the association of BCK to vesicles seems to implicate more than electrostatic interactions, posttranslational modifications may be required for this strong binding. This hypothesis is supported by the profile of BCK in IEF-SDS/PAGE-2D gels, which showed 4 aligned spots of the same molecular weight but different isoelectric points. It has also been reported in literature that vesicle-associated CK shows a migration different to soluble BCK during agarose electrophoresis (Friedhoff et al. 1977), indicating a shift in the isoelectric point of BCK. The existence of different charge variants located at different subcellular sites has already been shown in case of other proteins like tubulin (Zambito et al. 2002). The authors suggested that differences in pI might have an impact on palmitoylation efficiency of tubulin but failed to prove this hypothesis experimentally. A similar resistance to treatment with pH 11.5 as for BCK has been reported for vesicle-associated protein SNAP-25 (Veit et al. 1996, Chin et al. 2000). In this case, membrane association seems in fact to depend on SNAP-25 palmitoylation. S-palmitoylation is one of the several secondary protein modifications involving covalent attachment of a fatty acid such as palmitic acid, to cysteine residues. Such modifications enhance protein hydrophobicity and can function as membrane anchor. Palmitoylation, in contrast to e.g. myristoylation, is a reversible process (Sorek et al. 2009), which obviously plays a significant role in subcellular trafficking of proteins between membrane compartments, as well as in modulating protein-protein interactions. Recently, palmitoylation of several SNARE proteins (incl. VAMP2, SNAP-25 and synaptotagmin) and its role in membrane fusion has been reviewed (Prescott et al. 2009). These findings raise the question whether BCK could be linked to synaptic vesicles in a similar way. The BCK sequence includes four cysteine residues per monomer, which are conserved in vertebrates (rat, human, mouse, bovine, chicken). One is the conserved active site cysteine 283 (chicken BCK numbering), but at least one other cysteine is partially exposed at the protein surface (Cys141, see Figure 11-1) and, although not within a classical palmitoylation sequence (<http://csspalm.biocuckoo.org/>), may be partially palmitoylated. Further more elaborated experiments like metabolic incorporation of radiolabeled palmitate followed by BCK immunoprecipitation (Zambito et al. 2002) would be required to verify this hypothesis. Palmitoylation can also occur in yeast (Lobo et al. 2002, Roth et al. 2002, Roth et al. 2006). Very recently, Y2H experiments have shown that single cysteine mutations were sufficient to interrupt the interaction between human prostaglandin receptor and Rab11, which was shown to be regulated by palmitoylation (Reid et al. 2010). It might thus be interesting to test whether different BCK cysteine mutations could affect the detected BCK-VAMP interaction.

Independent of the concrete molecular mechanism by which a fraction of BCK interacts with the synaptic vesicles, this localization could allow local ATP supply to the manifold ATP-requiring processes involved in the trafficking of these vesicles. A link between the CK/PCr shuttle and neurotransmitter trafficking has been already suggested by earlier studies in the 80s. For instance, BCK was shown to be associated with acetylcholine receptor-rich membranes (Barrantes et al. 1985), and BCK activity seems to play a role in quantal release of acetylcholine in synaptosomes (Dunant et al. 1988). The herein reported direct link of BCK with the SNARE machinery necessary for membrane

fusion gives first molecular insight but raises even more questions on the functional role of BCK at synaptic vesicles. At this stage, we can only hypothesize that BCK is recruited to synaptic vesicles to provide ATP necessary for the energy demanding steps in endo- and exocytosis. Since it has been reported that PCr seems to facilitate vesicular glutamate uptake, albeit independent of BCK activity (Xu et al. 1996), it would be interesting to analyze whether BCK inhibition could influence glutamate release from synaptosomes, as this method has already been established and described for another project in the laboratory (Part V). Finally, it should be considered that BCK also interacted with VAMP3, a non-neuronal VAMP isoform, suggesting that BCK might be implicated in intracellular vesicle trafficking also in other tissues.

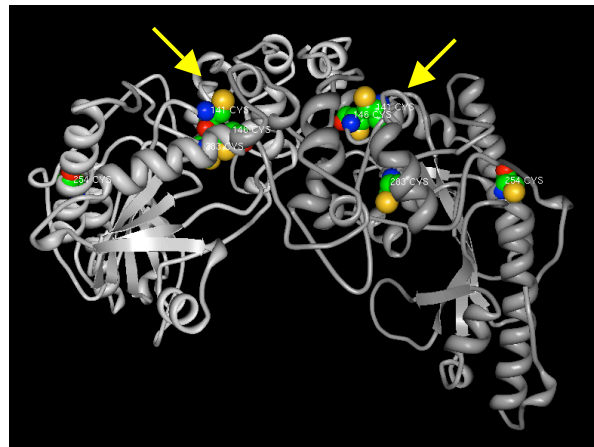


FIGURE 11-1. **Cysteine residues present in the dimeric BCK structure.** Cysteine residues conserved in vertebrates are represented in spacefill representation in the chicken BCK structure given in a schematic backbone representation (PDB ID: 1QH4) (Eder et al. 1999). Putative palmitoylated cysteines 141 are indicated with yellow arrows.

## 2. MITOCHONDRIA

A rather surprising finding of our study was the association of BCK with mitochondria. Although the CK/PCr system has been studied for decades, association of cytosolic CK with mitochondria has been rather based on anecdotal evidence. In mice deficient for mitochondrial sMtCK, oxidative phosphorylation of muscle mitochondria could still be stimulated by creatine (Boehm et al. 1998). The authors suggested relocation of cytosolic MCK to mitochondria to explain this phenotype. However, in wild type animals, mitochondria from skeletal muscle and heart are generally free of cytosolic CK, while for brain mitochondria there is published evidence for co-purification of appreciable amounts of cytosolic BCK (Tokarska-Schlattner et al. 2007).

In contrast to the BCK association to vesicles, the association to mitochondria seems to be of electrostatic character as high salt or high pH treatment led to dissociation of BCK. However,

recombinant BCK produced in *E. coli* was unable to associate to liver mitochondria or BCK-depleted brain mitochondria. These results suggest that mitochondria-associated BCK is either posttranslationally modified, as we suggest for the vesicle-bound fraction, or that the association requires the presence of a specific membrane receptor present in brain mitochondria. The only posttranslational modification reported in literature which would fit to the 4 spot-profile detected during 2D electrophoresis is phosphorylation. Actually, BCK autophosphorylation (Hemmer et al. 1995) and phosphorylation by protein kinase C (PKC) (Chida et al. 1990) and AMP activated protein kinase (AMPK) (Ponticos et al. 1998) have been reported. However at least BCK phosphorylated by AMPK does not co-localize with mitochondria (Ramirez-Rios et al. 2010). In addition, association of cytosolic creatine kinase to mitochondria seems to be specific for BCK, since MCK was not detected in isolated muscle mitochondria (M. Tokarska-Schlattner, unpublished data).

To further characterize the interaction of BCK with mitochondria, rebinding experiments with recombinant BCK could be repeated with, brain mitochondria from BCK *-/-* knock out mice. If this works out, a protein binding partner of BCK could be identified by pull-down of previously added GST-BCK fusion protein as in a previous study on BCK-GM130 interaction (Burklen et al. 2007) or by immunoprecipitation.

As for the function of surface-associated mitochondrial BCK, it may be speculated that this BCK pool functions in a similar way as uMtCK. It may have some preferential access to ATP generated in mitochondria, and thus increase mitochondrial PCr production. As mentioned above, in case of sMtCK knockout in muscle, it seems as if cytosolic MCK can functionally compensate the loss of sMtCK to some degree, consistent with relocation to mitochondria. In fact, expression levels of brain uMtCK are lower than those of sMtCK in muscle, and this may stimulate additional association of BCK with mitochondria. On the other hand, energy metabolism of skeletal and cardiac muscle is mostly oxidative whereas brain cell metabolism is more glycolytic. In glycolytic cells (e.g. brain cells), glycolytic hexokinase II (HKII) has been found associated to mitochondria (Smith 2000). HKII expression and mitochondrial association is also increased in cancer cells (Smith 2000), which typically switch from an oxidative to a glycolytic energy metabolism. In many cancers, BCK is also strongly overexpressed (Baggetto et al. 1992, Lillie et al. 1993, Zarghami et al. 1996). It might thus be possible that BCK competes or cooperates in some way with HKII for access to mitochondrially produced ATP. Further investigations have to show whether BCK is effectively also associated to mitochondria of cancer cells and whether mitochondrial HKII association is required for BCK association.

### 3. PLASMA MEMBRANE

In this study, the Y2H screen identified exclusively transmembrane proteins as putative BCK interaction partners in the plasma membrane and intracellular vesicles, with functions in intracellular transport (JWA, VAMP), ion channeling (SLC22A17), or cell adhesion (PCDHB10). However, these proteins do not directly function in an ATP-dependent manner. We propose that they recruit BCK to the plasma membrane to replenish the ATP pool in the unstirred surface layer of the membrane. Indeed, BCK location at the plasma membrane was also shown for acetylcholine receptor rich membranes (Wallimann et al. 1985) in association with transmembrane proteins such as the thrombin receptor PAR-1 and the potassium-chloride-channel KCC2 (Mahajan et al. 2000, Inoue et al. 2004). No direct interaction of CK with an ATP producing or consuming protein has been reported so far. In contrast, indirect association of CK to ATP production or consumption has already been characterized in detail for other CK enzymes, e.g. MCK coupling to myosin ATPases through M-band and myosin binding (Wallimann et al. 1984, Hornemann et al. 2003) and MtCK coupling to ANT through binding with cardiolipin (Schlattner et al. 2009). It might be possible that recruitment of CK to the membrane near ATPases is sufficient to constitute an energetically favorable environment, since it would allow two-dimensional diffusion of generated ATP along the membrane surface layer.

Altogether, the data presented in this work are consistent with the model of an intracellular CK/PCr shuttle, in which the CK/PCr shuttle links ATP-generating (mitochondria) to ATP consuming sites (synaptic vesicles, plasma membrane). A hypothetical integration of the identified putative BCK interaction partners within a CK/PCr shuttle is given in Figure 11-2.

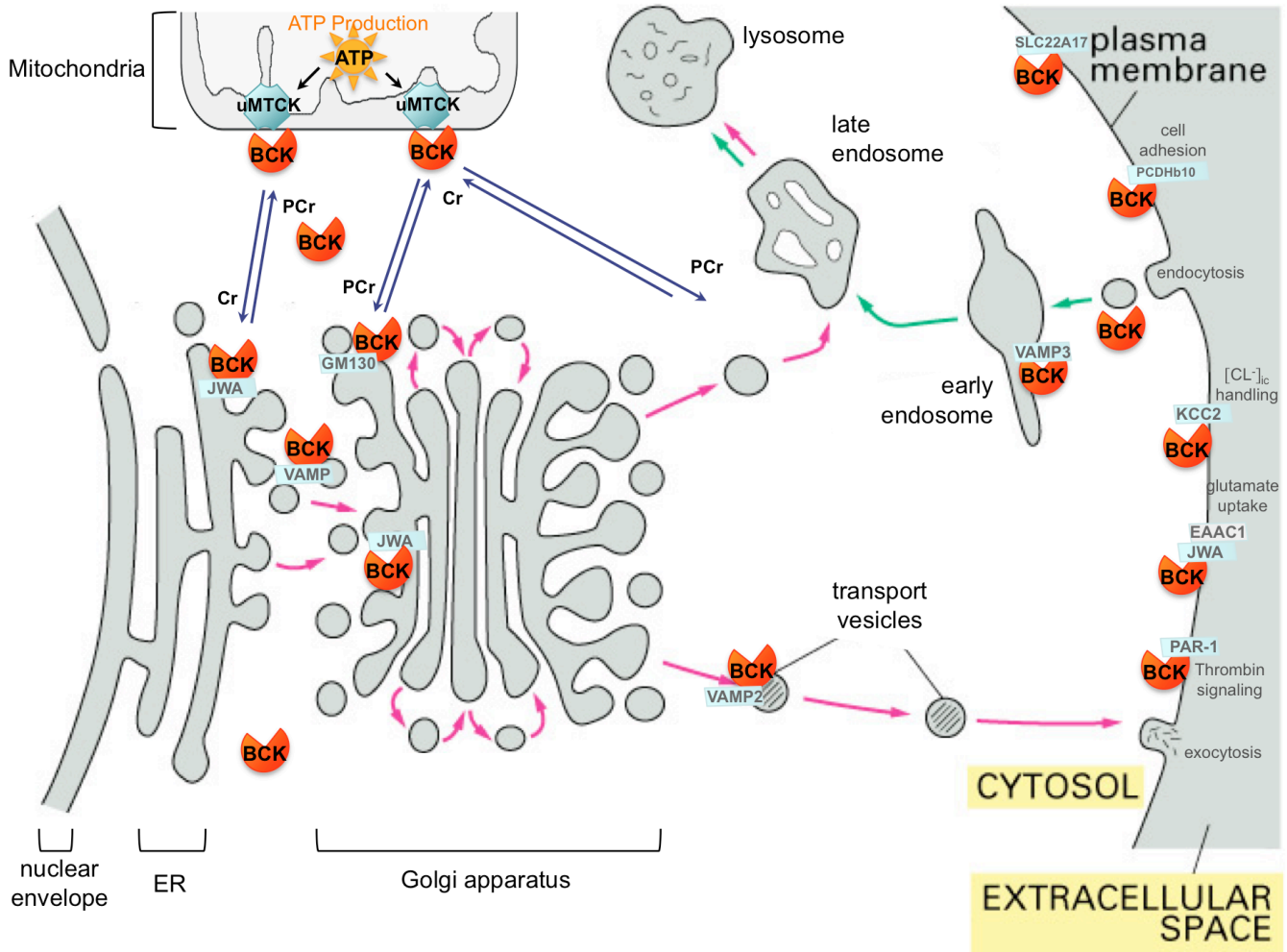


FIGURE 11-2. **Schematic model of membrane-based BCK compartmentation.** Based on subcellular BCK localization and BCK interaction partners published (GM130) or proposed in this study (Chapter 10), a hypothetical distribution of bound BCK within a schematic cell is depicted. The soluble fraction of BCK involved in global energy buffering, another important part of the CK/PCr system, is neglected in this scheme. PCr generated by uMtCK in mitochondria and possibly by BCK at the mitochondrial surface represent the providing end of the CK/PCr shuttle. At the receiving end of the shuttle, BCK is associated to various energy demanding membrane compartments where it functions as a local energy buffering system. Among the energy requiring processes are exocytosis, early states of endocytosis, and glutamate uptake. Selected BCK interaction partners are indicated in blue boxes. The color code for the arrows is: violet, Cr and PCr diffusion; pink, exocytotic pathways; green, endocytosis.

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# PART IV



FINAL CONCLUSIONS

&

FUTURE PERSPECTIVES

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1. *Final conclusions & future perspectives (english)*

2. *Conclusions finales & perspectives (français)*

## FINAL SUMMARY, CONCLUSIONS AND OUTLOOK

This thesis has investigated the interactome of two key enzymes in energy metabolism, AMP-activated protein kinase (AMPK) and creatine kinase (CK). The control of energy homeostasis is central for all biological systems (Hochachka 2003). Both enzymes ascertain energy homeostasis, albeit at different time scales and with different mechanisms. While AMPK integrates intra- and extracellular signals to orchestrate cell metabolism via a plethora of activating and inhibitory signaling pathways (Neumann et al. 2007), CK provides an immediate cellular energy buffering and transfer system (Wallimann et al. 2007). For both, signaling and energy transfer, specific protein-protein interactions seem to be important, as has been shown in detail for CK and can be also assumed for AMPK, following the example of other major protein kinases.

To analyze protein-protein interactions, partially novel interactomics approaches have been applied and different techniques were combined in order to obtain robust data. First, original cytosolic yeast two-hybrid (Y2H) systems, the split-ubiquitin based cyto-Y2H and the split-Trp1 based Y2H, allowed identification and analysis of binary PPIs. Second, surface plasmon resonance (SPR)-based, interaction assays allowed biophysical confirmation and extraction of affinities of certain protein-protein interactions or, coupled to *in vitro* AMPK phosphorylation assays, identified AMPK substrates preferentially phosphorylated by certain AMPK isoform combinations.

### 1. ANALYSIS OF THE AMPK INTERACTOME

From our approaches aiming at AMPK interaction partners, important conclusions can be drawn from the methodological and biological point of view.

Two different interactomics screening strategies were applied in this thesis to identify new protein interactions of different AMPK complexes. Firstly, a cyto-Y2H screen for new interaction partners of AMPK $\beta$ 1 and AMPK $\beta$ 2 (Chapter IV) led to the identification of 7 new putative candidates. With a large majority of membrane proteins identified, this approach revealed that cytosolic Y2H is able, in principle, to reveal such partners of AMPK. In a second, novel approach (Chapter V), an *in vitro* two-dimensional screening combined a biophysical interaction assay with phosphorylation assays to identify AMPK-interacting substrates. As anticipated, this strategy was able to identify rather AMPK isoform-specific AMPK substrates. The number of potentially interesting candidates was limited, but both strategies have potential to be further improved. Different AMPK baits could be screened for their potential to obtain interactors, possibly involving recombination with endogenous yeast AMPK

homologues, or yeast three- or four-hybrid approaches may be checked to express the full mammalian AMPK complex. For the two-dimensional screen, the purification step preceding the first screening as well as the gel separation subsequent to the *in vitro* phosphorylation in the second screening step can be further extended by adding a chromatography step or using 2D-PAGE. Finally, most of the identified AMPK substrates have still to be further confirmed as *bona fide in vivo* interaction partners of AMPK. As a model, how different interactomics methods like different Y2H techniques, SPR measurements, co-immunoprecipitation, and co-pull down can be combined for validation of a novel AMPK interacting protein, work on glutathion-S-transferases is presented in Chapter VI. These data illustrate the complementarity of the applied methods to obtain qualitative but also quantitative interaction data. In the era of emerging high-throughput interaction screens, it may be appropriate to emphasize the importance of such detailed approaches to obtain reliable interaction data.

As a result of the different applied strategies, several interesting AMPK interacting proteins have been identified that are worth being followed-up further. First, interaction of AMPK with VAMP3 and the close family member VAMP2 was independently observed in two different Y2H systems (i.e. cyto-Y2H and Split-Trp Y2H), and VAMP2 enriched with synaptic vesicles was co-immunoprecipitated with the heterotrimeric AMPK complex. This convincing evidence for AMPK interaction with VAMP2/3 contrasts with the failure of AMPK to phosphorylate these VAMPs, suggesting that they may function as scaffolding proteins. They may recruit AMPK to vesicles for some function within the endo- and exocytotic machinery. Consistent with this notion, specific VAMPs seem to participate in specific cellular processes. AMPK-regulated surface expression of nutrient transporters GLUT4 and CD36 involves VAMP3 and VAMP5, but not VAMP2 (Schwenk et al. 2010), while the AMPK-VAMP2 interaction that we detected on synaptic vesicles may play a role in the mobility and release of synaptic vesicles. It would thus be interesting to investigate the distribution of AMPK in different intracellular vesicle populations, e.g. by co-labeling different VAMPs and AMPK subunit isoforms to study their colocalization by FRET/confocal microscopy. Further, molecular mechanisms and the nature of the SNARE complexes involved in different processes like GLUT4 translocation and neurotransmitter release are quite different. Thus, identification of proteins co-immunoprecipitating with VAMP2 and VAMP3 in different cell types might reveal different multiprotein complexes involved. Taken together, we propose here the model of an at least partial spatio-temporal organization of AMPK signaling by multiprotein complexes, possibly constituted of scaffolding, regulatory and target proteins. The role of scaffolding proteins would be the recruitment of AMPK to specific subcellular sites where AMPK action, but possibly also AMPK activation, proceeds in a local manner, either via local changes in ATP/AMP ratios or local presence of upstream kinases.

Among the identified proteins in our two-dimensional screen, fumarate hydratase (FH, or fumarase) and possibly also fatty acid binding protein 1 (FABP1) represent *in vitro* AMPK targets, preferentially interacting with and phosphorylated by the AMPK $\alpha$ 2 isoform. Thus, the  $\alpha$ -subunit could at least in some cases determine some substrate-specificity of the AMPK complex. AMPK mediated

phosphorylation of FH led to significant enzymatic activation of this enzyme. We suggest, that AMPK mediated phosphorylation and activation of cytosolic FH could be involved in its role in the DNA damage response (Yogev et al. 2010). FH was phosphorylated with high stoichiometry, in contrast to FABP1, which will facilitate the identification of the AMPK phosphorylation site. Once the site determined, phosphosite-specific antibodies should be generated enabling detection of phosphorylated FH in cells *in vivo* and putative nuclear translocation of FH following AMPK activation.

Finally, identification of glutathion-S-transferases as AMPK interaction partner by a panel of complementary methods links AMPK for the first time to direct regulation of cellular oxidative stress defense. Both members of the main cytosolic GST families that were analyzed, GSTP1 and GSTM1, interact strongly with and are phosphorylated by AMPK, although only activity of GSTP1 was increased by this modification. Further studies have to elucidate whether there are additional functions of the strong AMPK/GST interaction possibly related to mitochondrial translocation or AMPK glutathionylation. Indeed, for the cytosolic isoform GSTA4 such a phosphorylation mediated mitochondrial translocation has already been described (Raza et al. 2002, Robin et al. 2003). Importantly, there are two notes of caution associated with the finding of an AMPK-GST interaction. First, activation of AMPK e.g. by a strategy applying AMPK activators to reinforce the tumor-suppressing LKB1-AMPK-mTOR pathway may be contraproductive. This would also increase activity of GSTP1, which is ubiquitously expressed and whose overexpression is associated with resistance to cancer therapy. In fact, it should be tested whether abolishing or mimicking GSTP1 phosphorylation (e.g. by mutagenesis of the phosphorylation site) could reduce/increase the resistance of cancer cells to chemotherapy. Second, AMPK also interacts with the popular GST-tag, a parasite worm ortholog of mammalian M-family GSTs. This may interfere with any *in vitro* study using GST-fused proteins in interaction assays with AMPK like e.g. co-immunoprecipitation.

## 2. ANALYSIS OF BCK COMPARTMENTALIZATION

Preliminary results obtained in this thesis by combining classic biochemical methods and an original cytosolic Y2H screen enlarge our knowledge on BCK compartmentalization in brain. Besides muscle, brain is one of the organs with the most fluctuating energy requirements. In muscle, cytosolic MCK has been extensively studied (Wallimann et al. 2007). It is functionally tightly coupled to energy requiring processes like myofibrillar and sarcoplasmic reticulum ATPases, providing them locally with ATP, without however directly interacting with these ATPases. This has favoured a concept in which other components, mainly cytoskeletal or membrane components, can function as receptors to bring CK in close vicinity of the ATP-consuming process. Such receptors were identified as myomesin and M-protein, recruiting MCK to the myofibril, or as cardiolipin, a phospholipid recruiting mitochondrial CK to the inner mitochondrial membrane. Much less is known on the brain homologue BCK. Earlier studies from the 70s associated brain BCK with synaptic vesicles and plasma membrane

(Friedhoff et al. 1977), while few more recent studies, although not on brain cells, identified true BCK receptor proteins in the plasma membrane (Mahajan et al. 2000, Inoue et al. 2004) and the Golgi apparatus (Burklen et al. 2007).

We confirmed localization of BCK at the surface of synaptic vesicles, involving a strong non-electrostatic interaction, possibly due to a hydrophobic component. We suggest VAMP2 which was identified as BCK interactor by different Y2H assays, to act as (one of the) BCK receptors to recruit BCK to the energy-requiring membrane fusion SNARE machinery prior to exocytosis of neurotransmitters. Inhibition of such a mechanism in the BCK knock-out mice may contribute to the pronounced deficits in learning, memory and spatial orientation observed in these animals. It might thus be interesting to compare the glutamate release from synaptosomes of control *versus* BCK knock-out mice. Since also the VAMP2 homolog VAMP3 which is present in vesicle populations of other cell types, interacts with BCK in the Y2H assays, it is conceivable that BCK supports also other endo- and exocytotic processes. These may include phagocytosis, membrane receptor recycling, or protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus and the cell surface.

We finally show for the first time an electrostatic association of BCK with isolated brain mitochondria. However, since recombinant BCK was unable to associate to liver or BCK-depleted brain mitochondria, interaction may include posttranslational modification of BCK or a brain-specific mitochondrial receptor that was detached in BCK-depleted brain mitochondria. Further studies have to show, whether decreased expression of mitochondrial MtCK in more glycolytic tissues like brain, tumors, or in an MtCK knockout model leads to a compensatory association of cytosolic BCK to the mitochondrial surface. It might be possible that this fraction of BCK, although not located in the mitochondrial intermembrane space as MtCK, can also be functionally coupled to oxidative ATP generation. This could be tested in permeabilized cells, using pyruvate kinase (PK)-phosphoenolpyruvate (PEP) to trap ADP and thus compete with mitochondrial respiration for BCK-generated ADP.

### 3. PUTATIVE INTERPLAY OF AMPK AND CREATINE KINASE

As a matter of fact, both AMPK and BCK were found to share VAMP2/3 and JWA as interaction partners. In addition, preliminary data show that both kinases co-purify with brain mitochondria, and that recombinant AMPK111 and AMPK221 are able to bind to such mitochondria (data not shown). Thus, co-localization of both enzymatic systems could be envisaged as a more widespread phenomenon. Indeed, Ponticos and colleagues (Ponticos et al. 1998) presented data on a close interaction and cellular co-localization of AMPK and muscle MCK that involved regulation of AMPK by PCr/Cr ratios and, *vice versa*, phosphorylation of CK and inhibition of its activity. Although regulation of AMPK and CK via PCr/Cr ratios and phosphorylation, respectively, could not be confirmed by subsequent studies (Ingwall 2002, Suter et al. 2006, Taylor et al. 2006), most recent data confirm *in vitro* and *in vivo* phosphorylation of at least BCK by AMPK. This involves an interaction

between both kinases that is however rather transient than of high affinity (Ramirez-Rios et al. 2010). The study confirmed that BCK phosphorylation did not affect enzymatic activity, but discovered a specific localization of phosphorylated BCK at the ER following AMPK activation (Ramirez-Rios et al. 2010). Possibly, the results of Ponticos et al. have to be re-interpreted in that phosphorylation of MCK triggers localization of AMPK at the myofibrillar M-band, together with MCK, as has been observed by these authors in immunostainings of rat muscle fibers (Ponticos et al. 1998). Based on our Y2H data and these considerations, we propose that in some cases BCK receptor complexes also act as or contain AMPK scaffolding proteins, thus leading to multiprotein complexes including both kinases. This could explain the stringent co-immunoprecipitation of MCK and AMPK (Ponticos et al. 1998) not observed in binary MCK/AMPK interaction assays (data not shown). We suggest as a rationale for AMPK/CK co-localization a local sensing of ATP/AMP ratios by AMPK. CK would face a primary fall in local ATP, but regenerate ATP by dragging on available PCr. Only in situations where supply of PCr becomes limiting, i.e. at the very onset of energy stress, local ATP will fall, triggering AMP increase by the adenylate kinase reaction. This would then be sensed locally by the co-localized AMPK, even if global, cellular [ATP] pools are still high, allowing an early activation of compensatory AMPK signaling.

In conclusion, screening for AMPK and CK interaction partners yielded substantial new information on their putative cellular functions. For AMPK, some interactors are substrates whose enzymatic properties are changed by phosphorylation (GSTP1, FH), others like VAMP2/3 may rather represent scaffolding proteins recruiting AMPK and possibly further proteins to specific sites for further phosphorylation events. For CK, identified proteins are typically receptor proteins that also recruit BCK to sites of high ATP requirement in order to preserve local ATP pools. Finally, co-localization of both, AMPK and BCK, as suggested by some common interactors, may involve hitherto unrecognized activation of AMPK by local, CK-controlled ATP/AMP ratios.

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## CONCLUSIONS FINALES & PERSPECTIVES

Au cours de cette thèse l'interactome de deux enzymes clés du métabolisme énergétique a été examiné en détail. Le contrôle de l'homéostasie énergétique est crucial pour tout organisme vivant (Hochachka 2003). La créatine kinase (CK) ainsi que la protéine kinase activée par l'AMP (AMPK) jouent un rôle central dans le maintien de celle-ci, bien qu'à une échelle temporelle et *via* des mécanismes différents. L'AMPK intègre des signaux intra- et extra-cellulaires afin de diriger le métabolisme cellulaire *via* l'activation et l'inhibition de certaines voies métaboliques (Neumann et al. 2007) alors que la CK constitue un système cellulaire de stockage et de transport d'énergie immédiat (Wallimann et al. 2007). Pour la CK et d'autres protéines kinases, les interactions protéine/protéine semblent avoir une fonction importante non seulement dans le transfert mais également dans la signalisation énergétique.

Afin d'analyser les interactions protéine/protéine des deux kinases, différentes approches interactomiques ont été utilisées et combinées afin d'obtenir de solides données. Premièrement, l'emploi de systèmes de double-hybride en levures (Y2H) novateurs a permis l'identification et l'analyse d'interactions binaires protéine/protéine. La détection d'interaction dans le cas du cyto-Y2H est basée sur la reconstitution d'ubiquitine divisée suivie de la libération d'un facteur de transcription et d'une réponse transcriptionnelle. Dans le cas du split-Trp, l'interaction conduit à la reconstitution de la protéine Trp1p ainsi qu'à la reconstitution de son activité enzymatique impliquée dans la synthèse du tryptophane. Deuxièmement, des essais d'interaction basés sur la résonance plasmonique de surface (SPR) ont permis de confirmer certaines interactions et de déterminer leurs constantes d'affinité. De plus, la SPR couplée à des essais de phosphorylation *in vitro* par l'AMPK a également mené à l'identification de substrats préférentiellement phosphorylés par une certaine isoforme de l'AMPK.

### 1. ANALYSE DE L'INTERACTOME DE L'AMPK

Deux stratégies différentes ont été employées dans cette thèse afin d'identifier de nouveaux partenaires d'interaction de différents complexes AMPK. Premièrement, le cyto-Y2H utilisé pour rechercher de nouveaux partenaires d'interaction d'AMPK $\beta$ 1 et d'AMPK $\beta$ 2 (Chapitre 4) a mené à l'identification de sept nouveaux candidats potentiels. La majorité de ces candidats sont des protéines membranaires démontrant la capacité d'un système de double-hybride cytosolique à détecter de tels partenaires de l'AMPK. Dans une deuxième approche, un criblage bidimensionnel combinant une



approche d'interaction biophysique (SPR) avec des essais de phosphorylation *in vitro* a permis l'identification de substrats interagissant avec l'AMPK. Même si le nombre de candidats intéressants identifiés est pour le moment limité, les deux stratégies ont démontré leur potentiel et peuvent être perfectionnées. Ainsi, différentes sous-unités de l'AMPK peuvent être utilisées comme appât pour l'identification de nouvelles cibles. En outre, une approche de triple- ou quadruple- hybride en levure pourrait être envisagée en exprimant l'hétérotrimère entier d'AMPK ou plusieurs sous-unités capables de former des complexes avec les sous-unités d'AMPK endogènes de levure. Dans le cas du crible bidimensionnel (SPR/ essai kinase), une amélioration de la résolution des protéines est envisageable soit en ajoutant des étapes supplémentaires de fractionnement (chromatographies) préalables au crible SPR soit en procédant à une séparation bidimensionnelle des protéines après l'essai kinase par 2D-PAGE. Finalement, une analyse plus poussée de la majorité des partenaires d'AMPK identifiés sera nécessaire pour confirmer qu'il s'agit de véritables substrats ou partenaires d'interaction *in vivo*. L'étude de la glutathion S-transférase (GST) décrite dans le chapitre 6 constitue un modèle de la combinaison nécessaire de différentes méthodes d'interactomique (Y2H, co-immunoprécipitation, SPR, co-pull down) pour valider les nouveaux partenaires d'interaction de l'AMPK. Ces données illustrent également la complémentarité de ces méthodes pour obtenir des données à la fois qualitatives et quantitatives sur l'interaction. A un moment où les cribles à haut débit pour la recherche de partenaires gagnent en importance, il semble effectivement approprié de souligner la portée et l'importance de telles approches pour l'obtention de données fiables.

L'utilisation des diverses stratégies présentées ici a mené à l'identification de plusieurs partenaires d'interaction de l'AMPK dont l'étude approfondie serait intéressante. Premièrement, l'interaction de l'AMPK avec VAMP3 et avec son homologue VAMP2 a été observée indépendamment avec deux techniques de double-hybride différentes (cyto-Y2H et split-Trp). De plus, sur des fractions de vésicules synaptiques VAMP2 a été co-immunoprécipité avec le complexe hétérotrimérique AMPK. Malgré l'évidence d'une interaction entre AMPK et VAMP2/3, il s'avère que les protéines VAMPs ne sont pas phosphorylées par AMPK *in vitro* ce qui laisse supposer qu'elles exercent une fonction d'échafaudage pour l'AMPK sur les vésicules synaptiques. Elles pourraient ainsi recruter l'AMPK aux vésicules pour y exercer une certaine fonction dans l'endo- et/ou l'exocytose. En effet, les différentes VAMPs semblent participer à des processus cellulaires spécifiques. Par exemple, la translocation à la membrane plasmique des vésicules de GLUT4 et de CD36, régulée par l'AMPK, semble impliquer VAMP3 et VAMP5 mais pas VAMP2 (Schwenk et al. 2010). L'interaction AMPK/VAMP2 que nous avons détectée sur les vésicules synaptiques pourrait jouer un rôle dans la mobilité et le relargage de ces derniers. Il serait donc intéressant d'analyser la distribution d'AMPK au sein de différentes populations vésiculaires, par exemple par colocalisation par transfert d'énergie par résonance de type Förster (FRET) de différentes isoformes d'AMPK et de VAMP co-marquées. De plus, les mécanismes moléculaires et la nature des complexes SNARE impliqués dans les différents processus tels la translocation de GLUT4 ou le relargage des neurotransmetteurs sont relativement différents. L'identification par co-immunoprécipitation de protéines associées à VAMP2 ou VAMP3 dans les

différents types cellulaires pourrait révéler l'implication de complexes multiprotéiques différents. Nous proposons ainsi un modèle de signalisation d'AMPK à travers la formation au moins partielle de complexes multiprotéiques, constitués par exemple de protéines d'échafaudage, de protéine de régulation et/ou de cibles. Le rôle des protéines d'échafaudage serait donc le recrutement de l'AMPK vers des compartiments cellulaires spécifiques où AMPK exercerait non seulement son rôle de kinase mais serait également activée de façon locale soit par des changements du ratio AMP/ATP local soit par la présence des kinases en amont.

Lors du crible bidimensionnel, nous avons identifié la fumarate hydratase (FH, ou fumarase) et la fatty acid binding protein 1 (FABP1) comme cibles potentielles d'AMPK. Les deux protéines semblent interagir préférentiellement avec AMPK $\alpha$ 2 et être préférentiellement phosphorylées par celle-ci. La phosphorylation de la FH par AMPK provoque un changement significatif de son activité enzymatique. Nous proposons que cette phosphorylation de FH et son activation pourraient être impliquées dans la réponse à l'endommagement de l'ADN (Yogev et al. 2010). Contrairement à FABP1, FH est phosphorylée avec une stœchiométrie élevée, ce qui facilitera l'identification du site de phosphorylation par l'AMPK. Une fois le site déterminé, des anticorps dirigés contre ce site pourront être générés et permettre de détecter la FH phosphorylée dans les cellules ainsi que d'analyser une éventuelle translocation dans le noyau suite à l'activation par l'AMPK.

Finalement l'identification, par des méthodes complémentaires, de la glutathion S-transférase comme partenaire d'interaction et de cible de l'AMPK relie pour la première fois l'AMPK à la régulation directe de la défense contre le stress oxydant cellulaire. Les deux membres majoritaires de la famille cytosolique des GST, GSTP1 et GSTM1, ont été analysés. Les deux interagissent fortement avec l'AMPK, ils sont tous deux phosphorylés par cette dernière, mais seule l'activité de GSTP1 est augmentée par cette phosphorylation. Des études supplémentaires devront montrer si l'interaction entre AMPK et GST pourrait avoir d'autres effets par exemples sur la translocation des GST à la mitochondrie ou sur la glutathionylation de l'AMPK. En effet, il a été montré, dans le cas de GSTA4, qu'une phosphorylation pouvait influencer la translocation mitochondriale de celle-ci (Raza et al. 2002, Robin et al. 2003).

Il est important de mentionner que la découverte de l'interaction AMPK/GST soulève deux problématiques. Premièrement, activer l'AMPK dans le but de renforcer la voie de signalisation LKB1-AMPK-mTOR pourrait être contre-productif car ceci augmenterait parallèlement l'activité de GST et donc la résistance aux anticancéreux. En effet, il faudrait tester si abolir ou mimer la phosphorylation de GSTP1 (par exemple par mutagenèse du site de phosphorylation) réduit ou augmente la résistance des cellules cancéreuses à la chimiothérapie. Deuxièmement, l'AMPK interagit également avec le tag GST, un orthologue parasite de la famille M des GSTs. Ceci pourrait interférer dans toute étude d'interaction *in vitro* avec l'AMPK et utilisant des protéines de fusion à la GST (exemple de la co-immunoprécipitation).

## 2. ANALYSE DE LA COMPARTIMENTALISATION DE LA BCK

Les résultats préliminaires obtenus dans cette thèse en combinant des méthodes biochimiques classiques et un crible double-hybride cytosolique novateur révèlent de nouvelles connaissances sur la compartimentalisation de la BCK dans le cerveau. Outre le muscle, le cerveau est l'organe ayant les demandes énergétiques les plus fluctuantes. Dans le muscle, la MCK cytosolique a été étudiée en détail (Wallimann et al. 2007) et il a été montré qu'elle est fonctionnellement couplée aux processus à fort besoin énergétique tel que les ATPases des myofibrilles et du réticulum sarcoplasmique. Dans ces cas, la MCK fournit localement l'ATP sans pour autant interagir directement avec les ATPases. Ces résultats ont mené au développement du concept de l'association de la CK à des compartiments, comme le cytosquelette ou des membranes cellulaires, *via* des récepteurs afin de la localiser à proximité des processus de consommation d'ATP. Ainsi, la myoméline et la M-protéine recrutent la MCK aux myofibrilles et la cardiolipine, un phospholipide, recrute la CK mitochondriale à la membrane intramitochondriale. Les connaissances sur l'homologue cytosolique du cerveau, la BCK, sont beaucoup plus limitées. Dans les années 70 des études ont suggérées une association de BCK aux vésicules synaptiques et à la membrane plasmique (Friedhoff et al. 1977). Des études plus récentes ont identifié des protéines réceptrices de BCK à la membrane plasmiques (Mahajan et al. 2000, Inoue et al. 2004) et à l'appareil de Golgi (Burklen et al. 2007).

Nous avons non seulement confirmé une localisation de BCK à la surface des vésicules synaptiques, mais avons également montré qu'elle y est associée par des interactions non électrostatiques, probablement par l'implication d'une composante hydrophobe. Nous suggérons que VAMP2, identifiée également comme partenaire de BCK par différents essais de double-hybride, fonctionne comme un des récepteurs recrutant BCK à la machinerie de fusion des membranes, SNARE, afin de fournir l'énergie nécessaire à l'exocytose des neurotransmetteurs. L'inhibition d'un tel mécanisme pourrait contribuer aux déficits d'apprentissages, de mémoire et d'orientation spatiale observés chez les souris knock-out (KO) pour BCK. Il pourrait ainsi être intéressant de comparer le relargage du glutamate des synaptosomes de souris control *versus* les KO BCK. Lors des essais de double-hybride, la BCK interagissait non seulement avec VAMP2 mais également avec son homologue VAMP3 présent dans des populations vésiculaires différentes et également dans d'autres types cellulaires. Il est donc concevable que la BCK soutient également d'autres processus d'endo- et d'exocytose impliqués par exemple dans la phagocytose, le recyclage de récepteurs membranaires ou dans le transport des protéines du réticulum endoplasmique (ER) à l'appareil de Golgi et à la surface cellulaire.

Nous avons également montré pour la première fois une association électrostatique de BCK avec des mitochondries de cerveau isolées. Cependant, cette interaction pourrait nécessiter soit des modifications post-traductionnelles soit un récepteur spécifique des mitochondries de cerveau car la

BCK recombinante est incapable de s'associer aux mitochondries de foie ou aux mitochondries de cerveau dont la BCK a préalablement été dissociée. Des études supplémentaires devront montrer si l'expression diminuée de la MtCK mitochondriale dans des tissus d'avantage glycolytiques comme le cerveau, les tumeurs ou des modèles de MtCK KO pourrait mener à une association compensatoire de la BCK cytosolique à la surface des mitochondries et si cette fraction de CK cytosolique pourrait être couplée fonctionnellement à la génération oxidative d'ATP. Cette hypothèse pourrait être testée sur des cellules perméabilisées utilisant la pyruvate kinase (PK) et le phosphoenolpyruvate (PEP) pour piéger l'ADP et ainsi provoquer une compétition avec la respiration mitochondriale pour l'ADP généré par BCK.

### 3. INTERACTION POTENTIELLE ENTRE L'AMPK ET LA BCK

Les deux enzymes, AMPK et BCK, interagissent avec VAMP2/3 et JWA en double-hybride. De plus, des données préliminaires montrent que les deux kinases sont co-purifiées avec les mitochondries du cerveau et que les isoformes recombinantes AMPK111 et AMPK221 sont capables de s'associer aux mitochondries (données non présentées). La colocalisation des deux enzymes peut donc être envisagée en tant que phénomène cellulaire répandu. En effet, Ponticos et ses collègues (Ponticos et al. 1998) ont présenté des données sur une interaction et une colocalisation cellulaire de l'AMPK avec la MCK musculaire impliquant une régulation d'AMPK par le ratio PCr/Cr ainsi qu'une phosphorylation de CK par AMPK provoquant l'inhibition de son activité enzymatique. Bien que la régulation d'AMPK et de CK par le ratio PCr/Cr et la phosphorylation respectivement n'a pas pu être confirmée par des études ultérieures (Ingwall 2002, Suter et al. 2006, Taylor et al. 2006), des données très récentes *in vitro* et *in vivo* confirment une phosphorylation au moins de BCK par l'AMPK. Cependant ceci implique une interaction plutôt transitoire entre la BCK et l'AMPK (Ramirez-Rios et al. 2010). Cette étude confirme que la phosphorylation de BCK n'affecte pas son activité enzymatique. Par contre elle montre que la BCK phosphorylée présente une localisation spécifique au ER suite à l'activation d'AMPK (Ramirez-Rios et al. 2010). Les résultats de Ponticos *et al.* devront peut-être être réinterprétés. La phosphorylation de MCK par AMPK pourrait en effet réguler la colocalisation de MCK et d'AMPK à la M-bande des myofibrilles tel qu'il a été observé par les auteurs lors d'immunomarquage de fibres musculaires de rat (Ponticos et al. 1998). Sur la base de nos résultats de double-hybride en levure et les considérations ci-dessus, nous proposons que dans certains cas des complexes récepteurs de BCK comportent également ou agissent en tant que protéines d'échafaudage de l'AMPK formant ainsi des complexes multiprotéiques comprenant les deux kinases. Ceci pourrait expliquer l'interaction forte détectée par co-immunoprécipitation de l'AMPK et MCK (Ponticos et al. 1998) alors qu'une telle interaction n'a pas pu être confirmée par des essais d'interaction binaires (données non présentées). La colocalisation de AMPK et CK pourrait faciliter la détection rapide de changements dans le ratio AMP/ATP. La CK ferait ainsi face à des chutes locales soudaines d'ATP en

régénérant de l'ATP à partir de la PCr. Dans le cas où le stock local de PCr baisserait, la concentration en ATP chuterait à son tour provoquant une augmentation locale d'AMP accrue par la réaction de l'adénylate kinase et ainsi activerait l'AMPK colocalisée avant que le stock cellulaire d'ATP est affecté.

En conclusion, le criblage de nouveaux partenaires d'interaction de l'AMPK et de la CK a livré de nouvelles informations sur leurs possibles fonctions cellulaires. Dans le cas de l'AMPK, certains partenaires d'interaction sont des substrats d'AMPK dont les propriétés enzymatiques sont modifiées par la phosphorylation (GSTP1, FH). Dans d'autres cas, ce sont des interacteurs tel que VAMP2/3 qui pourraient fonctionner comme protéines d'échafaudage recrutant l'AMPK et probablement aussi d'autres protéines à des sites spécifiques pour d'autres évènements de phosphorylation. Dans le cas de la CK, les protéines identifiées semblent fonctionner comme récepteurs recrutant BCK aux sites à fort besoin d'ATP afin de préserver le réservoir local d'ATP. Finalement la colocalisation d'AMPK et de BCK suggérée par différents partenaires d'interaction en commun pourrait impliquer une activation locale d'AMPK par des ratios ATP/AMP contrôlés par la CK.

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# PART V



## EXPERIMENTAL PROCEDURES

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## EXPERIMENTAL PROCEDURES

### 1. CLONING

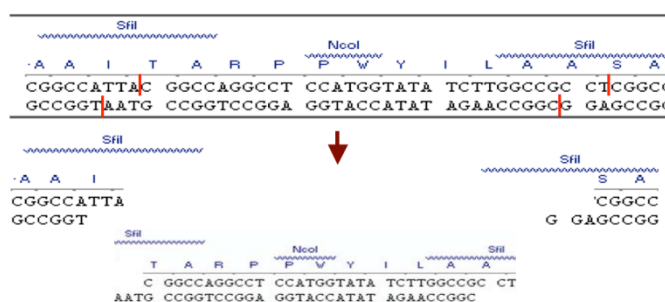
#### 1.1. Material

For pPolymerase chain reactions (PCR), dNTPs and Phusion DNA polymerase were from Finnzymes Oy (Espoo, Finland). All restriction enzymes, T4 DNA ligase and their corresponding buffers were from New England Biolabs (MA, USA). For DNA fragment and plasmid purification, NucleoSpin Extract II and NucleoSpin Plasmid Kit were from Macherey Nagel (Dürren, Germany). CytoY2H vectors pCab (bait vector) and pDSL (prey vector) or and SplitTrp vectors pI-Ctrp3 (bait vector) and pMet25-NTrp (prey vector) were gently provided by Dualsystems Biotech.

#### 1.2. Vector construction

All Yeast two-hybrid (Y2H) vectors contain two *SfiI*-sites used for cDNA introduction (Fig. 1). In order to facilitate further cloning procedures, all other vectors were modified to introduce the same *SfiI*-sites as in the Y2H vectors.

FIGURE 1. **Representation of Y2H-vector *SfiI*-cloning sites.** Cleavage with *SfiI* leads to formation of two incompatible cohesive ends. Inserts with the same end can be introduced in a oriented manner.



In brief, all vectors were constructed by amplification using primers with loose ends with *SfiI* sites and a second cleavage site used for ligation of the vector (Table 1). After amplification, PCR products were purified with NucleoSpin Extract II according to the provider's instructions. They were digested overnight with the corresponding restriction enzyme (e.g. *XhoI* for pAB-GSTn), purified again and ligated using T4 DNA ligase according to provider instructions. Ligation products were introduced into DMSO competent Mach1 *E. coli* cells for plasmid amplification. Each construct was verified by nucleotide sequencing (Genome Express, Meylan, France). The cyto-Y2H prey vector pDSL was modified by site directed mutagenesis to introduce a Stop-codon behind the second *SfiI*-site, thus allowing the use of cDNA inserts without such a codon.

Constitutively active mutant AMPK  $\alpha 2T172D\beta 2\gamma 1$  (221TD) was cloned by ligation of *DraIII*- and *SpeI*-digested fragments from tricistronic plasmids p $\gamma 1\beta 2His-\alpha 2$  and p $\gamma 1\beta 1His-\alpha 2T172D$ .

TABLE 1. Primers used for *Sfi*1-based vectors.

Name	Sense	Sequence	Concentration	Use
pET221-HA-fw	fw	GCCTCCATGGTATATCTTGGCCGCTCGGCCTACCCATACGATGTTCCAGATTACGCTTAGGATCCGGCTGCTAACAAAGC	100pmol/ul	construction of pAB-Ha with <i>Sfi</i> 1 Sites from pET221
pET221-rev	rev	CATACCATGGAGGCTGGCCGTAATGGCCTCTAGGAAACCGTTGGTCTCC	100pmol/ul	construction of pAB-Ha with <i>Sfi</i> 1 Sites from pET221
pET221-His-fw	fw	GCCTCCATGGTATATCTTGGCCGCTCGGCCTATCATCATCATCATTAGGATCCGGCTGCTAACAAAGCC	100pmol/ul	construction of pAB-His with <i>Sfi</i> 1 Sites from pET221
pAB-His2-fw	fw	GCCCATCATCATCATCATCATCGGGATCCGGCATCG	100pmol/ul	construction of pAB-His with <i>Sfi</i> 1 Sites from pET221
pAB-His2-rev	rev	CTTTCGGGCTTTGTTAGCAGCCTAGTGGTGATGGTGATGATGCGGGATCCGCTACATG	100pmol/ul	construction of pAB-His with <i>Sfi</i> 1 Sites from pET221
p52b- <i>Sfi</i> 1-f	fw	GACCTAAGCTTTATATCTTGGCCGCTCGGCCTAGTCTGTACAAGTCGACGC	100pmol/ul	Introduction of <i>Sfi</i> 1 Sites in pET-52B(+)
p52b- <i>Sfi</i> 1s-f	fw	GACCTAAGCTTTATATCTTGGCCGCTCGGCCTAGGATCCTGTACAAGTCGACGC	100pmol/ul	Introduction of <i>Sfi</i> 1 Sites and Stop before His-Tag in pET-52B(+)
p52b- <i>Sfi</i> 1-r	rev	CGATAAAGCTTAGGCCCTGGCCGTAATGGCCCGGGTCCCTGAAAGAGGACTTC	100pmol/ul	Introduction of <i>Sfi</i> 1 Sites in pET-52B(+)
pABSEMn-f	fw	GACTGGATCCATATCTTGGCCGCTCGGCCTAGGCGCCGCATAGATAAC	100pmol/ul	Introduction of <i>Sfi</i> 1 Sites in pSEMS-26m (SnapTag in Nt)
pABSEMn-r	rev	GATAGGATCCAGGCCCTGGCCGTAATGGCCCTATACCTGAGGACCCAGCC	100pmol/ul	Introduction of <i>Sfi</i> 1 Sites in pSEMS-26m (SnapTag in Nt)
pAB-GSTn-fw	fw	GACTCTCGAGTATATCTTGGCCGCTCGGCCTAGTCTGTACTGACTGAC	100pmol/ul	Introduction of <i>Sfi</i> 1 Sites in pGEX-4T (to construct pAB-GSTn)
pAB-GSTn-rv	rev	GATACTCGAGAGGCCCTGGCCGTAATGGCCGATCCACGCGGAACAGATC	100pmol/ul	Introduction of <i>Sfi</i> 1 Sites in pGEX-4T (to construct pAB-GSTn)
Mut-pDSL-fw	fw	CGCCTGGCCTAGCGAGAATTCGATATC	100pmol/ul	Introduction of Stop in pDSL (pDSL20 construct)
Mut-pDSL-rev	rev	GATATCGAATTCCTCGTAGGCCGAGGGC	100pmol/ul	Introduction of Stop in pDSL (pDSL20 construct)

Primer sequences for forward (fw) or reverse (rev) sense PCR, concentration and application are given. Precise maps of each vector can be found in Annex 5

### 1.3. Insert amplification

All AMPK subunits and truncated AMPK  $\beta$ -subunits ( $\Delta\beta$ , aa 1-54;  $\text{ct}\beta_2$ , aa 55-272;  $\text{GBD}\beta_2$ , aa 72-272) were amplified from  $\text{p}\gamma 1\beta 1\text{His-}\alpha 1$ ,  $\text{p}\gamma 1\beta 1\text{His-}\alpha 1\text{T172D}$ ,  $\text{p}\gamma 1\beta 2\text{His-}\alpha 2$  and  $\text{p}\gamma 1\beta 1\text{His-}\alpha 2\text{T172D}$  (Neumann et al. 2003) by PCR using forward and reverse primers (Table 2) with *Sfi*1-restriction sites. After *Sfi*1-digestion, they were introduced into Y2H vectors pCab, pDSL, pI-Ctrp3 and pMet25-NTrp.

VAMP3 was cloned from the isolated yeast clone using two successive PCRs with one forward primer reconstituting the missing N-terminal domain of VAMP3, followed by the use of a second forward primer and a reverse primer introducing *Sfi*1-Sites. The coding sequence from rat VAMP2 (GeneID: 24803) was amplified from rat muscle cDNA and introduced into yeast vectors via *Sfi*1 restriction sites.

Fumarate Hydratase 1 (FH1) (GeneID: 24368) and FABP1 (GeneID: 24360) were amplified from rat liver cDNA and introduced into pAB52s.

The coding sequences for rat GSTM1 (GeneID: 24423) and GSTP1 (GeneID : 24426) were amplified from rat muscle cDNA and introduced into pAB52s.

TABLE 2. Primers used for Sfi1-based inserts.

Name	Sense	Sequence	Concentration	Use
flVAMP3-fw1	fw	CGAAGACTTCAGCAGACACAAAATCAAGTAGATGAGGTGGTGACATAATGCGAGTTAAGTGGACAAGGT	100pmol/ul	construction of full length VAMP3
flVAMP3-fw2	fw	CAATGTATTGGCCATTACGGCCATGTCTACAGGTCCAACGTGCCACTGGCAGTAATCGAAGACTTCAGCAGACACA	100pmol/ul	construction of full length VAMP3
flVAMP3-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTGAAGAGACAACCCACACAGT	100pmol/ul	construction of full length VAMP3
ntVAMP3-Sfi1-f	fw	CAATGTATTGGCCATTACGGCCATGTCTACAGGTCCAACGT	100pmol/ul	soluble part of VAMP3 with Sfi1-Sites
ntVAMP3-Sfi1-r	rev	CAATACATTGCAGGCCGAGGCGGCCCTTGAATCTTCCACC	100pmol/ul	soluble part of VAMP3 with Sfi1-Sites
ntJWA-Sfi1-f	fw	CAATGTATTGGCCATTACGGCCATGGACCTAATATCGCCCCAC	100pmol/ul	soluble nt of JWA with Sfi1-sites
ntJWA-Sfi1-r	rev	CAATACATTGCAGGCCGAGGCGGCCCTTGAATCTTCCACC	100pmol/ul	soluble nt of JWA with Sfi1-sites
ctJWA-Sfi1-f	fw	CAATGTATTGGCCATTACGGCCATGTCATGTTGAGACTTCC	100pmol/ul	soluble ct of JWA with Sfi1-sites
ctJWA-Sfi1-r	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCCTCATTGTGATATAGTCACTG	100pmol/ul	soluble ct of JWA with Sfi1-sites
NgaiR-Xma1-f	fw	GACCTCCCGGGCAGTCTGCTGAGGATCC	100pmol/ul	soluble loop of SLC22A17 (included in clone 19)
NgaiR-No1-r	rev	ACATTGCAGGCCGCGCTAGATGTTGGGGATTTGAGGAG	100pmol/ul	soluble loop of SLC22A17 (included in clone 19)
ctNogo-Sfi1-f	fw	CAATGTATTGGCCATTACGGCCATGAACGGCCTACAGGCAC	100pmol/ul	soluble CT of Nogo with Sfi1-Sites
ctNogo-Sfi1-r	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCGCTCAATC	100pmol/ul	soluble CT of Nogo with Sfi1-Sites
ctPCb10-Sfi1-f	fw	CAATGTATTGGCCATTACGGCCATGTCGCGGCCGACTCAG	100pmol/ul	soluble CT of protocadherin b10 with Sfi1-Sites
ctPCb10-Sfi1-r	rev	CAATACATTGCAGGCCGAGGCGGCCCTTGAATATAAATCCAAAGCTATTTCGG	100pmol/ul	soluble CT of protocadherin b10 with Sfi1-Sites
A2-Sfi1-r	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	AMPK 211and 221 with Sfi1-Sites
A1-Sfi1-r	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	AMPK 111 with Sfi1-Sites
ALDH2-fw	fw	CGATGTATTGGCCATTACGGCCATGTCGCGGCCGACTCAG	100pmol/ul	ALDH2(rat) with Sfi1-Sites
ALDH2-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	ALDH2(rat) with Sfi1-Sites
FABP-fw	fw	CGATGTATTGGCCATTACGGCCATGAATCTTCCGCAAGTAC	100pmol/ul	FABP(rat) with Sfi1-Sites
FABP-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	FABP(rat) with Sfi1-Sites
VAMP2-fw	fw	CGATGTATTGGCCATTACGGCCATGTCGCGGCCGACTCAG	100pmol/ul	VAMP2(rat) with Sfi1-Sites
VAMP2-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	VAMP2(rat) with Sfi1-Sites
ActG1-fw	fw	CGATGTATTGGCCATTACGGCCATGGAAGAAAGAAATCGCCGCCCTC	100pmol/ul	ActG1 (rat) with Sfi1-Sites
ActG1-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	ActG1 (rat) with Sfi1-Sites
A2-fw	fw	CGATGTATTGGCCATTACGGCCATGGCTGAGAAGCAGAAGT	100pmol/ul	$\alpha$ 2(WT or TD) with Sfi1-Sites
A2-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	$\alpha$ 2(WT or TD) with Sfi1-Sites
vSNARE-fw	fw	CAATGTATTGGCCATTACGGCCATGAGACTCAGCAGACACAAAATC	100pmol/ul	SNARE with Sfi1-Sites
vSNARE-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	SNARE with Sfi1-Sites
hG2-fw	fw	CAATGTATTGGCCATTACGGCCATGGGAAGCGCGTATGG	100pmol/ul	h $\gamma$ 2(AMPK) with Sfi1-Sites
hG2-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	h $\gamma$ 2(AMPK) with Sfi1-Sites
hB2-fw	fw	CAATGTATTGGCCATTACGGCCATGGGAAGCAGCAGCAGC	100pmol/ul	h $\beta$ 2(AMPK) with Sfi1-Sites
hB2-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	h $\beta$ 2(AMPK) with Sfi1-Sites
Sfi1-b1-fw	fw	CAATGTATTGGCCATTACGGCCATGGGAAGCAGCAGCAGC	100pmol/ul	$\beta$ 1(AMPK) with Sfi1-Sites
B1-sf1-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	$\beta$ 1(AMPK) with Sfi1-Sites
Sfi1-a1-fw	fw	CAATGTATTGGCCATTACGGCCATGGGAAGCAGCAGCAGC	100pmol/ul	$\alpha$ 1(AMPK) with Sfi1-Sites
A1-Sfi1-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	$\alpha$ 1(AMPK) with Sfi1-Sites
ntV2-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	ntVAMP2(soluble) with Sfi1 Sites
GST-fw	fw	CGATGTATTGGCCATTACGGCCATGTCGCGGCCGACTCAG	100pmol/ul	GST with Sfi1-Sites
GST-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	GST with Sfi1-Sites
FuHy-fw	fw	CGATGTATTGGCCATTACGGCCATGAACCGGCCATTCTGTCTC	100pmol/ul	Fumarate Hydratase with Sfi1-Sites
FuHy-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	Fumarate Hydratase with Sfi1-Sites
GSTP1-fw	fw	CGATGTATTGGCCATTACGGCCATGTCGCGGCCGACTCAG	100pmol/ul	GSTP1 with Sfi1-Sites
GSTP1-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	GSTP1 with Sfi1-Sites
GSTM1-fw	fw	CGATGTATTGGCCATTACGGCCATGTCGCGGCCGACTCAG	100pmol/ul	GSTM1 with Sfi1-Sites
GSTM1-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	GSTM1 with Sfi1-Sites
GEC1-fw	fw	CGATGTATTGGCCATTACGGCCATGAAGTCCAGTCAAGGAG	100pmol/ul	GEC1 with Sfi1-Sites
Gec1-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	GEC1 with Sfi1-Sites
Gec1-rev2	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	GEC1 with Sfi1-Sites
CTB2-fw	fw	CAGTGTATTGGCCATTACGGCCCAAGAGATTTGTATC	100pmol/ul	CT- $\beta$ 2 w/o Screening NT with Sfi1-Sites
GBD-CT-B2-fw	fw	CAGTGTATTGGCCATTACGGCCCAAGAGATTTGTATC	100pmol/ul	GBD-CT- $\beta$ 2 with Sfi1-Sites
NTB2-rev	rev	CAATACATTGCAGGCCGAGGCGGCCCTTCCAGCTTTGCTGATATAGTCACTG	100pmol/ul	Nt- $\beta$ 2 until GBP domain with Sfi1-Sites
beta1-rev (DS)	rev	AATACATTGCAGGCCGAGGCGGCCCAATGCCTTGTATTCCTCGGA	100pmol/ul	Construction of $\Delta\beta$ 1 (aa1-54)
beta2-rev (DS)	rev	AATACATTGCAGGCCGAGGCGGCCCAAGGAGCTTGGAGTCACTG	100pmol/ul	Construction of $\Delta\beta$ 2 (aa1-54)

Primer sequences for forward (fw) or reverse (rev) sense PCR, concentration and application are given. (DS) Primers at Dualsystems Biotech. A Precise list of all available vectors is given in Annexe 6

## 2. PROTEIN EXPRESSION AND PURIFICATION

### 2.1. Purification of AMPK

Wild-type AMPK  $\alpha$ 2-His  $\beta$ 2  $\gamma$ 1 (221WT) and AMPK  $\alpha$ 2T172D-His  $\beta$ 2  $\gamma$ 1 (221TD) were bacterially expressed and purified as published previously (Neumann et al. 2003, Riek et al. 2009).

### 2.2. Purification of GSTs

GST from *Schistosoma japonicum* (GST-Sj, GenBank: M14654.1), has been expressed and purified from pGEX-4T-1 (GE Healthcare Life Sciences, Pittsburgh, PA, USA). GSTM1 and GSTP1 were expressed from pAB52s. All 3 GST constructs, as well as the GST-ACC fusion construct (kindly provided by G. Hardie, Univ. of Dundee, UK) (Scott et al. 2002), were transformed into competent *E. coli* BL21-Codon Plus (DE3)-RIL cells (Stratagene, La Jolla, CA, USA) and incubated overnight at 37°C on LB agar containing 100  $\mu$ g/ml ampicillin and 30  $\mu$ g/ml chloramphenicol. Cultures were

grown in LB containing antibiotics and 1% glucose at 37 °C with shaking until OD (600nm) 0.6-0.8. Cells were then cooled down to 30°C and protein expression was induced for 3 hours with 1 mM isopropyl  $\beta$ -D -thiogalactopyranoside (IPTG) final concentration. Cells were harvested and resuspended in lysis buffer (PBS (Phosphate Buffer Saline), 1% Triton X-100, 20% glycerol). After addition of 125 U benzonase (Merck, Whitehouse station, NJ, USA) and sonication, insoluble material was removed by centrifugation (25000 g, 40 min, 4°C). The supernatant was applied to a Glutathione Sepharose 4B (GE Healthcare Life Sciences) column and GST was purified according to the purification protocol provided by GE Healthcare. GSTs were then dialyzed overnight in 20 mM HEPES pH 7.4, 100 mM NaCl to eliminate the glutathione and 10% glycerol was added for storage

### **2.3. Purification of GST-CamKK**

CamKK $\beta$  was expressed from pGEX-PreS-CamKK $\beta$ , gently provided by H. Tokumitsu (Dept. of Signal Transduction Sciences, Kagawa Medical University). Expression was performed under the same conditions as for GST proteins (3 h at 30°C, 1 mM IPTG). Cells were lysed in PBS pH 7.4 with Complete Protease Inhibitor cocktail (1 tablet per 50 ml solution) (Roche Diagnostics, Mannheim, Germany) as described for GSTs and applied to the same Glutathione Sepharose column. After elution with 10 mM GSH, 50 mM Tris pH 8, 30 U PreScission Protease (GE Healthcare) were added and the proteins were dialyzed for 15 h at 5°C into 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT. After digestion, GST and uncleaved fusion protein were removed by a second application of the fraction to a Glutathione Sepharose column. The collected flow-through was passed another two times on the column. After addition of 2 mM CaCl<sub>2</sub> final concentration, the sample was applied to a Calmodulin Sepharose (GE Healthcare) column and purified according to the purification protocol provided by GE Healthcare.

### **2.4. Purification of other GST-fusion proteins**

GST-ntVAMP2, GST-ntVAMP3 and GST-ACC fusion construct (kindly provided by G. Hardie, Univ. of Dundee, UK) (Scott et al. 2002), were expressed in *E.coli* BL21-Codon Plus (DE3)-RIL cells (Stratagene, La Jolla, CA, USA) and purified as described for GSTs above.

### **2.5. Purification of Strep-fusion proteins**

Strep-FH1 and Strep-FABP1 were expressed *E.coli* BL21-Codon Plus (DE3)-RIL cells (Stratagene, La Jolla, CA, USA). Cultures were grown in LB containing 100  $\mu$ g/ml ampicillin, 30  $\mu$ g/ml chloramphenicol and 1% glucose at 37 °C with shaking until OD (600nm) 0.6-0.8. Cells were then cooled down to 30°C and protein expression was induced for 3,5 hours with 1 mM isopropyl  $\beta$ -D -thiogalactopyranoside (IPTG) final concentration. Cells were harvested and resuspended in lysis buffer (60 mM Tris pH 8, 15 % sucrose, 50 mM NaCl, 10 mM EDTA, 15 mM  $\beta$ -mercaptoethanol) After addition of 125 U benzonase (Merck, Whitehouse station, NJ, USA) and

sonication, insoluble material was removed by centrifugation (25000 g, 40 min, 4°C). The supernatant was applied to a Strep Tactin Superflow column (Merck KGaA, Darmstadt, Germany) and Strep-fusion proteins were purified according to the purification protocol provided by Merck KGaA.

## **2.6. Purification of BCK**

Human BCK was expressed and purified from pET42(+) (Novagen, Madison, USA) in *Escherichia coli* as follows. Expression was induced for 4 h at 37°C with 1 mM IPTG. BCK was purified as previously described (Eder et al. 1999) with some modifications. Briefly cells were lysed in 50 mM Tris-HCl, pH 8.0 containing 15% sucrose and 125 U benzonase (Merck, Whitehouse station, NJ, USA), insoluble material was removed by centrifugation (25000 g, 40 min, 4°C). The supernatant was adjusted to pH 5.8 and loaded onto the Blue Sepharose column (Pharmacia, Dübendorf, Switzerland). After washing BCK was eluted with 50 mM NaPi, pH 8.0 containing 10 mM MgADP. Samples were desalted in 25 mM Tris-HCl, pH 8 using a HiPrep 26/10 Desalting column, and subsequently concentrated on a Resource Q column using a 0-500 mM NaCl gradient. Proteins were eluted at ~ 240 mM NaCl. More details can be found in the PhD thesis of S. Ramirez-Rios.

## **3. PREPARATION OF SUBCELLULAR ORGANELLES FROM RAT**

### **3.1. Preparation of rat liver extract for biophysical interaction screening**

#### *3.1.1. Preparation of liver extract*

Total liver from one rat was snap-frozen in liquid nitrogen and homogenized in 15 ml ice-cold extraction buffer A (20 mM HEPES, pH 7.4, 100 mM NaCl, 50 µM EDTA) plus an anti-protease cocktail (1 tablet per 50 ml solution) (Roche Diagnostics, Basel, Switzerland) using a Polytron PT 3000 homogenizer at 24 000 rpm for 20 s. The sample was further centrifuged at 15000g for 30 min at 4 °C and the supernatant filtered through a 0.22 µm filter.

#### *3.1.2. Prefractionation of proteins by Ni-NTA and Gel Filtration Chromatographies.*

Three ml of liver extract were applied to a Ni-NTA column (2 ml bed volume; Qiagen) preequilibrated in buffer A. The column was then washed at a flow rate of 1 ml/min with 2 column volumes (CV) of buffer A. Proteins were eluted with imidazole buffer (20 mM HEPES, pH 7.4, 100 mM NaCl and imidazol 250 mM). Only the 5 first ml were collected. This process was repeated 3 times. Between each load, the column was washed with 4 CV of imidazole buffer, 1 CV of water, 1 CV NaOH 0.5 M and 5 CV buffer A. To reduce the volume, proteins collected were precipitated with 80 % of ammonium sulfate, 2 hours at 4°C. The pellet was resuspended in 5 ml buffer A and further centrifuged at 15 000 g for 10 min at 4 °C. The supernatant was filtered (0.22 mm filter) and applied to a gel filtration column (Superdex 200; separation range Mr 10 000-600 000; volume 120

ml; GE healthcare) preequilibrated in buffer A. Proteins (or complexes) were then separated according to their size, at a flow rate of 1 ml/min and collected in 12 fractions of 5 ml (namely S1 to S12) where an anti-protease cocktail (1 tablet per 50 ml solution) (Roche) was added.

The gel filtration column was calibrated with the following Mr markers: thyroglobulin (670 kDa), bovine  $\gamma$ -globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B12 (1.35 kDa).

### ***3.2. Preparation of Rat Liver Extract for GST pull downs***

Cytosolic liver extract was obtained from liver homogenate (10 mM HEPES pH 7.4, 220 mM mannitol, 70 mM sucrose, 0.1% BSA, 0.2 mM EDTA) after centrifugation at 1000 g for 10 min and a second centrifugation of the supernatant at 12 000 g for 10 min. The resulting supernatant was used for pull-down and immunoprecipitation of GST isoenzymes.

### ***3.3. Preparation of rat liver mitochondria and incubation with recombinant BCK***

Mitochondria were isolated from Wistar rat liver. First, liver tissue was homogenized by a Teflon/glass potter in Mannitol-Hepes-Sucrose buffer containing 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 0.1% bovine serum albumin and 0.2 mM EDTA. The homogenate was centrifuged for 10 min at 700 g to remove heavy debris as platelets and nuclei. The supernatant was then centrifuged for 10 min at 7000 g. The pellet containing mitochondria was resuspended in 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 100  $\mu$ M EGTA, 25% Percoll and centrifuged for 35 min at 100,000 g. Purified mitochondria were washed twice with 250 mM sucrose, 10mM HEPES, pH 7.4, 100  $\mu$ M EGTA by centrifugation at 7000 g for 10 min. Washed mitochondria were then recovered from the pellet and resuspended in washing buffer. Liver mitochondria (ca. 5 mg) was incubated with 0.5 mg purified recombinant BCK for 1 h at 37°C and washed 1-4 times with washing buffer before subjection to SDS-Page and immunoblotting using anti-BCK antibody (generated and characterized previously (Schlattner et al. 2002)).

### ***3.4. Preparation of synaptosomes and brain mitochondria***

Synaptosomes and brain mitochondria were isolated from Wistar rat forebrains as previously described (Booth et al. 1978). Where noted, mitochondria was treated with 100 mM Na<sub>2</sub>CO<sub>3</sub> pH 10, 125 mM KCl or with 100 mM Na<sub>2</sub>CO<sub>3</sub> pH 11.5 for 30 min at 37°C and then washed 3 times in isolation buffer to remove soluble proteins. Alternatively mitochondria were treated with 2 M KCl in 10 mM HEPES pH 7.4, 0.32 M sucrose, 1 mM EDTA as previously described (Carre et al. 2002). To analyze BCK attachment to brain mitochondria, treated mitochondria (0.65 mg) were incubated with 20  $\mu$ g recombinant BCK for 30 min at 37°C and BCK association was analyzed by immunoblotting using anti-BCK (generated and characterized previously (Schlattner et al. 2002)) primary antibody and anti-chicken secondary antibody (1:5000, 31401, Fisher Scientific).

### **3.5. Preparation of synaptic vesicles**

Synaptic vesicles were obtained from Wistar rat forebrains by differential centrifugation and a density gradient procedure (Whittaker et al. 1964). Rat forebrains were homogenized in sucrose buffer (0.32M sucrose, 4mM HEPES pH 7.4) and separated into fractions P1 (nuclei, large myelin fragments, tissue debris), P2 (mitochondria, synaptosomes, small myelin fragments, some microsomes) and S2 (microsomes, some small mitochondria and synaptosomes, soluble proteins) as shown in Fig.1. The synaptosomal fraction (P2) was washed two times with sucrose buffer and resuspended in water (2ml/mg tissue) to disrupt synaptosomal membranes and liberate synaptic vesicles. Synaptic vesicles were then purified on a discontinuous sucrose density gradient composed of 20 ml 0.6 M sucrose, 5 ml 0.4 M sucrose and 5 ml sample (Fig.1). After centrifugation for 2 h at 53 500 g (4°C), vesicles are located in the 0.4 M sucrose phase. Enrichment of synaptic vesicles was verified by immunoblotting using anti-VAMP2 primary antibody (1:3000, PA1-766, Pierce Biotechnology, Rockford, IL, USA) and anti-rabbit secondary antibody (1:5000, NA934, GE Healthcare Life Sciences, Pittsburgh, PA, USA). All protein concentrations of biological samples were determined using the BCA Protein Assay Kit according to provider's instructions (Thermo Scientific, Rockford, IL, USA). Protein concentrations of purified recombinant proteins were determined according to Bradford (Bradford 1976) with the Biorad microassay (Biorad, Reinach, Switzerland) and BSA as standard.

## **4. YEAST TWO-HYBRID PROCEDURES**

### **4.1. Binary interaction assays**

This chapter describes the analysis of binary interactions (confirmation assays). The dimerization of GST or BCK served as positive controls, and interactions of proteins of interest with Large T antigen (*Simian virus*) as negative control.

#### **4.1.1. Cyto-Y2H System**

Cytosolic yeast two-hybrid (Y2H) systems based on reconstitution of split proteins have been used for protein interaction screening. The cytoY2H (Mockli et al. 2007) (Dualsystems Biotech, Schlieren, Switzerland) is based on the split-ubiquitin system (Johnsson et al. 1994, Stagljar et al. 1998). The membrane-anchored bait is fused to a reporter cassette composed of the C-terminal ubiquitin half and the artificial transcription factor LexA-VP16 whereas the prey is fused to the N-terminal half of ubiquitin. Bait/prey interaction leads to ubiquitin reconstitution and cleavage by ubiquitin-specific proteases that liberate the transcription factor thus leading to a classical transcriptional read-out. While the original system used an ER-membrane anchor (Ost4P) for the bait, we applied here in addition a version using a plasma membrane anchor (A $\beta$ -domain). Cloning procedures using Sfi1 sites,

transformation of yeast cell line NMY51 (*MATa his3delta200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 ura3::(lexAop)8-lacZ (lexAop)8-ADE2 GAL4*) using the lithium acetate method (Gietz et al. 2006) and yeast spotting were performed as described earlier (Mockli et al. 2007). Different dilutions (1, 1/10, 1/100, 1/1000) of overnight yeast cultures were spotted on selective medium lacking tryptophan and leucine (SD-WL) for growth verification and on medium lacking tryptophan, leucine, adenine and histidine (SD-AHWL) for protein interaction analysis. In some cases up to 5 mM 3 Amino-triazole (3-AT; Applichem, Darmstadt, Germany) was added to increase selection stringency (see also Y2H cDNA library screening procedures). The spotted plates were incubated 48-72 h at 30°C.

#### 4.1.2. *Split-Trp System*

A second Y2H system, split-Trp, is based on splitted Trp1p protein (Tafelmeyer et al. 2004). A N-terminal part of Trp1p is fused to bait subunits and a C-terminal part of Trp1p is fused to the prey. Bait/prey interaction leads to reconstitution of Trp1p activity that directly triggers growth of the yeast Trp1p null mutant strain CRY1 (*MATa ura3-1 trp1-1 his3-11,15 leu2-3,112 ade2-1 can1-100 GAL*) on medium lacking tryptophane, thus avoiding any transcriptional step. Yeast transformation of CRY1 and spotting have been performed similar as for the cyto-Y2H methods. Yeast cells were spotted on selective medium lacking uracil and leucine (SD-UL) for growth verification and on medium lacking uracil, leucine, methionine and tryptophan with copper content (SD-UMWL+CuSO<sub>4</sub>) for protein interaction analysis at full bait and prey expression levels. The spotted plates were incubated up to 9 days at 27°C.

### 4.2. *Cytosolic Y2H cDNA library screen*

#### 4.2.1. *Self-activation test*

Bait vectors, encoding BCK, ΔBCK, Δβ<sub>1</sub> or Δβ<sub>2</sub> fusion proteins, were introduced into yeast strain NMY51 using the lithium acetate method (Gietz et al. 2006, Mockli et al. 2007). Transformed cells were plated on SD-W (selection for bait-vectors). Bait encoding yeast was then transformed with 7 μg empty cDNA library vector as described for cDNA library screens. Transformed yeast cells were plated on SD-WL (plasmid selection) and on SD-AHWL supplemented with 0, 1, 2.5, 5, 7.5, 10 mM 3-AT. Plates were incubated 72h at 30°C and grown colonies observed and counted. Transformation efficiency had to exceed 10<sup>5</sup> colonies/ μg prey plasmid to cover the cDNA library complexity. For cDNA library screening procedures, the 3-AT concentration was used for selection, which presented no colonies grown in the self-activation test.

#### 4.2.2. *Human brain cDNA library screening*

A human brain cDNA library in the prey vector pDSL (Dualsystems Biotech) was transformed into the yeast reporter strain NMY51 harboring pCab-Δβ<sub>1</sub> or pCab-Δβ<sub>2</sub> using a high-efficiency



transformation protocol based on the lithium acetate method (Gietz et al. 2006). After transformation, the cells were allowed to recover for 90 minutes in 2xYPAD complete medium. Cells were resuspended in 4.8 ml NaCl 0.9 % and 0.3 ml of the cell suspension was plated on 16 round 150 mm diameter plates, containing the medium defined in the self-activation test, SD-AHWL for  $\Delta$ BCK and BCK baits and SD-AHWL + 2.5 mM 3-AT for  $\Delta\beta$ 1 and  $\Delta\beta$ 2. Transformants were selected after 2-3 days incubation at 30°C.

### **4.3. Yeast plasmid rescue**

Restreaked selected transformants were grown overnight in 5 ml ST-WL. Yeast DNA was extracted using the NucleoSpin Plasmid extraction kit (Macherey-Nagel) according to standard procedures with following changes. Cells were resuspended in 0.2 ml buffer A1 and 0.1 ml lyticase solution (1.2 M Sorbitol, 0.1 M NaPO<sub>4</sub> buffer, pH 7.4, 5 mg/ml Lyticase) was added. The spin columns were washed with both guanidine hydrochloride (AW) and ethanol (A4) before yeast DNA was eluted from the column. Finally, 5  $\mu$ l of the eluate were transformed into *E. coli* to amplify the rescued prey plasmid. DNA was extracted from two different *E. coli* colonies per clone using the NucleoSpin Plasmid extraction kit. For insert size analysis of the prey cDNA, 2  $\mu$ l were digested with *Sfi* I.

### **4.4. Bait dependency test**

The plasmids that contained an insert were further processed by a bait-dependency test. For this purpose, the individual prey plasmids were co-transformed with  $\Delta\beta$ 1,  $\Delta\beta$ 2 or LT (negative control) bait into NMY51 and spotted as described above on medium selective for the plasmids or for protein interaction. Positive clones were sequenced. The identity of cDNA inserts was determined by performing Basic Local Alignment Search Tool (BLAST) searches (Altschul et al. 1990) against GenBank®, the reading frame was verified using Vector NTI (Invitrogen, Carlsbad, CA 92008, USA). Co-immunoprecipitation and co-pull down assays

## **5. SURFACE PLASMON RESONANCE (SPR) ASSAYS**

### **5.1. Surface Plasmon Resonance Screening for AMPK 221 interactors (Chapter V)**

The SPR screening was performed with a BIAcore 1000 (GE Healthcare). Histidine-tagged AMPK221 isoform was immobilized on a NTA sensor chip (GE Healthcare). The running buffer used was 10 mM HEPES pH 7.4, 100 mM NaCl, 50  $\mu$ M EDTA and 0.005 % Surfactant P20. For each fraction, the surface was first activated by 1 min-pulse of 500  $\mu$ M NiCl<sub>2</sub>, then 50 nM AMPK221 were injected at 5  $\mu$ L/min until reaching 4000 response units (RU). A 2 min-pulse of each fraction was injected on the AMP221 surface at 20  $\mu$ L/min. After 2 min of dissociation, the protein-free surface

was regenerated by the injection of 10 mM HEPES, pH 8.3, 150 mM NaCl, 350 mM EDTA and 0.005 % Surfactant P20.

## ***5.2. Analysis of GST-AMPK interaction (Chapter VI)***

For SPR with BIAcore (GE Healthcare), GST proteins were covalently immobilized by standard amine coupling (GE Healthcare) on the carboxylic functions of two different chips. Gold chips functionalized by mixed self-assembled monolayers as described (Boireau et al. 2009) were kindly provided by Wilfrid Boireau (FEMTO-ST, CNRS Besançon, France). They used 97% 11-mercapto-1-undecanol to reduce non-specific adsorption of proteins to the surface, and 3% 16-mercaptohexadecanoic acid for protein immobilization to obtain well controlled, low ligand densities as convenient for initial experiments with GST-Sj. CM5 chips (GE Healthcare) allowing higher ligand surface density were necessary for sequential analyte injections without regeneration as used for kinetic analysis of GSTM1/P1. GST preparations (30 µg/ml) in 10 mM acetate buffer pH 6 (GST-Sj), pH 5 (GSTM1) or pH 4 (GSTP1) were injected at 5 µl/min to yield  $\approx 2.5$  ng GST/mm<sup>2</sup>. AMPK diluted to different concentrations just prior to measurements was injected onto the GST surfaces for 180 to 300 s at 20 or 30 µl/min, which excludes mass transfer limitations (not shown). Experimental curves were corrected for bulk refractive index changes and kinetic analysis was performed by fitting association and dissociation curves with BIAevaluation software.

## **6. CO-IMMUNOPRECIPITATION AND CO-PULL DOWN ASSAYS**

### ***6.1. Co-immunoprecipitation of AMPK and VAMP2 (Chapter IV)***

VAMP2 was immunoprecipitated from synaptic vesicle fractions (20 µg protein) incubated with 1 µg recombinant His-tagged AMPK (221TD) using anti-His-tag antibody (1:200, 2366, Cell Signaling Technology, Danvers, MA, USA) and Protein A Sepharose (10 %) in IP-buffer (10 mM HEPES pH 7.3, 100 mM NaCl, 6 g/L BSA, 0.5 % dodecylmaltoside) overnight at 4°C. The Sepharose was washed 4 times with wash-buffer (10 mM HEPES pH 7.3, 100 mM NaCl, 0.1 % Tween 20), re-suspended in SDS-PAGE sample buffer, and the solubilized denaturated protein was subjected to SDS-PAGE and immunoblotting using anti-VAMP2 antibody.

### ***6.2. Co-pull down of AMPK and VAMP2 (Chapter IV)***

20 µg vesicle fraction were incubated with or without 1 µg recombinant AMPK (221TD) overnight at 4°C in IP-buffer containing 10 mM imidazole and 10 % nickel-nitrilotriacetic acid (NTA) Sepharose (Qiagen, Hilden, Germany). The Sepharose was washed 5 times with wash-buffer containing 10 mM imidazole and proteins bound to the Sepharose beads were directly re-suspended

and denaturated in SDS-PAGE sample buffer and subjected to SDS-PAGE and immunoblotting as described above.

### **6.3. Co-immunoprecipitation of GSTs and AMPK (Chapter VI)**

GST was immunoprecipitated from liver extract (1 mg protein) using anti-GSTM (ab53942, Abcam) or GSTP1 (ab53943, Abcam) antibody (1:240) in PD-Buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 2,5 mM MgCl<sub>2</sub>, 10% glycerol, 6 g/L BSA, 0,5% Tween 20, 0,02% NaN<sub>3</sub>) overnight at 4°C. After addition of Protein A Sepharose (50% in PD-Buffer), samples were incubated for another hour at 4°C. The Sepharose was washed 8 times in PF-buffer, resuspended in SDS-PAGE sample buffer, and solubilized denaturated protein was subjected to SDS-PAGE and immunoblotting as above.

### **6.4. Co-pull down of AMPK and GSTs (Chapter VI)**

Either 30 µg of purified recombinant protein (GST-Sj, rat GSTM1 or GSTP1), or 1 mg liver extract were incubated with 30 µg recombinant AMPK (221WT or 221TD) for 1 h in PD-buffer before addition of Glutathione Sepharose beads (50% suspension in PD-buffer) and incubation for an additional hour at 4°C. Washed proteins bound to the Sepharose beads were directly resuspended and denaturated in SDS-PAGE sample buffer and subjected to SDS-PAGE and immunoblotting using anti-AMPK $\alpha$  primary antibody (dilution 1: 1000, 2532, Cell Signaling Technology, Danvers, MA, USA) and anti-rabbit secondary antibody (1: 5000, NA934, GE Healthcare) allowing luminescent detection. Alternatively, proteins stained on PAGE gels with colloidal Coomassie Blue were identified by MALDI-TOF/TOF mass spectrometry.

## **7. AMPK PHOSPHORYLATION ASSAYS**

### **7.1. Phosphorylation assays of recombinant VAMP2/3 & synaptic fractions (Chapter IV)**

AMPK 221TD was activated by incubation for 20 min at 30°C in kinase buffer with cold ATP. Purified Strep-ntVAMP3, GST-ntVAMP, GST-ntVAMP3 (200 pmol), synaptosomal proteins (20 µg) or vesicular proteins (1.3 or 2.6 µg) were then incubated for 2-8 min at 37°C in the presence or absence of 30 pmol 221TD in kinase buffer containing 200 µM [ $\gamma$ -<sup>32</sup>P]ATP (specific activity 400 mCi/mmol ATP), 50 µM AMP, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 10 mM HEPES (pH 7.4). For negative controls, proteins were incubated without AMPK. Kinase reactions were stopped by addition of SDS-PAGE sample buffer and subjected to SDS-PAGE and autoradiography or Typhoon phosphoimager (GE Healthcare).

## 7.2. Screening for AMPK Substrates (Chapter V).

### 7.2.1. Phosphorylation assays of protein fractions used for SPR measurements

AMPK assays were performed with 12.5  $\mu\text{L}$  of various fractions by incubation with or without recombinant constitutively active AMPK (50 pmol) at 37 °C in kinase buffer (200  $\mu\text{M}$  [ $\gamma\text{-}^{32}\text{P}$ ]ATP (specific activity of 400 mCi/mmol ATP), 50  $\mu\text{M}$  AMP, 5 mM  $\text{MgCl}_2$ , 1 mM DTT and 10 mM HEPES pH 7.4) at a final volume of 25  $\mu\text{L}$ . The kinase reactions were then stopped after 2 min by addition of 10  $\mu\text{L}$  of SDS sample buffer (105 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 15% (v/v) glycerol, 1.2 M  $\beta$ -mercaptoethanol, and 0.02% (w/v) bromophenol blue), heated to 95 °C for 5 min, and separated by SDS-PAGE. Following colloidal Coomassie staining, gels were air-dried and exposed to autoradiography films (GE Healthcare) for up to 2 weeks to reveal potential substrates of AMPK.

### 7.2.2. Trypsin Digestion and Mass Spectrometry.

After development, the autoradiography films were realigned to the dried 1-D gels. Radioactively labeled AMPK-specific bands in the gel were marked by piercing through film and gel. To avoid contamination by keratin and dust particles, the following procedures, including in-gel digestion, were performed in a laminar flow sterile bench. Marked bands were excised, the Whatmann paper layer was removed, and the gel pieces were subjected to in-gel digestion with trypsin (Promega) as described previously (Benvenuti et al. 2002). Extracted peptides were lyophilized and stored at -80°C for 4-6 weeks to reduce radioactivity levels. Peptides were reconstituted in 5  $\mu\text{L}$  of 10% (v/v) acetonitrile (ACN) supplemented with 0.1% (v/v) trifluoroacetic acid (TFA) and desalted using ZipTip C18 microcolumns. Peptides were eluted from the ZipTip in 3  $\mu\text{L}$  of 50% (v/v) ACN/10% (v/v) TFA. An aliquot was mixed with an equal volume of  $\alpha$ -cyano-hydroxycinnamic acid (10 mg/mL in 50:50 acetonitrile:water supplemented with 0.1% (v/v) TFA), spotted on a gold coated-MALDI target and allowed to dry at room temperature. All spectra were obtained by MALDI MS and MALDI MS/MS using an Ultraflex TOF/TOF II (Bruker Daltonics, Bremen, Germany). The mass spectrometer was operated in the positive ion reflector mode with 150 ns delayed extraction time, the nitrogen laser (337 nm) was set to a repetition rate of 50 Hz and the ion acceleration voltage was 25 kV. Mass measurements were performed automatically through fuzzy logic-based software to accumulate 500-800 single laser shot spectra or manually to accumulate 2000 single laser shot spectra. All spectra were calibrated externally with the mass signals of the peptide calibrant standard II. Routinely, prior to automate acquisition, two spots were used for signal and parameter optimization. The first monoisotopic signals in the spectra were assigned automatically using the peak detection algorithm SNAP (Bruker Daltonics, Bremen, Germany). The smoothing algorithm of Savitzky-Golay was applied (width 0.2 m/z, cycle number 1). Processed MS and MS/MS spectra were combined through BioTools software (Bruker Daltonics, Bremen, Germany) to search the Uniref100 database (release 6.0), non restricted to the taxonomy, using MASCOT software v. 2.0 (Matrix Science, London, UK).

Mass errors of 150 ppm for both, the peptide mass fingerprints and the precursor ions selected for fragmentation were allowed. A maximal tolerance of 0.2 Da was allowed for fragment ions. In addition, the search parameters tolerated oxidation of methionine, carbamidomethylation of cysteine, phosphorylation in threonine and serine as variable modifications were included up to one missed cleavage for trypsin. Probability-based MOWSE scores greater than 50 were considered significant and not a random event.

### *7.2.3. In vitro analysis of target phosphorylation by different AMPK isoforms.*

AMPK 221WT was activated by incubation with 1 pmol CamKK $\beta$  for 20 min at 30°C in kinase buffer with cold ATP. Purified FABP or FH1 (200 pmol) were then incubated for 8 min at 37°C in the presence or absence of 3 pmol previously activated AMPK in kinase buffer containing 200  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (specific activity 650 mCi/mmol ATP), 50  $\mu$ M AMP, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 10 mM HEPES (pH 7.4). For negative controls, targets were incubated with 1 pmol CamKK $\beta$  alone without AMPK. Kinase reactions were stopped by addition of SDS-PAGE sample buffer and subjected to SDS-PAGE and Typhoon phosphoimager (GE Healthcare).

### *7.3. AMPK phosphorylation assays of GSTs and control target (Chapter VI).*

AMPK 221WT was activated by incubation with 1 pmol CamKK $\beta$  for 20 min at 30°C in kinase buffer with cold ATP. Purified GSTs and GST-ACC (200 pmol) were then incubated for 3 min at 37°C in the presence or absence of 3.5 pmol previously activated 221WT in kinase buffer containing 200  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (specific activity 400 mCi/mmol ATP), 50  $\mu$ M AMP, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 10 mM HEPES (pH 7.4). For negative controls, GSTs were incubated with 1 pmol CamKK $\beta$  alone without AMPK. Alternatively, to analyse effects of GST on AMPK *in vitro* phosphorylation activity, 4 pmol AMPK 221TD were preincubated without or with 8 pmol BSA or 8 pmol GSTM1 in kinase buffer (without [ $\gamma$ -<sup>32</sup>P]ATP) during 20 min at 30°C before addition of 200 pmol target protein and [ $\gamma$ -<sup>32</sup>P]ATP, followed by incubation for 5 min at 37°C. Kinase reactions were stopped by addition of SDS-PAGE sample buffer and subjected to SDS-PAGE and autoradiography or Typhoon phosphoimager (GE Healthcare).

### *7.4. AMPK activation by CamKK $\beta$ (Chapter VI).*

AMPK 221WT (25 nM) was incubated for 10 min at 30°C in absence or presence of GSTM1 or GSTP1 (50 or 125 nM) in kinase buffer. Recombinant CamKK $\beta$  (1.25 nM) was added and samples were incubated for 3 min at 30 °C. AMPK activity was probed by immunoblot analysis of Thr-172 phosphorylation using anti-phospho-AMPK $\alpha$  primary antibody (1: 1000, 2531, Cell Signaling Technology, Danvers, MA, USA) and anti-rabbit secondary antibody allowing luminescent detection.

## 8. MEASUREMENT OF ENZYMATIC ACTIVITIES

### 8.1. *Fumarate Hydratase enzyme activity (Chapter V)*

AMPK 221WT was activated by CamKK $\beta$  as described above and incubated with FH1 and ATP for 30 min at 37°C prior to activity measurements. Enzyme activity of FH1 and phospho-FH1 was then determined at 25°C by a spectrophotometric assay measuring fumarate formation (250 nm) in 0.1M potassium phosphate buffer pH 7.6 using 0.5 to 8 mM malate. Data were analyzed by direct fitting to Michaelis-Menten kinetics and secondary plots using SigmaPlot 10 (Systat Software, USA).

### 8.2. *GST enzyme activity (Chapter VI)*

AMPK 221WT was activated by CamKK $\beta$  as described above and incubated with GSTP1 and ATP for 30 min at 37°C prior to activity measurements. Enzyme activity of GST and phospho-GST was then determined at 25°C by a spectrophotometric assay using 2 mM (GSTP1) or 10mM (GSTM1) glutathione and 0.1 to 1 mM 1-chloro-2,4-dinitrobenzene as described (Habig et al. 1974). Data were analyzed by direct fitting to Michaelis-Menten kinetics and secondary plots using SigmaPlot 10 (Systat Software, USA).

### 8.3. *Determination of BCK activity in synaptic vesicle fraction (Chapter IX)*

Enzymatic activity of BCK on synaptic vesicles was determined using a coupled enzyme assay as described previously (Wallimann et al. 1977). Briefly, ATP production (reverse reaction) was coupled by hexokinase (399 U/ml) and glucose-6-phosphate dehydrogenase (150 U/ml) to NADPH production, using 2 mM ADP, 5 mM MgCl<sub>2</sub>, 20 mM PCr, 40 mM D-glucose and 1 mM NADP in 4 mM HEPES pH 7.4, 0.32 M sucrose. Changes in the redox state of pyridine nucleotides were followed at 340 nm in a Specord 210 spectrophotometer (Analytikjena, Germany) at 25°C. In order to determine if BCK was outside or inside synaptic vesicles, those were permeabilized by saponine treatment (0.18 mg/ml) or broken by freezing/thawing cycles and BCK activity was compared to these of intact vesicles.

## 9. OTHER METHODS

### 1.1. *Glutamate release assay from synaptosomes (Chapter IV)*

The glutamate release assay was performed using enzyme-linked fluorescence detection of released glutamate (Nicholls et al. 1986, Cousin et al. 1998). This assay relies on the generation of  $\alpha$ -ketoglutarate from released glutamate by glutamate dehydrogenase accompanied by the oxidation of NADP into NADPH. NADPH accumulation is measured fluorometrically. In brief, synaptosomes were re-suspended in either plus (1.2 mM CaCl<sub>2</sub>) or minus (1 mM EGTA) Krebs-like solution (118.5

mM NaCl, 4.7 mM KCl, 0.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM HEPES, 10 mM glucose, pH 7.4) at 37°C. Where indicated, synaptosomes were treated with A-769662 compound (50 μM final) (Abbott Laboratories) for 30 min at 37°C. Experiments were started after addition of 1 mM NADP. Forty units of glutamate dehydrogenase was added after 1 min, and the synaptosome suspension was stimulated after 4-5 min with 30 mM KCl. Increases in fluorescence due to production of NADPH were monitored using a Hitachi F-2500 spectrofluorometer at 340 nm excitation and 460 nm emission. Experiments were standardized by the addition of 10 nmol of glutamate.

### **9.1. 2D- electrophoresis of brain mitochondria and synaptic vesicles (Chapter IX)**

Proteins from brain mitochondria or synaptic vesicles were precipitated with 10 % trichloroacetic acid (1 h, -20°C). Samples were centrifuged 10 min at 18 200 g. The pellet was washed 3 times with acetone and dried before resuspension in 2D lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 1.0% v/v IPG buffer, 0.5% v/v Triton X-100, protease inhibitor mix (Roche Diagnostics, Mannheim, Germany). Protein content was determined by the 2D-Quant Kit (GE Healthcare). Approximately 0.5 mg proteins were separated in the first dimension using EttanIPGphor 3 and immobilized gel strips with a nonlinear gradient pH 3-10 (24 cm, GE Healthcare). For isoelectric focusing, voltage was gradually increased from 0 V to 10 000 V during 12 h and then kept constant for 4 h to achieve about 64 500 V in total. Strips were then equilibrated in 75 mM Tris-HCl pH 8.8, 6 M urea, 29.3% glycerol, 2% SDS, and 0.002% bromophenol blue, first in presence of 1% DTT (w/v) and then with 2.5% iodoacetamide (w/v) (15 min each). For protein separation in the second dimension, strips were applied to large 10% SDS-PAGE gels. Gels were then subjected to semi-dry western blotting and immunolabeled with primary anti-BCK antibody and secondary anti-chicken antibody.

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# ANNEXES



## **ANNEX 1:**

*Abbreviations*

## **ANNEX 2:**

*AMPK substrates identified by individual studies or by phosphoscreening*

## **ANNEX 3:**

*Putative AMPK interacting proteins*

## **ANNEX 4:**

*Expression vectors based on SfiI cloning strategy*

## **ANNEX 5:**

*Summary of all constructs available for further investigations*

## **ANNEX 6:**

*Curriculum vitae*

A $\beta$	amyloid beta peptide
ACC	acetylCoA carboxylase
AD	Alzheimer disease
ADP	adenosine diphosphate
AGAT	arginine:glycine aminotransferase
AICAR	5-aminoimidazole-4-carboxamide riboside
AIS	autoinhibitory sequence
AK	adenylate kinase
AKAP	A-kinase anchoring protein
ALS	amyotrophic lateral sclerosis
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANT	adenine nucleotide translocator
APLP2	APP family member amyloid-like protein 2
APP	amyloid precursor protein
Asb-9	ankyrin repeat and SOCS box protein 9
ATP	adenosine triphosphate
BCK / BBCK	brain type creatine kinase
CamKK	Ca <sup>2+</sup> /calmodulin dependent kinase kinase
cAMP	cyclic adenosine monophosphate
CBS	cystathionine beta-synthetase
CFTR	cystic fibrosis transmembrane conductance regulator Cl <sup>-</sup> channel
CK	creatine kinase
CLDND1	claudin domain-containing protein 1
CNS	central nervous system
CoIP	co-immunoprecipitation
CPT	carnitine palmitoyltransferase
Cr	creatine
CrT1	creatine transporter
DNA	deoxyribonucleic acid
eEF2K	eukaryotic elongation factor 2 kinase
EGFR	epidermal growth factor receptor
eNaC	epithelial sodium channel
Erg28p	probable ergosterol biosynthetic protein 28
FA	fatty acid
FABP	fatty acid binding protein
FAT	fatty acid transproter
FH	fumarate hydratase
GABA	gamma-aminobutyric acid
GABARAP	GABA receptor-associated protein
GAMT	guanidinoacetate methyltransferase
GBD	glucogen binding domain
GLUT	glucose transporter
GlutDH	glutamate dehydrogenase
GM130	Golgi matrix protein 130
GPAT	glycerol-3-phosphate acyl transferase
HD	Huntignton disease
HDAC	histone deacetylase
HIF	hypoxia inducible factor
HK	hexokinase
HMGR	3-hydroxy-3-methylglutary-CoA reductase
HSL	hormone sensitive lipase
IGF-I	insulin-like growth factor I
ITM2B	integral membrane protein 2B
JNK	c-Jun N-terminal kinase

KCC2	electroneutral potassium-chloride cotransporter 2
LC-ESI-MS	liquid chromatography-electrospray ionisation-mass spectrometry
LT	antigen Large T
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MAPK	mitogen activated protein kinase
MCK /MMCK	muscle type creatine kinase
MEF	myocyte enhancer factor
MEF cells	mouse embryonic fibroblast cells
MIP-T3	TRAF3-interacting protein 1
mTORC	mammalian target of rapamycin complex
NDPK	nucleoside diphosphate kinase
NKCC2	Na <sup>+</sup> -K <sup>+</sup> -2Cl <sup>-</sup> cotransporter
NSF	N-ethylmaleimide-sensitive-factor
OxPhos	oxidative phosphorylation
PAR-1	proteinase-activated receptor 1
PCDHB10	protocadherin beta-10
PCr	phosphocreatine
PD	Parkinson disease
PEPCK	phosphoenol-pyruvate carboxylase
PFK	6-phosphofructokinase
PGC-1 $\alpha$	proliferator-activated receptor gamma co-activator-1 alpha
PHD	HIF alpha prolyl hydroxylase
PK	pyruvate kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PP1	protein phosphatase 1
PP2C $\alpha$	protein phosphatase 2C alpha
PPAR	peroxisome proliferator activated receptors
PPI	protein-protein interaction
pVHL	von Hippel-Lindau disease tumor suppressor
RNA	ribonucleic acid
RTN4	reticulon 4 or Nogo-A
SLC22A17	Solute carrier family member 17
sMtCK	sarcomeric mitochondrial creatine kinase
SNAP-25	synaptosomal-associated protein 25
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment receptor
SPARC	secreted protein acidic and rich in cysteine
SPR	surface plasmon resonance
TAK1	transforming growth factor-beta activated kinase 1
TCA	tricarboxic acid
TG	triacylglycerol
TRIP6	thyroid receptor-interacting protein 6
TSC	tuberous sclerosis protein
uMtCK	ubiquitous mitochondrial creatine kinase
VAMP	vesicle associated membrane protein
VDAC	voltage dependent anion channel
Y2H	yeast two-hybrid

## AMPK SUBSTRATES IDENTIFIED BY INDIVIDUAL STUDIES

Approach	Target	SwissProt ID	PubMedID	Role	Localization
Individual study	6-phosphofruktokinase 2 (PFK2)	O60825	11069105/12065600	Carbohydrate metabolism	cytosol
Individual study	Acetyl CoA carboxylase 1 (ACC1)	P11497	1346520	Lipid metabolism	cytosol
Individual study	Acetyl CoA carboxylase 2 (ACC2)	O00763	9029219	Lipid metabolism	cytosol
Individual study	AICAR responsive element binding protein (AREBP)		17097062	Transcription	nucleus
Individual study	Akt substrate 160kDa (AS160)	Q8BYJ6	12417310	Carbohydrate metabolism	cytosol
Individual study	ATP-sensitive inward rectifier potassium channel 11 (Kir6.2)	P70673	19357830	Ion transport	cell membrane
Individual study	Calcium-activated potassium channel protein 4 (KCa3.1)	O15554	19052260	ion transport	cell membrane
Individual study	Calcium-activated potassium channel subunit alpha-1 (KCa1.1)	Q62976	17179156	Ion transport	cell membrane
Individual study	Carbohydrate responsive element binding protein (CREB)	Q8VIP2	11724780	Transcription	cytosol/nucleus
Individual study	Casein kinase I isoform epsilon	P49674	17525164	Circadian rhythm/Protein degradation	cytosol/nucleus
Individual study	CREB-regulated transcription coactivator 2 (TORC2)	Q53ET0	16148943	Transcription	cytosol/nucleus
Individual study	Cryptochrome-1	Q16526	19833968	Circadian rhythm	cytosol/nucleus
Individual study	Cyclin-dependent kinase inhibitor 1B (p27(kip1))	P46527	17237771	Cell cycle	cytosol/nucleus
Individual study	Cystic fibrosis transmembrane conductance regulator (CFTR)	P13569	19419994	ion transport	endosome/cell membrane
Individual study	cytosolic creatine kinase (M and B)	P12277	9501090	Creatine metabolism/ATP synthesis	cytosol
Individual study	E3 ubiquitin-protein ligase NEDD4-like (Nedd4-2)	Q96PU5	16844684	Ubiquitin conjugation pathway	cytosol
Individual study	Eukaryotic elongation factor 2 kinase (eEF2K)	O00418	14709557	Translation Elongation	cytosol
Individual study	FOXO3	O43524	17711846	Transcription	cytosol/nucleus
Individual study	GABA type B receptor subunit 1	Q9Z0U4	17224405	ion transport	cell membrane
Individual study	Golgi-specific brefeldin A resistance factor 1 (GBF1)	Q92538	18063581	Vesicle transport	Golgi
Individual study	Glucosamine-fructose-6-phosphate aminotransferase 1 (GFAT1)	Q06210	19170765	Carbohydrate metabolism	cytosol
Individual study	Glycogen synthase (GS)	Q9Z1E4	2567185/15561936	Carbohydrate metabolism	cytosol
Individual study	Hepatocyte nuclear factor 4-alpha (HNF4- $\alpha$ )	P41235	11423471/12740371	Transcription	nucleus
Individual study	Histone acetyltransferase p300	Q09472	11518699	Transcription	nucleus
Individual study	Histone deacetylase 5 (HDAC5)	Q9UQL6	18184930	Transcription	nucleus
Individual study	HMG-CoA reductase (HMGR)	P51639	2369897	Lipid metabolism	ER
Individual study	hormone-sensitive lipase	P15304	15878856	Lipid metabolism	Cell membrane/cytosol
Individual study	Importin alpha 1	P52294	15342649	Nuclear pore	cytosol/nucleus
Individual study	Insulin receptor substrate 1 (IRS1)	P35570	11598104	cell signaling	cytosol/nucleus
Individual study	Kidney-specific Na-K-Cl symporter (NKCC2)	Q13621	17341212	ion transport	cell membrane
Individual study	laforin	O95278	19171932	Ubiquitin conjugation pathway	Cytosol
Individual study	mTOR	P42345	14970221	Cell growth	ER/Golgi/Mito/Membrane
Individual study	Myosin light chain kinase, smooth muscle (MLCK)	Q15746	18426792	Muscle contraction	cytosol
Individual study	Nitric oxide synthase, endothelial (eNOS)	Q62600	10025949	Oxidoreductase	Cell membrane/cytosol/Golgi
Individual study	p53	P04637	15866171	Cell cycle / Apoptosis	Cytosol/ER/Nucleus
Individual study	PGC1a	Q9UBK2	17609368	Transcription	nucleus
Individual study	poly(ADP-ribose) polymerase (PARP)	isoform dependent	16480959	DNA repair	nucleus
Individual study	Proteasome 26S non-ATPase subunit 11 (PSMD11)	O00231	19616115	Protein degradation	Proteasome
Individual study	protein targeting to glycogen R5 (R5/PTG)	Q9UQK1	19171932	Carbohydrate metabolism	cytosol
Individual study	RAF proto-oncogene serine/threonine-protein kinase (RAF-1)	P04049	9091312	Apoptosis	Cell membrane/cytosol/mito
Individual study	Regulatory-associated protein of mTOR (Raptor)	Q8N122	18439900	Cell growth	cytosol
Individual study	reitinblastoma protein	P13405	19217427	Transcription	nucleus
Individual study	RNA polymerase I (Pol I)-associated transcription factor	Q9NYV6	19815529	Transcription	nucleus
Individual study	TBC1D1	Q7Z6H2	17995453	Carbohydrate metabolism	cytosol
Individual study	Thyroid receptor-interacting protein 6 (TRIP6)	Q15654	16624523	Transcription	cytosol/nucleus
Individual study	Tuberin (TSC2)	P49815	14651849	Protein Transport	Cell membrane/cytosol
Individual study	Vasodilator-stimulated phosphoprotein (VASP)	P50552	17082196	Cytoskeleton organization	Cell membrane/cytosol

## AMPK SUBSTRATES IDENTIFIED BY PHOSPHOSCREENING

Approach	Target	SwissProt ID	PubMedID	Role	Localization
Phosphoscreen	alpha-actin	P68134	17608512	Cytoskeleton organization	cytosol/cytoskeleton
Phosphoscreen	Adenylate kinase isoenzyme 1 (AK1)	P00568	20599718	ATP metabolism	cytosol/nucleus
Phosphoscreen	Band 3 anion transport protein	P02730	20599718	Ion transport	cell membrane
Phosphoscreen	Beta-synuclein	Q91Z23	17608512	Dopamine metabolism	cytosol
Phosphoscreen	Bisphosphoglycerate mutase	P07738	20599718	Carbohydrate metabolism	cytosol
Phosphoscreen	Carbonic anhydrase 1	P00915	20599718	Once-carbon metabolism	cytosol
Phosphoscreen	Catalase	P04040	20599718	Oxidoreduction	peroxisome
Phosphoscreen	Citrate synthase, mitochondrial	Q9CZU6	17608512	Carbohydrate metabolism	mito
Phosphoscreen	Dihydropyrimidinase-related protein 2	O08553	17608512	Cell differentiation	cytosol
Phosphoscreen	Flavin reductase	P30043	20599718	Oxidoreduction	cytosol
Phosphoscreen	gamma-actin	Q3T5B7	17608512	Cytoskeleton organization	cytosol/cytoskeleton
Phosphoscreen	Glial fibrillary acidic protein	Q3USS4	17608512	Cell differentiation	cytosol
Phosphoscreen	Glutamate dehydrogenase 1, mitochondrial	P26443	17608512	Oxidoreduction	mito
Phosphoscreen	Glutamine synthetase	Q91VC6	17608512	Cell proliferation	cytosol/mito
Phosphoscreen	Glutathione S-transferase omega-1	P78417	20599718	Glutathione transferase	cytosol
Phosphoscreen	Glutathione synthetase	P48637	20599718	Glutathione metabolism	cytosol
Phosphoscreen	Glyceraldehyde-3-phosphate dehydrogenase	P16858	17608512	Carbohydrate metabolism	cytosol
Phosphoscreen	Malate dehydrogenase, cytoplasmic	P40925	20599718	Carbohydrate metabolism	cytosol
Phosphoscreen	Peroxisiredoxin-6	P30041	20599718	Lipid metabolism	cytosol/vesicles/lysosome
Phosphoscreen	Phosphoglycerate kinase 1	P00558	20599718	Carbohydrate metabolism	cytosol
Phosphoscreen	Prostaglandin E synthase 3	Q9R0Q7	17608512	Lipid metabolism	cytosol
Phosphoscreen	Protein kinase C and casein kinase substrate in neurons protein 1	Q61644	17608512	Endocytosis	cytosol
Phosphoscreen	Purine nucleoside phosphorylase	P00491	20599718	Immune response	cytosol/cytoskeleton
Phosphoscreen	Selenium-binding protein 1	Q96GX7	20599718	Protein Transport	cytosol/nucleus
Phosphoscreen	Synapsin-1	O88935	17608512	Neurotransmitter release	Cell membrane/Golgi
Phosphoscreen	Thioredoxin-like protein 1	O43396	20599718	Electron transport	cytosol
Phosphoscreen	Transaldolase	P37837	20599718	Carbohydrate metabolism	cytosol
Phosphoscreen	Transferrin	Q06AH7	20599718	Iron ion homeostasis	cytosol/extracellular
Phosphoscreen	Transitional endoplasmic reticulum ATPase	Q0V924	20599718	Ubiquitin conjugation pathway	cytosol/nucleus
Phosphoscreen	Tubulin beta-2B chain	Q3TG26	17608512	Cytoskeleton organization	cytosol/cytoskeleton
Phosphoscreen	Ubiquitin carboxyl-terminal hydrolase 14	P54578	20599718	Ubiquitin conjugation pathway	Cell membrane/cytosol/proteasome
	interacting AMPK substrates				
	other isoforms of AMPK substrates				

Database	Bait	Name	SwissProt	Method	PubmedID	Localization	Role
IntAct	hAMPK B1	116 kDa U5 small nuclear ribonucleoprotein component	Q15029	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	26S protease regulatory subunit 6A	P17980	Anti-bait CoIP	17353931	Proteasome	Protein Degradation
IntAct	hAMPK B1	26S protease regulatory subunit 6B	P43686	Anti-bait CoIP	17353931	Proteasome	Protein Degradation
IntAct	hAMPK B1	26S proteasome non-ATPase regulatory subunit 1	Q99460	Anti-bait CoIP	17353931	Cytosol/Nucleus/Proteasome	Protein Degradation
IntAct	hAMPK B1	26S proteasome non-ATPase regulatory subunit 14	O00487	Anti-bait CoIP	17353931	Proteasome	Protein Degradation
IntAct	hAMPK B1	26S proteasome non-ATPase regulatory subunit 6	Q15008	Anti-bait CoIP	17353931	Proteasome	Protein Degradation
IntAct	hAMPK B1	28S ribosomal protein S16, mitochondrial	Q9Y3D3	Anti-bait CoIP	17353931	Mito	Protein Biosynthesis
IntAct	hAMPK B1	40S ribosomal protein S9	P46781	Anti-bait CoIP	17353931	Ribosome	Protein Biosynthesis
IntAct	hAMPK A1	5'-3' exoribonuclease 2	Q9DBR1	Y2H	20368287	Ribosome	Protein Biosynthesis
IntAct	hAMPK B1	6-phosphofructokinase type C	Q01813	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	6-phosphogluconate dehydrogenase, decarboxylating	P52209	Anti-bait CoIP	17353931	Cytosol	Carbohydrate metabolism
IntAct	hAMPK B1	60S ribosomal protein L3	P39023	Anti-bait CoIP	17353931	Cytosol	Carbohydrate metabolism
IntAct	hAMPK B1	60S ribosomal protein L31	P62899	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	hAMPK B1	60S ribosomal protein L35a	P18077	Anti-bait CoIP	17353931	Ribosome	Protein transport
IntAct	hAMPK B1	60S ribosomal protein L36	Q9Y3U8	Anti-bait CoIP	17353931	Ribosome	Protein Biosynthesis
IntAct	hAMPK A1	78 kDa glucose-regulated protein	P11021	Anti-bait CoIP	17353931	Ribosome	Protein Biosynthesis
IntAct	hAMPK A1	Abi interactor 1	Q8CBW3	Affinity purification	16306228	ER	Protein Biosynthesis
IntAct	hAMPK B1	Acetyl-CoA acetyltransferase, cytosolic	Q9BWD1	Y2H	20368287	Cytosol/Cytoskeleton/Nucleus	Vesicle transport
IntAct	hAMPK A1	Adenine phosphoribosyltransferase	P08030	Anti-bait CoIP	17353931	Cytosol	Lipid metabolism
IntAct	hAMPK B1	ADP-sugar pyrophosphatase	Q9UUK9	Y2H	20368287	Cytosol	Purine metabolism
IntAct	hAMPK A1	ADP/ATP translocase 2	P05141	Anti-bait CoIP	17353931	Mito	Purine metabolism
PubMed	hAMPK B2	Adrenodoxin, mitochondrial	P10109	Affinity purification	19616115	Mito	ATP synthesis
PubMed	hAMPK G3	Alpha-crystallin B chain	P02511	Y2H	19616115	Mito	Electron transport
IntAct	hAMPK B1	Apoptosis inhibitor 5	Q9BZZ5	Y2H	17353931	Mito	Apoptosis/Protein folding
IntAct	hAMPK B1	Aspartate aminotransferase, mitochondrial	P00505	Anti-bait CoIP	17353931	Cytosol/Nucleus	Apoptosis
IntAct	hAMPK B1	ATP synthase subunit f, mitochondrial	P56134	Anti-bait CoIP	17353931	Mitochondria	Lipid Transport
IntAct	hAMPK B1	ATP synthase subunit O, mitochondrial	P48047	Anti-bait CoIP	17353931	Mito	ATP synthesis
IntAct	hAMPK B1	ATP-binding cassette sub-family F member 1	Q8NE71	Anti-bait CoIP	17353931	Mitochondria	ATP synthesis
IntAct	hAMPK B1	ATP-citrate synthase	P53396	Anti-bait CoIP	17353931	Ribosome	Protein Biosynthesis
IntAct	hAMPK B1	Band 4.1-like protein 5	Q9HCN4	Anti-bait CoIP	17353931	Cytosol	Lipid metabolism
PubMed	hAMPK G3	Brain protein I3	Q95415	Y2H	19616115	Cytosol	Cell adhesion
IntAct	hAMPK B1	BRCA2 and CDKN1A-interacting protein	Q9P287	Anti-bait CoIP	17353931	Nucleus	Cell cycle
IntAct	hAMPK A1	Calcium homeostasis endoplasmic reticulum protein	Q8CGZ0	Y2H	20368287	Cytosol/ER	Protein Biosynthesis
IntAct	hAMPK B1	Calycin-binding protein	Q9HB71	Anti-bait CoIP	17353931	Cytosol/Nucleus	Ubiquitin conjugation pathway
IntAct	hAMPK B1	Carbonyl reductase [NADPH] 1	P16152	Anti-bait CoIP	17353931	Cytosol	Oxidoreductase
IntAct	hAMPK A1	Carboxypeptidase E	Q00493	Y2H	20368287	Cytosol	Protein Degradation
PubMed	hAMPK B2	Casein Kinase II subunit beta	P67870	Y2H	19616115	Cytosol	Cell proliferation
IntAct	hAMPK B1	CDGSH iron-sulfur domain-containing protein 1	Q9NZ45	Anti-bait CoIP	17353931	Mito	OxPhos regulation
IntAct	hAMPK B1	CDK5 regulatory subunit-associated protein 3	Q96J85	Anti-bait CoIP	17353931	Mito	Cell proliferation
MINT	hAMPK B1	Cell death activator CIDF-A	O70302	Anti-bait CoIP/Colocalization	18480843	Mito/Nucleus	Apoptosis
IntAct	hAMPK B1	CHC1 protein	Q5T081	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
IntAct	hAMPK A1	Class E basic helix-loop-helix protein 40	O35185	Y2H	20368287	Nucleus	Protein Transport
IntAct	hAMPK B1	Clathrin heavy chain 1	Q00610	Anti-bait CoIP	17353931	Membrane/Vesicles	Cytoskeleton organization
IntAct	hAMPK B1	Coactosin-like protein	Q14019	Anti-bait CoIP	17353931	Cytosol/Cytoskeleton	Cytoskeleton organization
IntAct	hAMPK B1	Coagulation factor X	P00742	Y2H	16169070	extracellular	Coagulation
MINT	hAMPK B1	Coagulation factor X	Q5JVE8	Y2H	16169070	extracellular	Coagulation
IntAct	hAMPK B1	Coatomer subunit beta	P35606	Anti-bait CoIP	17353931	ER/Golgi/Vesicles	Protein Transport
IntAct	hAMPK B1	Cold shock domain-containing protein E1	O75534	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis

Database	Bait	Name	SwissProt	Method	PubmedID	Localization	Role
IntAct	hAMPK B1	CTP synthase 1	P17812	Anti-bait CoIP	17353931		Pyrimidine biosynthesis
PubMed	hAMPK A1/A2	Cystic fibrosis transmembrane conductance regulator	P13569	Y2H, CoIP	10862786	Endosome	ABC transporter
IntAct	hAMPK B1	Cytochrome c	P99999	Anti-bait CoIP	17353931	Mito	Apoptosis
IntAct	hAMPK B1	DNA damage-binding protein 1	Q96003	Anti-bait CoIP	17353931	Cytosol/Nucleus	DNA repair
PubMed	hAMPK B2	DNA damage-inducible transcript 4-like protein	Q16531	Y2H	19616115	Cytosol	Regulation of signal transduction
IntAct	hAMPK B1	DNA replication licensing factor MCM5	P33992	Anti-bait CoIP	17353931	Nucleus	DNA replication
IntAct	hAMPK B1	DNA replication licensing factor MCM7	P33993	Anti-bait CoIP	17353931	Nucleus	Cell cycle
IntAct	hAMPK B1	Dynammin-2	P50570	Anti-bait CoIP	17353931	Cytosol/Cytoskeleton	Endocytosis
IntAct	hAMPK B1	Electron transfer flavoprotein subunit alpha, mitochondrial	P13804	Anti-bait CoIP	17353931	Mito	Electron transport
IntAct	hAMPK B1	Elongation factor 1-delta	P29692	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	hAMPK A1	EMILIN-1	Q99K41	Y2H	20368287	extracellular	Cell adhesion
IntAct	hAMPK B1	Endogenous Borna-like N element-2	Q6P217	Anti-bait CoIP	17353931		
PubMed	AMPK	endothelial NOS	Q62600	CoIP	10025949	Cell membrane/Cytoskeleton/Golgi	Oxidoreductase
IntAct	hAMPK B1	Enhancer of rudimentary homolog	P84090	Anti-bait CoIP	17353931		Cell cycle
IntAct	hAMPK B1	Enoyl-CoA hydratase, mitochondrial	P30084	Anti-bait CoIP	17353931	Mitochondria	Lipid metabolism
IntAct	hAMPK B1	Eukaryotic peptide chain release factor GTP-binding subunit ERF3A	P15170	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	hAMPK B1	Eukaryotic translation initiation factor 2 subunit 3	P41091	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	hAMPK B1	Eukaryotic translation initiation factor 3 subunit E	P60228	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	Eukaryotic translation initiation factor 3 subunit H	O15372	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	hAMPK B1	Eukaryotic translation initiation factor 3 subunit K	Q9UBQ5	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	Eukaryotic translation initiation factor 3 subunit M	Q7L2H7	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	hAMPK B1	Eukaryotic translation initiation factor 4 gamma 1	Q04637	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	hAMPK B1	Eukaryotic translation initiation factor 6	P56537	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	Ezrin	P15311	Anti-bait CoIP	17353931	Cell membrane/Cytoskeleton	Cytoskeleton organization
IntAct	hAMPK B1	F-actin-capping protein subunit alpha-1	P52907	Anti-bait CoIP	17353931	Cytosol/Cytoskeleton	Cytoskeleton organization
IntAct	hAMPK B1	Fascin	Q16658	Anti-bait CoIP	17353931	Cytosol	Cell proliferation
IntAct	hAMPK B1	Fatty acid-binding protein, epidermal (FABP5)	Q01469	Anti-bait CoIP	17353931	Cytosol	Lipid Transport
PubMed	hAMPK B2	Filamin-C	Q14315	Anti-bait CoIP	19616115	Cytosol/Cytoskeleton/Membrane	Cytoskeleton organization
GRID	hAMPK B1	Folliculin-interacting protein 1	Q8TF40	?	17028174	Cytosol	
IntAct	hAMPK B1	Four and a half LIM domains protein 1	Q13642	Anti-bait CoIP	17353931	Cytosol/Nucleus	Cell differentiation
IntAct	hAMPK B1	Fumarate hydratase	P07954	Anti-bait CoIP	17353931	Mito/Cytosol	Carbohydrate metabolism
PubMed	rAMPK A1	GABA type B receptor subunit 1	Q9Z0U4	CoIP, colocalization, Y2H	17224405	Cell membrane	Ion transport
IntAct	hAMPK B1	Girdin	Q3V6T2	Anti-bait CoIP	17353931	Membrane/Cytosol/vesicles	Cytoskeleton organization
IntAct	hAMPK B1	Glutaminyl-peptide cyclotransferase-like protein	Q9NXS2	Anti-bait CoIP	17353931	Membrane	Protein Degradation
HPRD	hAMPK B1	Glycogen debranching enzyme	P35573	Pull down	15886229	Cytosol	Carbohydrate metabolism
IntAct	hAMPK B1	Glycogen synthase, muscle	P13807	Anti-bait CoIP	17353931	Cytosol	Carbohydrate metabolism
IntAct	hAMPK B1	GMP synthase [glutamine-hydrolyzing]	P49915	Anti-bait CoIP	17353931	Cytosol	Purine metabolism
IntAct	hAMPK A1	Granulins	P28798	Y2H	20368287	Mito/extracellular	Cytokine
IntAct	hAMPK B1	Growth factor receptor-bound protein 2	P62993	Y2H/Anti-bait CoIP	18624398/18624398	Golgi	Apoptosis
IntAct	hAMPK B1	GrpE protein homolog 1, mitochondrial	Q9HAV7	Anti-bait CoIP	17353931	Mito	Protein import to mitochondria
PubMed	hAMPK B2	Guanine nucleotide-binding protein subunit beta-2-like 1	P63244	Y2H	19616115	Cell membrane	Receptor binding
IntAct	hAMPK B1	Heat shock 70 kDa protein 4L	O95757	Anti-bait CoIP	17353931	Cytosol/Nucleus	Chaperone (Stress response)
IntAct	hAMPK A1	Heat shock protein beta-1	P14602	Y2H	20368287	Nucleus/Plasma membrane	Chaperone (Stress response)
IntAct	hAMPK B1	Heterogeneous nuclear ribonucleoprotein Q	O60506	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	Heterogeneous nuclear ribonucleoprotein U-like protein 1	Q9BUJ2	Anti-bait CoIP	17353931	ER/Cytosol/Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	Hsc70-interacting protein	P50502	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	hAMPK A1/B1	Hsp90 co-chaperone Cdc37	Q16543	Anti-bait CoIP	17353931/16306228	Cytosol	Cell cycle
IntAct	hAMPK B1	Hypoxanthine-guanine phosphoribosyltransferase	P00492	Anti-bait CoIP	17353931	Cytosol	Purine metabolism
IntAct	hAMPK B1	Importin-9	Q96P70	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Transport
PubMed	h AMPK G1	Intermediate conductance calcium-activated potassium channel protein 4	O15554	CoIP, colocalization, pull down	19052260	Cell membrane	Ion transport

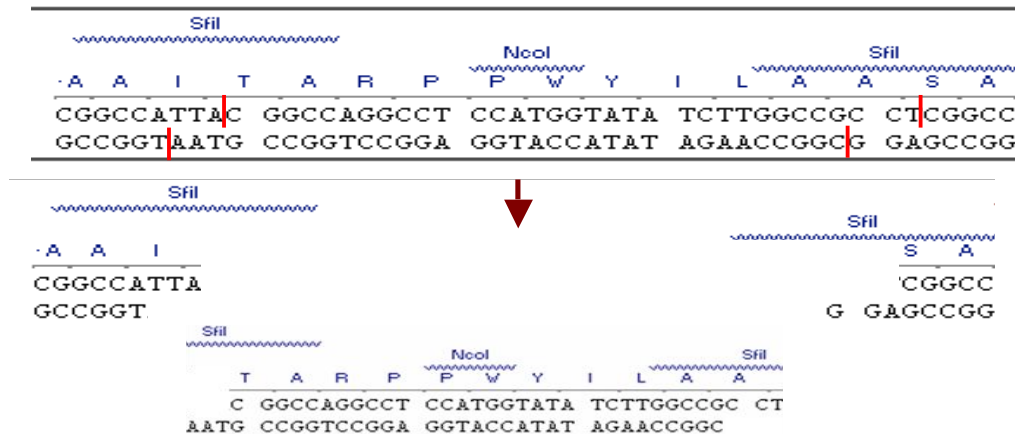
Database	Bait	Name	SwissProt	Method	PubmedID	Localization	Role
PubMed	AMPK	<b>Kidney-specific Na<sup>+</sup>-K<sup>+</sup> symporter (NKCC2)</b>	<b>Q13621</b>	<b>CoIP_colocalization</b>	<b>17341212</b>	<b>Cell membrane</b>	<b>Ion transport</b>
IntAct	HAMPK B1	Kinesin-1 heavy chain	P31176	Anti-bait CoIP	17353931	Cytosol/Cytoskeleton	Vesicle Transport
IntAct	HAMPK A1	Kinesin-like protein KIF1C	O35071	Y2H	20368287	Golgi/microtubule	Vesicle transport
PubMed	HAMPK A2/B2	<b>lactoferrin</b>	<b>O95278</b>	<b>Y2H_CoIP</b>	<b>19171932</b>	<b>Cytosol</b>	<b>Ubiquitin conjugation pathway</b>
IntAct	HAMPK A1	Leucine zipper putative tumor suppressor 2	Q91YU6	Y2H	20368287	Cytosol/Cytoskeleton	Cell cycle
IntAct	HAMPK B1	Leucine-rich repeat-containing protein 59	Q96AG4	Anti-bait CoIP	17353931	ER/microsomes	Protein Biosynthesis
PubMed	HAMPK G3	LIM and cysteine-rich domains protein 1	Q9NZU5	Y2H	19616115	Cytosol/Nucleus	Protein Biosynthesis
IntAct	HAMPK B1	Lupus La protein	P05455	Anti-bait CoIP	17353931	Cytosol/Nucleus	Cytokine
IntAct	HAMPK A1	Macrophage migration inhibitory factor	P14174	Anti-bait CoIP	17353931	Cytosol/secreted	Apoptosis
IntAct	HAMPK A1	Melanoma-associated antigen D1	Q9QYH6	Y2H	20368287	Cytosol/Plasma membrane	Apoptosis
IntAct	HAMPK B1	Methionyl-tRNA synthetase, cytoplasmic	P56192	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	HAMPK B1	Mitochondrial dicarboxylate carrier	Q9UBX3	Anti-bait CoIP	17353931	Mito	Mitochondrial carrier
HPRD	HAMPK A1	Mitochondrial import receptor subunit TOM34	Q15785	Y2H	11913976	Cytosol/Membrane/Mito	Protein import to mitochondria
IntAct	HAMPK B1	Moesin	P26038	Anti-bait CoIP	17353931	Cytosol/Cytoskeleton/Membrane	Cytoskeleton organization
IntAct	HAMPK B1	Myosin regulatory light chain 12A	P19105	Anti-bait CoIP	17353931	Cytoskeleton	Cytoskeleton organization
IntAct	HAMPK B1	Myosin-10	P35580	Anti-bait CoIP	17353931	Cytosol/Cytoskeleton	Cytoskeleton organization
IntAct	HAMPK B1	Myotrophin	P58546	Anti-bait CoIP	17353931	Cytosol	Cell differentiation
PubMed	HAMPK a2TD	Myozemin-1	Q9NP98	Y2H	19616115	Nucleus	Cytoskeleton organization
IntAct	HAMPK B1	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	O95182	Anti-bait CoIP/Y2H	17353931/16169070	Nucleus	Oxphos
IntAct	HAMPK B1	NADH-cytochrome b5 reductase 3	P00387	Anti-bait CoIP	17353931	Mitochondria	Lipid metabolism
PubMed	HAMPK a2TD	Nebulin-related anchoring protein	O86V77	Y2H	19616115	Cytoskeleton	Cytoskeleton organization
PubMed	HAMPK G3	Neugrin	Q9NPE2	Y2H	19616115	Nucleus	Cell differentiation
IntAct	HAMPK A1	NGF1-A-binding protein 2	Q61127	Y2H	20368287	Nucleus	Protein Biosynthesis
IntAct	HAMPK B1	NTF2-related export protein 1 (p15)	Q9UKK6	Anti-bait CoIP	17353931	Cytosol	Cell cycle
HPRD	HAMPK B1/B2	<b>NUAK family SNF1-like kinase 1</b>	<b>O60285</b>	<b>Affinity purification</b>	<b>16306228</b>	<b>Cytosol/Nucleus</b>	<b>Protein Transport</b>
IntAct	HAMPK B1	Nuclear migration protein nudC	Q9Y266	Anti-bait CoIP	17353931	Nucleus	Cell cycle
IntAct	HAMPK B1	Nuclear pore complex protein Nup93	Q8N1F7	Anti-bait CoIP	17353931	Nucleus	Protein Transport
IntAct	HAMPK A1	Nuclear receptor-binding factor 2	Q8VCQ3	Pull down	20368287	Nucleus	Protein Transport
IntAct	HAMPK B1	Nucleolar RNA helicase 2	Q9NR30	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Biosynthesis
IntAct	HAMPK B1	Ornithine aminotransferase, mitochondrial	P04181	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
IntAct	HAMPK B1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1	Q13526	Anti-bait CoIP	17353931	Mito	Amino-acid biosynthesis
IntAct	HAMPK A1/A2	Peroxisome proliferator-activated receptor delta	P35396	Anti-bait CoIP	18674809	Nucleus	Cell cycle
IntAct	AMPK	Peroxisome proliferator-activated receptor delta	P35396	CoIP	18674809	Nucleus	Protein Biosynthesis
IntAct	HAMPK B1	Platelet-activating factor acetylhydrolase IB subunit beta	P68402	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
IntAct	HAMPK B1	Poly [ADP-ribose] polymerase 1	P09874	Anti-bait CoIP	17353931	Cytosol	Lipid metabolism
PubMed	HAMPK	<b>poly(ADP-ribose) polymerase (PARP)</b>	<b>isoform depend</b>	<b>CoIP</b>	<b>16480959</b>	<b>Nucleus</b>	<b>DNA repair</b>
IntAct	HAMPK B1	Polyadenylate-binding protein 1	P11940	Anti-bait CoIP	17353931	Nucleus	DNA repair
IntAct	HAMPK B1	Polycomb group RING finger protein 6	Q9BYE7	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
PubMed	HAMPK a2TD	<b>Pre-b-cell leukemia homeobox interacting protein1</b>	<b>Q5T173</b>	<b>Y2H</b>	<b>19616115</b>	<b>Cytosol</b>	<b>Protein Biosynthesis</b>
IntAct	HAMPK B1	Pre-mRNA branch site protein p14	Q9Y3B4	Anti-bait CoIP	17353931	Nucleus	Ribosome biogenesis
IntAct	HAMPK B1	Pre-rRNA-processing protein TSR1 homolog	Q2NL82	Anti-bait CoIP	17353931	Nucleus	Ribosome biogenesis
IntAct	HAMPK B1	Prefoldin subunit 2	Q9UHV9	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
IntAct	HAMPK B1	Probable ATP-dependent RNA helicase DDX20	Q9UHI6	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Biosynthesis
IntAct	HAMPK B1	Probable ATP-dependent RNA helicase DHX36	Q9HZU1	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Biosynthesis
IntAct	HAMPK B1	probable threonyl-tRNA synthetase 2, cytoplasmic	A2RTX5	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	HAMPK B1	Profilin-2	P35080	Anti-bait CoIP	17353931	Cytosol	Cytoskeleton organization
IntAct	HAMPK B1	Proliferation-associated protein 2G4	Q9UQ80	Anti-bait CoIP	17353931	Cytosol/Cytoskeleton	Cytoskeleton organization
IntAct	HAMPK B1	Prolyl endopeptidase	P48147	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Biosynthesis
PubMed	HAMPK B2	<b>Proteasome 26S non-ATPase subunit 11</b>	<b>O00231</b>	<b>Y2H_CoIP</b>	<b>19616115</b>	<b>Proteasome</b>	<b>Regulation of peptide hormones</b>
IntAct	HAMPK B1	Proteasome subunit alpha type-6	P60900	Anti-bait CoIP	17353931	Proteasome	Protein Degradation
IntAct	HAMPK B1	Proteasome subunit alpha type-7	O14818	Anti-bait CoIP	17353931	Proteasome	Protein Degradation
IntAct	HAMPK B1	Proteasome subunit beta type-3	P49720	Anti-bait CoIP	17353931	Cytosol/Nucleus/Proteasome	Protein Degradation
IntAct	HAMPK B1	Protein cornichon homolog 4	Q9P003	Anti-bait CoIP	17353931	ER	Protein Transport



Database	Bait	Name	SwissProt	Method	PubmedID	Localization	Role
IntAct	hAMPK B1	Protein disulfide-isomerase A4	P13667	Anti-bait CoIP	17353931	ER	Protein Secretion
IntAct	hAMPK B1	Protein disulfide-isomerase A6	Q15084	Anti-bait CoIP	17353931	ER/plasma membrane	Protein Biosynthesis
IntAct	hAMPK A1	Protein FAM54A	Q8VED8	Y2H	20368287		
IntAct	hAMPK B1	Protein FAM98B	Q52LJ0	Anti-bait CoIP	17353931		
IntAct	hAMPK A1	Protein NEDD1	P33215	Y2H	20368287	Cytosol/Cytoskeleton	Cell cycle
IntAct	hAMPK B1	Protein RCC2	Q9P258	Anti-bait CoIP	17353931	Cytosol/Cytoskeleton/Nucleus	Cell cycle
IntAct	hAMPK B1	Protein SET	Q01105	Anti-bait CoIP	17353931	Cytosol/ER/Nucleus	Apoptosis
PubMed	hAMPK	protein targeting to glycogen R5	Q9U0K1	Y2H, CoIP	19171932	Cytosol	Carbohydrate metabolism
IntAct	hAMPK B1	Protein transport protein Sec61 subunit alpha isoform 1	P61619	Anti-bait CoIP	17353931	ER	Protein Transport
IntAct	hAMPK B1	Proteolipid protein 2	Q04941	Anti-bait CoIP	17353931	Plasma/ER membrane	Cell differentiation
IntAct	hAMPK B1	Puromycin-sensitive aminopeptidase	P55786	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Degradation
IntAct	hAMPK B1	Pyridoxal kinase	O00764	Anti-bait CoIP	17353931	Cytosol	Cell proliferation
IntAct	hAMPK B1	Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial	P08559	Anti-bait CoIP	17353931	Mito	Carbohydrate metabolism
IntAct	hAMPK B1	Rab GDP dissociation inhibitor beta	P50395	Anti-bait CoIP	17353931	Cytosol/Membrane	Protein Transport
IntAct	hAMPK B1	Ras-related protein Rab-7a	P51149	Anti-bait CoIP	17353931	Lysosome/Late endosome/Vesicles	Protein Transport
IntAct	hAMPK B1	Reticulon-4 (RTN4)	Q9NOC3	Anti-bait CoIP	17353931	ER/plasma membrane	Apoptosis
IntAct	hAMPK A1	Rho GTPase-activating protein 22	Q8BL80	Y2H	20368287	Cytosol/Nucleus	Cell differentiation
IntAct	hAMPK B1	Ribonucleoside-diphosphate reductase large subunit	P23921	Anti-bait CoIP	17353931	Cytosol	DNA replication
IntAct	hAMPK B1	RNA methyltransferase-like protein 1	Q9HC36	Anti-bait CoIP	17353931	Cytosol/Nucleus/Membrane	Protein Biosynthesis
IntAct	hAMPK B1	RNA-binding protein EWS	Q01844	Anti-bait CoIP	17353931	Cytosol/Nucleus/Membrane	Protein Biosynthesis
IntAct	hAMPK A1	Roquin	Q4VGL6	Y2H	20368287	Cytosol	Cell differentiation
IntAct	hAMPK B1	RuvB-like 2	Q9Y230	Anti-bait CoIP	17353931	Cytosol/Nucleus/Membrane	Protein Biosynthesis
IntAct	hAMPK B1	S-adenosylmethionine synthase isoform type-2	P31153	Anti-bait CoIP	17353931		One-carbon metabolism
IntAct	hAMPK B1	Serine hydroxymethyltransferase, mitochondrial	P34897	Anti-bait CoIP	17353931	Mito	One-carbon metabolism
IntAct	hAMPK B1	Serine/threonine kinase receptor-associated protein	Q9Y3F4	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Biosynthesis
BIND	hAMPK	Serine/threonine-protein kinase LKB1	Q15831	CoIP	14985505	Cytosol/Nucleus	AMPK kinase
IntAct	hAMPK B1	Signal recognition particle 14 kDa protein	P37108	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
PubMed	hAMPK a2TD	SNW domain containing 1	Q13573	Y2H	19616115	Nucleus	Protein Biosynthesis
IntAct	hAMPK A1	Sorbin and SH3 domain-containing protein 1	Q62417	Y2H	20368287	Cytosol/Cytoskeleton/Membrane	insulin-stimulated glucose transport
PubMed	hAMPK A1	SPARC	P09486	Y2H, CoIP, Pull down	20460104	Extracellular	Regulation of cell adhesion, proliferation
IntAct	hAMPK B1	Splicing factor 3A subunit 1	Q15459	Anti-bait CoIP	17353931	Nucleus/Spliceosome	Protein Biosynthesis
PubMed	hAMPK a2TD	SUMO-conjugating enzyme UBC9	P63279	Y2H	19616115	Nucleus	Ubiquitin conjugation pathway
IntAct	hAMPK A1	TCF3 fusion partner homolog	Q3UJ11	Y2H	20368287	Nucleus	DNA repair
IntAct	hAMPK A1	THAP domain-containing protein 7	Q8VCZ3	Y2H	20368287	Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	Threonyl-tRNA synthetase, cytoplasmic	P26639	Anti-bait CoIP	17353931	Cytosol	Protein Biosynthesis
IntAct	hAMPK A1	Thyroid receptor-interacting protein 6	Q9Z1Y9	Y2H	20368287	Cytosol/Cytoskeleton	Cell adhesion
HRPD	hAMPK A2	Thyroid receptor-interacting protein 6	Q15654	CoIP, Two-hybrid, Y2H	16624523,19616115	Cytosol/Cytoskeleton/Nucleus	Cytoskeleton organization
IntAct	hAMPK B1	Transcription intermediary factor 1-beta	Q13263	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	Translin	Q15613	Anti-bait CoIP	17353931	Cytosol/Nucleus	DNA recombination
IntAct	hAMPK B1	Translocon-associated protein subunit delta	P51571	Anti-bait CoIP	17353931	Cytosol/Nucleus	Protein Transport
IntAct	hAMPK B1	Trifunctional enzyme subunit alpha, mitochondrial	P40939	Anti-bait CoIP	17353931	ER/plasma membrane	Lipid metabolism
IntAct	hAMPK B1	tRNA methyltransferase 112 homolog	Q9UI30	Anti-bait CoIP	17353931	Mito	Protein Biosynthesis
PubMed	hAMPK a2TD	Tropomodulin 1	P28289	Y2H	19616115	Cytosol/Cytoskeleton	Cytoskeleton organization
GRID	hAMPK	Tuberm (TSC2)	P49815	CoIP	14651849	Cytosol/Membrane	mTOR signaling
IntAct	hAMPK B1	U5 small nuclear ribonucleoprotein 200 kDa helicase	Q75643	Anti-bait CoIP	17353931	Nucleus	Protein Biosynthesis
IntAct	hAMPK B1	Ubiquitin carboxyl-terminal hydrolase 5	P45974	Anti-bait CoIP	17353931	Lysosome	Inhibition of protein degradation
IntAct	hAMPK B1	Ubiquitin thioesterase OTUB1	Q96FW1	Anti-bait CoIP	17353931	Cytosol	Ubiquitin conjugation pathway
IntAct	hAMPK B1	Ubiquitin-conjugating enzyme E2 D3	P61077	Anti-bait CoIP	17353931	Cytosol	Ubiquitin conjugation pathway
IntAct	hAMPK B1	Ubiquitin-conjugating enzyme E2 L3	P68036	Anti-bait CoIP	17353931	Cytosol	Ubiquitin conjugation pathway
IntAct	hAMPK B1	Ubiquitin-fold modifier 1	P61960	Anti-bait CoIP	17353931	Cytosol/Nucleus	Ubiquitin conjugation pathway
IntAct	hAMPK B1	UMP-CMP kinase	P30085	Anti-bait CoIP	17353931	Cytosol/Nucleus	Pyrimidine biosynthesis
IntAct	hAMPK B1	Uncharacterized methyltransferase WBSRC22	O43709	Anti-bait CoIP	17353931	Nucleus	DNA methylation
IntAct	hAMPK A1	UPF0667 protein Clorf55 homolog	Q8K1J5	Y2H	20368287		
IntAct	hAMPK B1	Vacuolar ATPase assembly integral membrane protein VMA21	Q3Z4Q7	Anti-bait CoIP	17353931	ER/vesicles/membrane	Vacuolar proton transport
IntAct	hAMPK B1	VAMP3 protein sorting-associated protein 35	Q96QK1	Anti-bait CoIP	17353931	Cytosol/Membrane	Protein Transport
IntAct	hAMPK B1	VAMP3 protein	Q6FFG2	Anti-bait CoIP	17353931	Vesicles	Vesicle transport, endo/exocytosis
IntAct	hAMPK A1	WD repeat-containing protein 62	Q3U3T8	Y2H	20368287		
		interacting AMPK substrates					
		other isoforms of AMPK substrates					
		confirmed AMPK non-substrate interactors					

## THE CLONING SYSTEM

### 1. PRINCIPLE



### 2. THE VECTORS

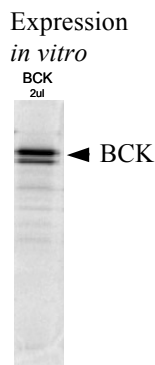
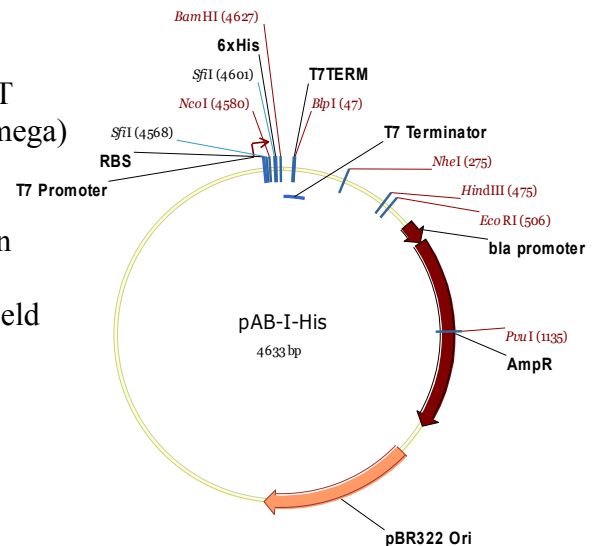
#### a. Expression *in vitro*

name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by
pAB-I-His	Amp	---	T7	---	His	Expression	anti-His	AB
pAB-I-HA	Amp	---	T7	---	HA	Expression	anti-HA	AB

They were used to express proteins *in vitro* using the TNT T7 Quick coupled Transcription/Traduction system (Promega) and Redivue<sup>TM</sup> L-[<sup>35</sup>S] Methionine (Amersham).

They are derived from vectors used for AMPK expression which are modified pET-3 vectors, They can also be used for expression in *E. coli*, but the yield is slow (tested with BCK)

Use : Co-Immunoprecipitation *in vitro*

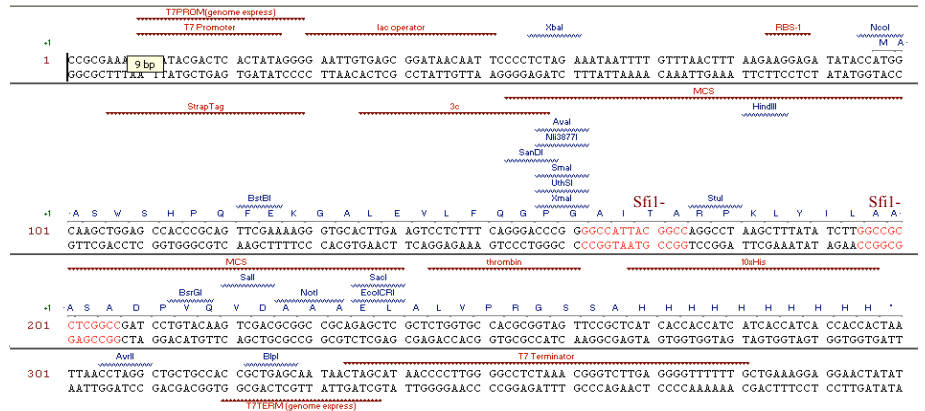


Autoradiograph

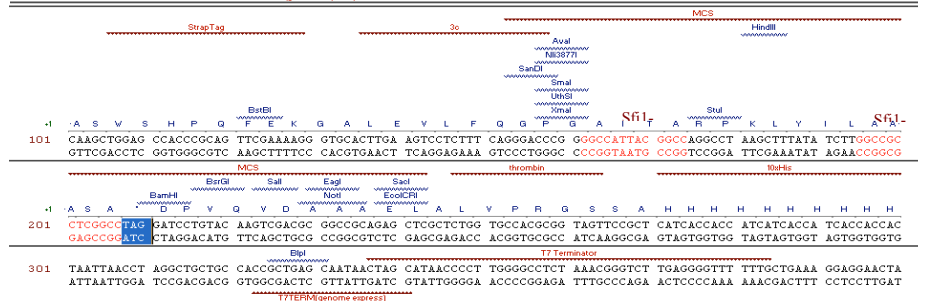
**b. Expression in *E. coli***

name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by
pET52b+	Amp	---	T7lac	---	Strep-His	Expression	anti-His	Novagen
pAB52	Amp	---	T7lac	---	Strep-His	Expression	anti-His	AB
pAB52s	Amp	---	T7lac	---	Strep	Expression	anti-Strep	AB

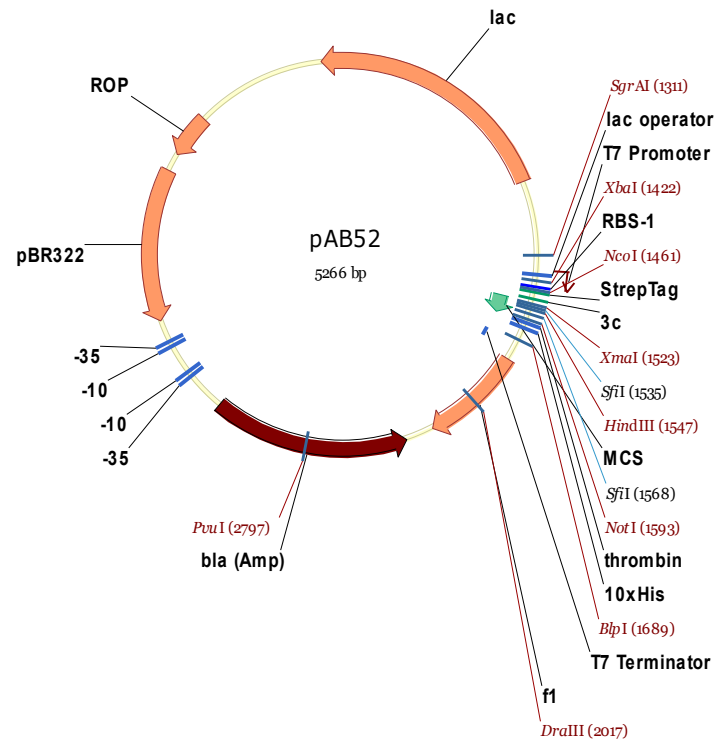
**pAB52**



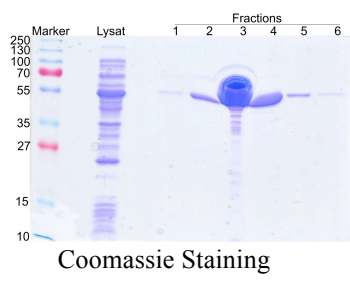
**pAB52s**



Derived from pET52b(+), these vectors allow recombinant expression of tagged protein in *E. coli*  
 - pAB52s : Proteins with Strep-tag in Nt  
 - pAB52 : Proteins with Strep-tag in Nt AND His-Tag in Ct

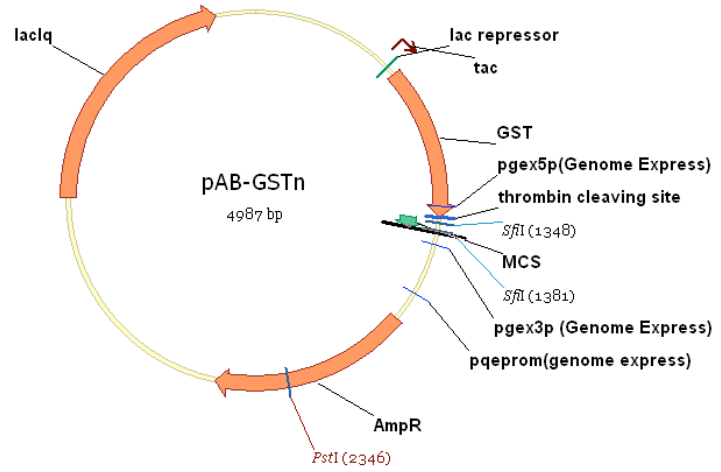


Purification of Strep-BCK-His (>5mg/ml)



name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by
pGex-4T-1	Amp	---	lacIq	---	GST	Expression		Amerham
pAB-GSTn	Amp	---	Tac	---	GST	Expression		AB

pAB-GSTn is derived from pGex-4T-1 and allows the expression of recombinant proteins in *E.coli* with a GST-tag at the Nt-end.



pAB-GSTn

GST		pgex5p (Genome Express)	
-3	· K K R I E A I P Q I D K Y L K S S K Y I A V P L Q G V Q A T F G G G ·		
1201	AAAAACGTAT TGAAGCTATC CCACAAATTG ATAAGTACTT GAAATCCAGC AAGTATATAG CATGGCCITT GCAGGCCCTG CAAGCCACGT TTGGTGGTGG		
	TTTTTGCATA ACTTCGATAG GGTGTTTAA C TATTCAATGAA CTTTAGGTCG TTCATATATC GTACCCGAAA CGTCCCGACC GTTCGGTGC A ACCCACCACC		
GST		MCS	
-3	· G D H P P K S D L V P R G S A I T A R P L E Y I L A A S A P H R D ·		
1301	CGACCATCCT CCAAAATCGG ATCTGGTTC GCGTGGATCG GCCATTACGG CCAGGCCTCT CGAGTATATC TTGGCCGCCT CGGCCCCGCA TCGTACTGA		
	GCTGGTAGGA GGTTTTAGCC TAGACCAAGG CGCACCTAGC CGGTAATGCC GGTCCGGAGA GCTCATATAG AACCCGCGGA GCCGGGGCGT AGCACTGACT		
-3	· L T I C L A R F G D D G E N L · H M Q L P E T V T A C C L · A D A G S ·		
1401	CTGACGATCT GCCTCGCGCG TTTCGGTGAT GACGGTGA A ACCTCTGACA CATGCAGCTC CCGGAGACGG TCACAGCTTG TCTGTAAGCG GATGCCGGGA		
	GACTGCTAGA CGGAGCGCGC AAAGCCACTA CTGCCACTTT TGGAGACTGT GTACGTCGAG GGCCTCTGCC AGTGTGGAAC AGACATTGCG CTACGGCCCT		
pgex3p (Genome Express)			
-3	· S R Q A R Q G A S A G V Y G G C R G A A M T Q S R S D S G V Y N S ·		
1501	GCAGACAAGC CGCTCAGGC GCGTCAGCG GTGTTGGCG GTGTCGGCG CACGCCATGA CCCAGTCACG TAGCGATAG GAGTGTATA ATTCTTGAAG		
	CGTCTGTTG GGCAGTCCG CGCAGTCGC CACAACCGC CACAGCCCG COTCGTACT GGGTCAGTC ATCGCTATCG CCTCACATAT TAAGAACTTC		

### c. Transfection

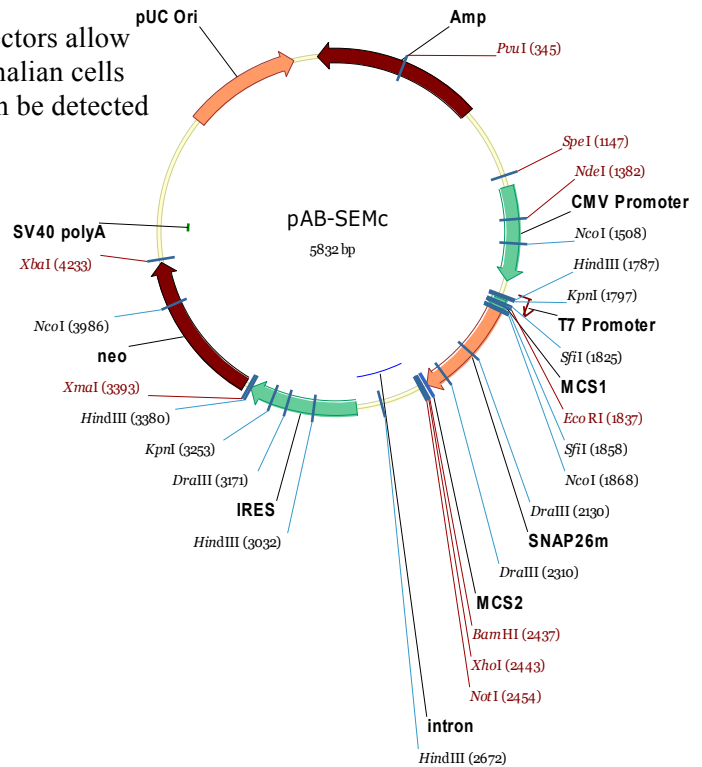
name	Resistance	Selection Marker	Promoter	Insert	Expressed	Use	Antibody	Created by
pSEMS-26m	Amp	neo	CMV	---	SNAP26m	Transfection		Covalys
pSEMS-26m'	Amp	neo	CMV	---	SNAP26m	Transfection		AB
pAB-SEMn	Amp	neo	CMV	---	SNAP26m	Transfection		AB
pAB-SEMc	Amp	neo	CMV	---	SNAP26m	Transfection		AB

Derived from pSEMS-26m (Covalys), These vectors allow the expression of recombinant proteins in mammalian cells as Snap-tag fusion proteins. Their expression can be detected by immunofluorescences.

pAB-SEMc has 2 Sfi1 sites at the Nt of the Snap-tag  
 pAB-SEMn (with Sfi1 sites at the Ct of the Snap-tag is not yet constructed)

#### Use :

Localisation of proteins of interest in mammalian cells by confocal microscopy.



## 1. Y2H-VECTORS

### 1.1. cyto-Y2H vectors

name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by	Box	Place
<i>Bait-vectors</i>										
pASO	Kan	Leu	ADH	---	Ost4-Cub	cyto-ER-Y2H	anti-LexA	DS	cyto-Y2H	A1
pASO-BCK	Kan	Leu	ADH	BCK	Ost4-BCK-Cub	cyto-ER-Y2H	anti-LexA	DS	cyto-Y2H	A3
pASO-ABCK	Kan	Leu	ADH	ABCK	Ost4-ABCK-Cub	cyto-ER-Y2H	anti-LexA	DS	cyto-Y2H	A5
pASO-ΔGM130	Kan	Leu	ADH	ΔGM130	Ost4-ΔGM130-Cub	cyto-ER-Y2H	anti-LexA	DS	cyto-Y2H	A7
pASO-Gal80	Kan	Leu	ADH	Gal80	Ost4-Gal80-Cub	cyto-ER-Y2H	anti-LexA	DS	cyto-Y2H	B2
pASO-Large1	Kan	Leu	ADH	Ag Large1	Ost4-Large1-Cub	cyto-ER-Y2H	anti-LexA	DS	cyto-Y2H	A9
pASO-p53	Kan	Leu	ADH	fl p53	Ost4-p53-Cub	cyto-ER-Y2H	anti-LexA	DS	cyto-Y2H	B1
pASO-VAMP3	Kan	Leu	ADH	flVAMP3	Ost4-VAMP3-Cub	cyto-ER-Y2H	anti-LexA	AB	cyto-Y2H	B6
pASO-JWA	Kan	Leu	ADH	flJWA	Ost4-JWA-Cub	cyto-ER-Y2H	anti-LexA	AB	cyto-Y2H	B7
pASO-fly2	Kan	Leu	ADH	fly2	Ost4-fly2-Cub	cyto-ER-Y2H	anti-LexA	AB	cyto-Y2H	B8
pCab	Kan	Leu	ADH	---	Aβ-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	A2
pCab-BCK	Kan	Leu	ADH	BCK	Aβ-BCK-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	A4
pCab-Δβ1	Kan	Leu	ADH	Δβ1	Aβ-Δβ1-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	B3
pCab-Δβ2	Kan	Leu	ADH	Δβ2	Aβ-Δβ2-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	B4
pCab-ABCK	Kan	Leu	ADH	ABCK	Aβ-ABCK-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	A6
pCab-Δy2	Kan	Leu	ADH	Δy2	Aβ-Δy2-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	B5
pCab-ΔGM130	Kan	Leu	ADH	ΔGM130	Aβ-ΔGM130-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	A8
pCab-VAMP3	Kan	Leu	ADH	VAMP3	Aβ-VAMP3-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	C1
pCab-fly2	Kan	Leu	ADH	fly2	Aβ-fly2-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	C2
pCab-LT	Kan	Leu	ADH	LT	Aβ-LT-Cub	cyto-M-Y2H	anti-LexA	Syl20	cyto-Y2H	B9
pCab-VAMP2	Kan	Leu	ADH	VAMP2(rat)	Aβ-VAMP2-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	C3
pCab-flα1	Kan	Leu	ADH	flα1	Aβ-flα1-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	C8
pCab-flβ2	Kan	Leu	ADH	flβ2	Aβ-flβ2-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	C4
pCab-ALDH2	Kan	Leu	ADH	ALDH2(rat)	Aβ-ALDH2-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	C5
pCab-ActG1	Kan	Leu	ADH	ActG1(rat)	Aβ-ActG1-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	C6
pCab-FABP	Kan	Leu	ADH	FABP(rat)	Aβ-FABP-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	C7
pCab-flβ1	Kan	Leu	ADH	flβ1	Aβ-flβ1-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	C9
pCab-flα2	Kan	Leu	ADH	flα2	Aβ-flα2-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	D1
pCab-flα2 TD	Kan	Leu	ADH	flα2 TD	Aβ-flα2TD-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	D2
pCab-GST	Kan	Leu	ADH	GST (bacteria)	Aβ-GST-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	D3
pCab-BCK S6D	Kan	Leu	ADH	BCK S6D	Aβ-BCK-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	D4
pCab-BCK T35D	Kan	Leu	ADH	BCK T35D	Aβ-BCK-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	D5
pCab-BCK DM	Kan	Leu	ADH	BCK S6D T35D	Aβ-BCK-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	D6
pCab-GBDctf2	Kan	Leu	ADH	GBDctf2	Aβ-GBDctf2-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	D7
pCab-ctf2	Kan	Leu	ADH	ctf2	Aβ-ctf2-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	D8
pCab-ctV3	Kan	Leu	ADH	ctVAMP3	Aβ-ctV3-Cub	cyto-M-Y2H	anti-LexA	AB	cyto-Y2H	D9
<i>Prey-vectors</i>										
pDSL-Nx	Amp	Trp	CYC	---	NubG	cyto-Y2H	anti-HA	DS	cyto-Y2H	F1
pDSL20	Amp	Trp	CYC	---	NubG-Stp	cyto-Y2H	anti-HA	Syl20	cyto-Y2H	G8
pDSL-BCK	Amp	Trp	CYC	BCK	NubG-BCK	cyto-Y2H	anti-HA	AB	cyto-Y2H	F2
pDSL-ABCK	Amp	Trp	CYC	ABCK	NubG-ABCK	cyto-Y2H	anti-HA	DS	cyto-Y2H	F3
pDSL-ΔGM130	Amp	Trp	CYC	ΔGM130	NubG-ΔGM130	cyto-Y2H	anti-HA	DS	cyto-Y2H	F4
pDSL-Δβ1	Amp	Trp	CYC	Δβ1	NubG-Δβ1	cyto-Y2H	anti-HA	AB	cyto-Y2H	F5
pDSL-Δβ2	Amp	Trp	CYC	Δβ2	NubG-Δβ2	cyto-Y2H	anti-HA	AB	cyto-Y2H	F6
pDSL-Δy2	Amp	Trp	CYC	Δy2	NubG-Δy2	cyto-Y2H	anti-HA	AB	cyto-Y2H	F7
pDSL-Nub1	Amp	Trp	CYC	Ost1	Nub1-Ost1	cyto-Y2H	anti-HA	DS	cyto-Y2H	F8
pDSL-NubG	Amp	Trp	CYC	Ost2	NubG-Ost1	cyto-Y2H	anti-HA	DS	cyto-Y2H	F9
pDSL-Rpb5	Amp	Trp	CYC	Rpb5	NubG-Rpb5	cyto-Y2H	anti-HA	DS	cyto-Y2H	G1
pDSL-LargeT	Amp	Trp	CYC	Ag LargeT	NubG-LargeT	cyto-Y2H	anti-HA	DS	cyto-Y2H	G2
pDSL-Gal80	Amp	Trp	CYC	Gal80	NubG-Gal80	cyto-Y2H	anti-HA	DS	cyto-Y2H	G3
pDSL-β2	Amp	Trp	CYC	fl β2	NubG-β2	cyto-Y2H	anti-HA	Syl20	cyto-Y2H	G5
pDSL-VAMP3	Amp	Trp	CYC	VAMP3	NubG-VAMP3	cyto-Y2H	anti-HA	AB	cyto-Y2H	G6
pDSL-JWA	Amp	Trp	CYC	JWA	NubG-JWA	cyto-Y2H	anti-HA	AB	cyto-Y2H	G7
pFur4-NubG	Amp	Trp	???	Fur4	NubG-Fur4	cyto-Y2H	anti-HA	DS	cyto-Y2H	G4
pDSL-flα1	Amp	Trp	CYC	fl α1	NubG-α1	cyto-Y2H	anti-HA	AB	cyto-Y2H	H6
pDSL-α1KD	Amp	Trp	CYC	α1KD	NubG-α1KD	cyto-Y2H	anti-HA	AB	cyto-Y2H	H7
pDSL-flβ1	Amp	Trp	CYC	fl β1	NubG-β1	cyto-Y2H	anti-HA	AB	cyto-Y2H	H5
pDSL20-flβ2	Amp	Trp	CYC	fl β2	NubG-β2	cyto-Y2H	anti-HA	AB	cyto-Y2H	G9
pDSL20-SNARE	Amp	Trp	CYC	Snare(h)	NubG-SNARE	cyto-Y2H	anti-HA	AB	cyto-Y2H	H1
pDSL20-VAMP2	Amp	Trp	CYC	VAMP2(rat)	NubG-VAMP2	cyto-Y2H	anti-HA	AB	cyto-Y2H	H2
pDSL20-ALDH2	Amp	Trp	CYC	ALDH2(rat)	NubG-ALDH2	cyto-Y2H	anti-HA	AB	cyto-Y2H	H3
pDSL20-FABP	Amp	Trp	CYC	FABP(liver rat)	NubG-FABP	cyto-Y2H	anti-HA	AB	cyto-Y2H	H4
pDSL20-ActG1	Amp	Trp	CYC	ActG1(rat)	NubG-ActG1	cyto-Y2H	anti-HA	AB	cyto-Y2H	H8
pDSL-FABP	Amp	Trp	CYC	FABP(liver rat)	NubG-FABP	cyto-Y2H	anti-HA	AB	cyto-Y2H	H9
pDSL-VAMP2	Amp	Trp	CYC	VAMP2(rat)	NubG-VAMP2	cyto-Y2H	anti-HA	AB	cyto-Y2H	I1
pDSL20-GS1	Amp	Trp	CYC	GS1 (bacteria)	NubG-GS1	cyto-Y2H	anti-HA	AB	cyto-Y2H	I2
pDSL20-a2WT	Amp	Trp	CYC	AMPK a2 (rat)	NubG-a2WT	cyto-Y2H	anti-HA	AB	cyto-Y2H	I3
pDSL-RNF41	Amp	Trp	CYC	RNF41 (rat)	NubG-RNF41 (clone)	cyto-Y2H	anti-HA	AB	cyto-Y2H	I4
pDSL20-a2TD	Amp	Trp	CYC	AMPK a2 (rat)	NubG-a2WT	cyto-Y2H	anti-HA	AB	cyto-Y2H	I5
pDSL20-BCK S6D	Amp	Trp	CYC	BCK S6D (human)	NubG-BCK S6D	cyto-Y2H	anti-HA	AB	cyto-Y2H	I6
pDSL20-BCK T35D	Amp	Trp	CYC	BCK T35D (human)	NubG-BCK T35D	cyto-Y2H	anti-HA	AB	cyto-Y2H	I7
pDSL20-BCK DM	Amp	Trp	CYC	BCK DM (human)	NubG-BCK S6D T35D	cyto-Y2H	anti-HA	AB	cyto-Y2H	I8
pDSL-a1 WT KD	Amp	Trp	CYC	AMPK a1 KD (rat)	NubG-a1WT KD	cyto-Y2H	anti-HA	AB	cyto-Y2H	I9
pDSL20-FH1	Amp	Trp	CYC	sol fumarase (rat)	NubG-FH1	cyto-Y2H	anti-HA	AB	cyto-Y2H	E9

## 1.2. Split-Trp vectors

name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by	Box	Place
<i>bait vectors</i>										
p-BCK-CTrp3	Kan	Ura	CUP1	BCK	BCK-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	A1
pG-BCK-CTrp3	Kan	Ura	GPD	BCK	BCK-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	A2
p-ABCK-CTrp3	Kan	Ura	CUP1	ABCK	ABCK-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	A3
pG-ABCK-CTrp3	Kan	Ura	GPD	ABCK	ABCK-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	A4
p-ΔGM130-CTrp3	Kan	Ura	CUP1	ΔGM130	ΔGM130-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	A5
pG-ΔGM130-CTrp3	Kan	Ura	GPD	ΔGM130	ΔGM130-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	A6
p-Δβ1-CTrp3	Kan	Ura	CUP1	Δβ1	Δβ1-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	A7
pG-Δβ1-CTrp3	Kan	Ura	GPD	Δβ1	Δβ1-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	A8
p-Δβ2-CTrp3	Kan	Ura	CUP1	Δβ2	Δβ2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	A9
pG-Δβ2-CTrp3	Kan	Ura	GPD	Δβ2	Δβ2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	B1
p-Δγ2-CTrp3	Kan	Ura	CUP1	Δγ2	Δγ2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	B2
pG-Δγ2-CTrp3	Kan	Ura	GPD	Δγ2	Δγ2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	B3
p-Gal80-CTrp3	Kan	Ura	CUP1	Gal80	Gal80-CTrp	SplitTrp-Y2H	anti-V5	DS	SplitTrp	B4
pG-Gal80-CTrp3	Kan	Ura	GPD	Gal80	Gal80-CTrp	SplitTrp-Y2H	anti-V5	DS	SplitTrp	B5
p-LtAg-CTrp3	Kan	Ura	CUP1	Ag LargeT	LtAg-CTrp	SplitTrp-Y2H	anti-V5	DS	SplitTrp	B6
pG-LtAg-CTrp3	Kan	Ura	GPD	Ag LargeT	LtAg-CTrp	SplitTrp-Y2H	anti-V5	DS	SplitTrp	B7
pG-p53-CTrp3	Kan	Ura	GPD	p53	p53-CTrp	SplitTrp-Y2H	anti-V5	DS	SplitTrp	B8
pG-VAMP3-CTrp3	Kan	Ura	GPD	VAMP3(h)	VAMP3-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	B9
pG-VAMP2-CTrp3	Kan	Ura	GPD	VAMP2(rat)	VAMP2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	C1
pG-JWA-CTrp3	Kan	Ura	GPD	JWA(h)	JWA-CTrp	SplitTrp-Y2H	anti-V5	DS	SplitTrp	C2
pG-α1-CTrp3	Kan	Ura	GPD	α1	α1-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	C3
pG-β2-CTrp3	Kan	Ura	GPD	β2	β2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	C4
pG-β1-CTrp3	Kan	Ura	GPD	β1	β1-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	C5
pG-FABP-CTrp3	Kan	Ura	GPD	FABP(rat)	FABP-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	C6
pG-ALDH2-CTrp3	Kan	Ura	GPD	ALDH2(rat)	ALDH2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	C7
p-α1-CTrp3	Kan	Ura	CUP1	α1	α1-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	C8
p-β2-CTrp3	Kan	Ura	CUP1	β2	β2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	C9
p-β1-CTrp3	Kan	Ura	CUP1	β1	β1-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	D1
p-FABP-CTrp3	Kan	Ura	CUP1	FABP(rat)	FABP-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	D2
p-ALDH2-CTrp3	Kan	Ura	CUP1	ALDH2(rat)	ALDH2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	D3
p-VAMP2-CTrp3	Kan	Ura	CUP1	VAMP2(rat)	VAMP2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	D4
p-ActG1-CTrp3	Kan	Ura	CUP1	ActG1(rat)	ActG1-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	D5
p-BCK S6D-CTrp3	Kan	Ura	CUP1	BCK S6D (human)	BCK S6D-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	D6
pBCK T35D-CTrp3	Kan	Ura	CUP1	BCK T35D (human)	BCK T35D-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	D7
p-BCK DM-CTrp3	Kan	Ura	CUP1	BCK DM (human)	BCK DM-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	D8
p-GST-CTrp3	Kan	Ura	CUP1	GST (bacteria)	GST-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	D9
p-γA-CTrp3	Kan	Ura	CUP1	γ-actin (rat)	γA-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	I1
p-FH1-CTrp3	Kan	Ura	CUP1	sol fumarase (rat)	FH1-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	I2
p-ctV3-CTrp3	Kan	Ura	CUP1	ctVAMP3 (rat)	ctV3-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	I3
p-ctβ2-CTrp3	Kan	Ura	CUP1	ctβ2 (rat)	ctβ2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	I4
p-GBDctβ2-CTrp3	Kan	Ura	CUP1	GBDctβ2 (rat)	GBDctβ2-CTrp	SplitTrp-Y2H	anti-V5	AB	SplitTrp	I5
<i>prey vectors</i>										
p-NTrp-BCK	Amp	Leu	CUP1	BCK	NTrp-BCK	SplitTrp-Y2H	anti-HA	AB	SplitTrp	E1
pG-NTrp-BCK	Amp	Leu	GPD	BCK	NTrp-BCK	SplitTrp-Y2H	anti-HA	AB	SplitTrp	E2
pM-NTrp-BCK	Amp	Leu	Met25	BCK	NTrp-BCK	SplitTrp-Y2H	anti-HA	AB	SplitTrp	E3
p-NTrp-ABCK	Amp	Leu	CUP1	ABCK	NTrp-ABCK	SplitTrp-Y2H	anti-HA	AB	SplitTrp	E4
pG-NTrp-ABCK	Amp	Leu	GPD	ABCK	NTrp-ABCK	SplitTrp-Y2H	anti-HA	AB	SplitTrp	E5
pM-NTrp-ABCK	Amp	Leu	Met25	ABCK	NTrp-ABCK	SplitTrp-Y2H	anti-HA	AB	SplitTrp	E6
p-NTrp-ΔGM130	Amp	Leu	CUP1	ΔGM130	NTrp-ΔGM130	SplitTrp-Y2H	anti-HA	AB	SplitTrp	E7
pG-NTrp-ΔGM130	Amp	Leu	GPD	ΔGM130	NTrp-ΔGM130	SplitTrp-Y2H	anti-HA	AB	SplitTrp	E8
pM-NTrp-ΔGM130	Amp	Leu	Met25	ΔGM130	NTrp-ΔGM130	SplitTrp-Y2H	anti-HA	AB	SplitTrp	E9
p-NTrp-Δβ1	Amp	Leu	CUP1	Δβ1	NTrp-Δβ1	SplitTrp-Y2H	anti-HA	AB	SplitTrp	F1
pG-NTrp-Δβ1	Amp	Leu	GPD	Δβ1	NTrp-Δβ1	SplitTrp-Y2H	anti-HA	AB	SplitTrp	F2
p-NTrp-Δβ2	Amp	Leu	CUP1	Δβ2	NTrp-Δβ2	SplitTrp-Y2H	anti-HA	AB	SplitTrp	F3
pG-NTrp-Δβ2	Amp	Leu	GPD	Δβ2	NTrp-Δβ2	SplitTrp-Y2H	anti-HA	DS	SplitTrp	F4
p-NTrp-Δγ2	Amp	Leu	CUP1	Δγ2	NTrp-Δγ2	SplitTrp-Y2H	anti-HA	DS	SplitTrp	F5
pG-NTrp-Δγ2	Amp	Leu	GPD	Δγ2	NTrp-Δγ2	SplitTrp-Y2H	anti-HA	DS	SplitTrp	F6
p-NTrp-LtAg	Amp	Leu	CUP1	Ag LargeT	NTrp-LtAg	SplitTrp-Y2H	anti-HA	AB	SplitTrp	F7
pG-NTrp-Gal80	Amp	Leu	GPD	Gal80	NTrp-Gal80	SplitTrp-Y2H	anti-HA	AB	SplitTrp	F8
p-NTrp-p53	Amp	Leu	CUP1	p53	NTrp-p53	SplitTrp-Y2H	anti-HA	AB	SplitTrp	F9
p-NTrp-Fe65	Amp	Leu	CUP1	Fe65	NTrp-Fe65	SplitTrp-Y2H	anti-HA	AB	SplitTrp	G1
pCup-L505c	Amp	Leu	CUP1	GFP	NTrp-p53	SplitTrp-Y2H	anti-HA	AB	SplitTrp	G2
pM-NTrp-VAMP3	Amp	Leu	Met25	VAMP3(h)	NTrp-VAMP3	SplitTrp-Y2H	anti-HA	AB	SplitTrp	G3
pM-NTrp-JWA	Amp	Leu	Met25	JWA(h)	NTrp-JWA	SplitTrp-Y2H	anti-HA	AB	SplitTrp	G4
pM-NTrp-β2	Amp	Leu	Met25	β2	NTrp-β2	SplitTrp-Y2H	anti-HA	AB	SplitTrp	G5
pM-NTrp-β1	Amp	Leu	Met25	β1	NTrp-β1	SplitTrp-Y2H	anti-HA	AB	SplitTrp	G6
pM-NTrp-β1α1	Amp	Leu	Met25	β1α1	NTrp-β1α1	SplitTrp-Y2H	anti-HA	AB	SplitTrp	G7
pM-NTrp-β1α2WT	Amp	Leu	Met25	β1α2WT	NTrp-β1α2WT	SplitTrp-Y2H	anti-HA	AB	SplitTrp	G8
pM-NTrp-SNARE	Amp	Leu	Met25	Snare(h)	NTrp-SNARE	SplitTrp-Y2H	anti-HA	AB	SplitTrp	G9
pM-NTrp-VAMP2	Amp	Leu	Met25	VAMP2(rat)	NTrp-VAMP2	SplitTrp-Y2H	anti-HA	AB	SplitTrp	H1
pM-NTrp-FABP	Amp	Leu	Met25	FABP(rat)	NTrp-FABP	SplitTrp-Y2H	anti-HA	AB	SplitTrp	H2
pM-NTrp-ALDH2	Amp	Leu	Met25	ALDH2(rat)	NTrp-ALDH2	SplitTrp-Y2H	anti-HA	AB	SplitTrp	H3
pM-NTrp-BCK S6D	Amp	Leu	Met25	BCK S6D (human)	NTrp-BCK S6D	SplitTrp-Y2H	anti-HA	AB	SplitTrp	H4
pM-NTrp-BCK T35D	Amp	Leu	Met25	BCK T35D (human)	NTrp-BCK T35D	SplitTrp-Y2H	anti-HA	AB	SplitTrp	H5
pM-NTrp-BCK DM	Amp	Leu	Met25	BCK DM (human)	NTrp-BCK DM	SplitTrp-Y2H	anti-HA	AB	SplitTrp	H6
pM-NTrp-β1α2TD	Amp	Leu	Met25	β1α2TD	NTrp-β1α2TD	SplitTrp-Y2H	anti-HA	AB	SplitTrp	H7
pM-NTrp-γA	Amp	Leu	Met25	γ-actin (rat)	NTrp-γA	SplitTrp-Y2H	anti-HA	AB	SplitTrp	H8
pM-NTrp-FH1	Amp	Leu	Met25	sol fumarase (rat)	NTrp-FH1	SplitTrp-Y2H	anti-HA	AB	SplitTrp	H9

## 2. TRANSFECTION OF EUKARYOTIC CELLS

name	Resistance	Selection Marker	Promoter	Insert	Expressed	Use	Antibody	Created by	Box	Place
pCI-Neo-PLAP-V5	Amp	neo	CMV	PLAP-V5	PLAP-V5	Transfection			TF	A1
pCI-Neo-IRAP	Amp	neo	CMV	IRAP (PLAP)	IRAP (PLAP)	Transfection			TF	A2
pSEMS-26m	Amp	neo	CMV	---	SNAP26m	Transfection		Novagen	TF	A3
pSEMS-26m-Cox8a	Amp	neo	CMV	Cox8a	Cox8a-SNAP26m	Transfection		Novagen	TF	A4
pAB-SEMc	Amp	neo	CMV	---	SNAP26m	Transfection		AB	TF	A5

### 3. PROCARIOTYC AND *IN VITRO* EXPRESSION VECTORS

AMPK										
name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by	Box	Place
py1	Amp	---	T7	$\gamma 1$ (AMPK) - rat	$\gamma 1$ (AMPK) - rat	Expression		Dietbert	AMPK	A1
py2	Amp	---	T7	$\gamma 2$ (AMPK) - human	$\gamma 2$ (AMPK) - human	Expression		Dietbert	AMPK	A2
py3	Amp	---	T7	$\gamma 3$ (AMPK) - human	$\gamma 3$ (AMPK) - human	Expression		Dietbert	AMPK	A3
p111WT	Amp	---	T7	His- $\alpha 1\beta 1\gamma 1$	AMPK(111)-His active	Expression		Dietbert	AMPK	B1
p111TD	Amp	---	T7	His $\alpha 1\beta 1\gamma 1$	AMPK(111)-His TD	Expression		Dietbert	AMPK	B2
p111 TE SD	Amp	---	T7	His $\alpha 1\beta 1\gamma 1$	AMPK(111)-His TE SD	Expression		Dietbert	AMPK	B3
p111 TE TD SD	Amp	---	T7	His $\alpha 1\beta 1\gamma 1$	AMPK(111)-His TE TD SD	Expression		Dietbert	AMPK	B4
p211WT	Amp	---	T7	His $\alpha 2\beta 1\gamma 1$	AMPK(211)-His	Expression		Dietbert	AMPK	C1
p211TD	Amp	---	T7	His $\alpha 2\beta 1\gamma 1$	AMPK(111)-His TD	Expression		Dietbert	AMPK	C2
p221WT	Amp	---	T7	His $\alpha 2\beta 2\gamma 1$	AMPK(111)-His	Expression		Dietbert	AMPK	D1
p221TD	Amp	---	T7	His $\alpha 2\beta 2\gamma 1$	AMPK(211)-His TD	Expression		AB	AMPK	D2
Creatine Kinase										
name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by	Box	Place
pET14b-huMtCK	Amp	---	T7	huMtCK	human uMtCK	Expression			CK	A1
pUS04-huMtCK	Amp	---		huMtCK	human uMtCK	Expression		Uwe S	CK	A2
pGFM-3Z-hsMtCK	Amp	---		hsMtCK	human sMtCK	Expression			CK	A3
pFluo1-hsMtCK	Amp	---		hsMtCK	human sMtCK	Expression			CK	A4
pUS01	Amp	---		---	---	Expression		Uwe S	CK	A5
pUS03-hsMtCK	Amp	---		hsMtCK	human sMtCK	Expression		Uwe S	CK	A6
pUS01-hsMtCK	Amp	---		hsMtCK	human sMtCK	Expression		Uwe S	CK	A7
pRF59-cMtCK	Amp	---		cMtCK	chicken M <sub>1</sub> CK	Expression		TS	CK	A8
pT23-BCK	Amp	---		cBBCK	chicken BBCK	Expression			CK	A9
pET3b-cBCK	Amp	---	T7	cBBCK	chicken BBCK	Expression			CK	B1
pET3b-hBCK	Amp	---	T7	hBBCK	human BBCK	Expression			CK	B2
pET14b-chick His-BCK	Amp	---	T7	His-cBBCK	His-chicken BBCK	Expression			CK	B3
pET-14b chick His-MCK	Amp	---	T7	His-cMMCK	His-chicken MMCK	Expression			CK	B4
Expression in vitro										
name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by	Box	Place
pAB-I-His	Amp	---	T7	---	His	Expression	anti-His	AB	ABeiv	A1
pAR-I-HA	Amp	---	T7	---	HA	Expression	anti-HA	AB	ABeiv	A2
pAB-BCK-His	Amp	---	T7	BCK	BCK-His	Expression	anti-His	AB	ABeiv	A3
pAB-BCK-HA	Amp	---	T7	BCK	BCK-HA	Expression	anti-HA	AB	ABeiv	A4
pAB- $\Delta$ BCK-His	Amp	---	T7	$\Delta$ BCK	$\Delta$ BCK-His	Expression	anti-His	AB	ABeiv	A5
pAB- $\Delta$ BCK-HA	Amp	---	T7	$\Delta$ BCK	$\Delta$ BCK-HA	Expression	anti-HA	AB	ABeiv	A6
pAB-VAMP3-His	Amp	---	T7	VAMP3	VAMP3-His	Expression	anti-His	AB	ABeiv	A7
pAB-VAMP3-HA	Amp	---	T7	VAMP3	VAMP3-HA	Expression	anti-HA	AB	ABeiv	A8
pAB-SNARE-His	Amp	---	T7	SNARE	SNARE-His	Expression	anti-His	AB	ABeiv	A9
pAB-SNARE-HA	Amp	---	T7	SNARE	SNARE-HA	Expression	anti-HA	AB	ABeiv	B1
pAR-JWA-His	Amp	---	T7	JWA	JWA-His	Expression	anti-His	AR	ABeiv	B2
pAB-JWA-HA	Amp	---	T7	JWA	JWA-HA	Expression	anti-HA	AB	ABeiv	B3
pAB-LT-His	Amp	---	T7	LT	LT-His	Expression	anti-His	AB	ABeiv	B4
pAB-LT-HA	Amp	---	T7	LT	LT-HA	Expression	anti-HA	AB	ABeiv	B5
Note : all vectors derived from AMPK-vector (modified pET)										
Expression with Strep-Tag										
name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by	Box	Place
pET152b+	Amp	---	T7/lac	---	Strep-His	Expression	anti-His	AB	ABstrep	A1
pAB52	Amp	---	T7	---	Strep-His	Expression	anti-His	AB	ABstrep	A2
pAB52s	Amp	---	T7	---	Strep	Expression	anti-Strep	AB	ABstrep	A3
pAB52-BCK	Amp	---	T7	BCK	Strep-BCK-His	Expression	anti-His	AB	ABstrep	A4
pAB52s-BCK	Amp	---	T7	$\Delta$ BCK	Strep-BCK	Expression	anti-His	AB	ABstrep	A5
pAB52s-ntVAMP3	Amp	---	T7	ntVAMP3	Strep-ntVAMP3	Expression	anti-Strep	AB	ABstrep	A6
pAB52s-ntJWA	Amp	---	T7	ntJWA	Strep-ntJWA	Expression	anti-Strep	AB	ABstrep	A7
pAB52-ctJWA	Amp	---	T7	ctJWA	Strep-ctJWA-His	Expression	anti-His	AB	ABstrep	A8
pAB52-ctNogo	Amp	---	T7	ctNogo	Strep-ctNogo-His	Expression	anti-His	AB	ABstrep	A9
pAB52-ctPCDb10	Amp	---	T7	ctPCDb10	Strep-ctPCDb10-His	Expression	anti-His	AB	ABstrep	B1
pET52-NgalRloop	Amp	---	T7	NgalRloop	Strep-NgalRloop-His	Expression	anti-His	AB	ABstrep	B2
pAB52s-CLDND	Amp	---	T7	CLDND	Strep-CLDND	Expression	anti-Strep	Syl20	ABstrep	B3
pAB52s-RNF41	Amp	---	T7	RNF41	Strep-RNF41	Expression	anti-Strep	Syl20	ABstrep	B4
pAB52s-GSTM1	Amp	---	T7	GSTM1 (rat)	Strep-GSTM1	Expression	anti-Strep	Syl20	ABstrep	B5
pAB52s-GSTP1	Amp	---	T7	GSTP1 (rat)	Strep-GSTP1	Expression	anti-Strep	Syl20	ABstrep	B6
Note : all vectors derived from pET152b(+) - Novagen										
Expression with GST										
name	Resistance	Auxotrophy	Promoter	Insert	Expressed	Use	Antibody	Created by	Box	Place
pGEX-4T-1	Amp	---	Tac	---	GST (S. japonicum)	Expression	anti-GST	GE Healthcare	AB-GST	A1
pAB-GSTn	Amp	---	Tac	---	GST (S. japonicum)	Expression	anti-GST	AB	AB-GST	A2
pAB-GSTn-VAMP2	Amp	---	Tac	---	GST-VAMP2 (human)	Expression	anti-GST	AB	AB-GST	A3
pAB-GSTn-VAMP3	Amp	---	Tac	---	GST-VAMP3 (human)	Expression	anti-GST	AB	AB-GST	A4
pAB-GSTn-SNARE	Amp	---	Tac	---	GST-SNARE (VAMP3)	Expression	anti-GST	AB	AB-GST	A5
pAB-GSIn-FABP	Amp	---	Iac	---	GST-FABP (rat)	Expression	anti-GS1	AB	AB-GS1	A6
pAB-GSTn-ALDH2	Amp	---	Tac	---	GST-ALDH2 (rat)	Expression	anti-GST	AB	AB-GST	A7
pAB-GSTn-ActG1	Amp	---	Tac	---	GST-ActG1 (rat)	Expression	anti-GST	AB	AB-GST	A8
pAB-GSTn-JWA	Amp	---	Tac	---	GST-JWA (human)	Expression	anti-GST	AB	AB-GST	A9
pAR-mGSTn	Amp	---	Tac	---	mGST (S. japonicum)	Expression	anti-GST	AR	AB-GST	B1
pGEX-GST-CamKK $\beta$	Amp	---	Tac	---	GST-CamKK $\beta$	Expression	anti-GST	H Tokumitso	AB-GST	B2
pGEX-GST- $\Delta$ CamKK $\beta$	Amp	---	Tac	---	GST-CamKK $\beta$ (84-434)	Expression	anti-GST	H Tokumitso	AB-GST	B3
pGEX-GST-ACC	Amp	---	Tac	---	GST-ACC	Expression	anti-GST	G Hardie	AB-GST	B4
pAB-GSTn-FH1	Amp	---	Tac	---	GST-FH1 (rat)	Expression	anti-GST	AB	AB-GST	B5





**CURRICULUM VITAE**  
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**Personal information:**

Place of Birth: Berlin, Germany  
 Date of Birth: August 05, 1982  
 Citizenship: German  
 Civil status: Married, 1 child (age: 1,5 years)

### RESEARCH EXPERIENCES AND SKILLS

**PhD Student at the Laboratory of Fundamental and Applied Bioenergetics in Grenoble, France**

11/2006 – 03/2011: Identification of new interaction partners of Creatine kinase and AMP-activated protein kinase in human brain by yeast two-hybrid screening. Confirmation of identified interactions by complementary biochemical methods. Analysis of the functional role of identified interactions. Publication and oral presentation of results in English and French.

Methodological Skills

*Molecular biology:* vector design; cloning (PCR, RNA isolation, cDNA synthesis, etc.) and yeast two-hybrid techniques.

*Biochemistry:* expression of recombinant proteins in *Escherichia coli* and *in vitro*; purification of proteins by chromatography; electrophoresis of SDS-Page and native gels; immuno-techniques; protein kinase assays (radioactive); enzymatic activity tests; basics in surface plasmon resonance spectroscopy (Biacore), 2D gel electrophoresis

*Cellular biology:* isolation of mitochondria, synaptosomes and synaptic vesicles from rat brain, glutamate release assay from synaptosomes by fluorimetry, cell culture, basics in oxygraphy

**Trainee at Dualsystems Biotech AG in Zurich, Switzerland**

12/2006 – 02/2007: Application and comparison of innovative (cytosolic) yeast two-hybrid techniques. Screening and analysis of protein-protein interactions.

Methodological Skills

*Molecular biology:* cloning and yeast two-hybrid techniques.

**Graduate Student at the Institute of Biochemistry, Charité in Berlin, Germany**

01/2006 – 07/2006: Determination of expression and purification conditions of different antibody Fab fragments.

Methodological Skills

*Molecular biology:* cloning

*Biochemistry:* expression of recombinant antibody fragments in *Escherichia coli*; purification of proteins by chromatography; SDS-Page; immuno-techniques (western blot, ELISA).

**Student Trainee in the Department of Research and Development at Berlin-Chemie in Berlin, Germany**

07/2005 – 08/2005: Separation and purification of nebivolol enantiomers by High-Performance Liquid Chromatography (HPLC).

### EDUCATION

- 2010: **PhD in Cellular Biology**, Laboratory of Fundamental and Applied Bioenergetics (LBFA) – Joseph Fourier University, Grenoble (France)  
Thesis research topic: Interactomics of key kinases in energy metabolism: characterizing interactions of AMP-activated protein kinase and B-type creatine kinase
- 2006: **Master's Degree in Bioscience Engineering**, National Institute of Applied Science (INSA), Lyon (France)  
**Master's Degree in Structural and Functional Biochemistry**, Claude Bernard Lyon I University, Lyon (France)  
Master thesis research topic: Construction and expression of recombinant antibody Fab fragments in *Escherichia coli*.
- 2003: **Vordiplom** in Chemistry, Freie Universität Berlin
- 2001: **Baccalauréat économique et social** and **Abitur**, Französisches Gymnasium, Berlin

## Complementary Education

July 2008	<b>Systems Biology</b> , J. Fourier University, Grenoble, France
June 2008	<b>Introduction to photon microscopy in biology</b> , Albert Bonniot Institute, Grenoble, France
July 2007	<b>Bioenergetics and metabolism</b> , J. Fourier University, Grenoble, France
April 2007	<b>Enzymatic catalysis and metabolic control</b> J. Fourier University, Grenoble

## PUBLICATIONS

- **Klaus A.**, Polge C., Zorman S., Auchli Y., Brunisholz R., Schlattner U. (2012) A two-dimensional screen for AMPK substrates identifies tumor suppressor fumarate hydratase as a preferential AMPK $\alpha$ 2 substrate. *J Proteomics* 75 (11): 3304-13
- **Brückner A.**, Polge C., Lentze N., Auerbach D., Schlattner U. (2009) Yeast Two-Hybrid, a powerful tool for systems biology. *Int J Mol Sci* 10: 2763-88
- Guerrero K., Monge C., **Brückner A.**, Puurand Ü., Kadaja L., Käämbre T., Seppet E. and Saks V. (2009) Study of possible interactions of tubulin, microtubular network and STOP protein with mitochondria in muscle cells. *Mol Cell Biochem* 337 (1-2): 239-49
- Schlattner U. ; Tokarska-Schlattner M., Ramirez S., **Brückner A.**, Kay L., Polge C., Epand R.F., Lacombe M.-L., Lee R.M. and Epand R.M. (2009) Mitochondrial kinases and their molecular interaction with cardiolipin. *Biochim Biophys Acta* 1788(10): 2032-47

### Submitted

- **Klaus A.**, Zorman S., Berthier A., Polge C., Ramirez S., Michelland S., Sève M., Vertommen D., Rider M., Lentze N., Auerbach D., Schlattner U. (2013) Glutathione S-Transferases interact with AMP-activated protein kinase: evidence for S-glutathionylation and activation in vitro. *PLoS One* (resubmission of revised manuscript)

## POSTER AND ORAL PRESENTATIONS

### Oral Presentations

- Combined interactomics approaches reveal new AMPK partners.**  
EXGENESIS Meeting (European consortium), Paris (France), 11<sup>th</sup> May 2009

### Poster Presentations

- Protein-protein interactions in cell signaling**, Brückner A, Polge C, Ramirez S, Schlattner U ;  
Journée Recherche en Biologie, Grenoble (France), 7<sup>th</sup> November 2007

### Participations to congresses

- Meeting of the french Exocytosis-Endocytosis club (Villard-de-Lans, 26- 28/10/2007)
- EXGENESIS Meeting (Edinburgh, 5-6/10/2009, Padua, 21-22/04/2008 and Grenoble 09/03/2007)

## LANGUAGE SKILLS

**German** (maternal language)  
**French** (fluent, bilingual)  
**English** (fluent)

## IT SKILLS

MS Office: Word, Exel, Powerpoint  
Special software: Sigma Plot, Vector NTI

## REFERENCE

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## Interactomics of key kinases in energy metabolism

A key property of complex biological systems is the presence of interaction networks crucial for all levels of cellular function, including the regulation of cellular energy. Two enzymes take center stage in the regulation of cellular and whole body energy metabolism. Creatine kinase (CK) functions as an intracellular energy storage and transport system playing a crucial role in the acute response to increasing energy demands. AMP-activated protein kinase (AMPK) regulates cellular and whole body energy delivery and consumption. We first applied an original cytosolic Y2H screen to identify new protein-protein interactions of cytosolic brain type CK (BCK) and AMPK in human brain. Various interaction candidates were identified, among them vesicle associated membrane proteins (VAMPs) interacting with both kinases. AMPK-VAMP interaction was confirmed by co-immunoprecipitation from synaptic vesicles, but did not lead to VAMP phosphorylation, suggesting that VAMP rather recruits AMPK for a regulation of exo- and endocytotic processes. A second strategy combining a biophysical interaction screen (Surface Plasmon Resonance, SPR) with an *in vitro* phosphorylation assay revealed rather AMPK isoform-specific targets. One is fumarate hydratase, which is preferentially phosphorylated by AMPK221, leading to an increase in enzyme activity *in vitro*. Finally, one class of interaction candidates, the glutathione S-transferases GSTM1 and -P1, were characterized in detail by a panel of interactomics methods (Y2H, SPR, co-immunoprecipitation) as reliable, high affinity interactors and identified as new AMPK substrates. In case of GSTP1, this leads to an increase in its enzymatic activity, suggesting a direct role of AMPK signaling in oxidative stress defense.

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## Interactomique de protéines clé du métabolisme énergétique

Une propriété clé des systèmes biologiques est la présence d'un réseau d'interactions protéiques, crucial pour toute fonction cellulaire comme par exemple la régulation du métabolisme énergétique. Deux enzymes clé impliquées dans cette régulation sont la créatine kinase (CK), dont la fonction consiste dans la gestion du stock et du transfert d'énergie, et la protéine kinase activée par l'AMP (AMPK), qui régule l'homéostasie énergétique au sein de la cellule et de l'organisme entier. Dans un premier temps un crible de double hybride en levure original fut appliquée afin d'identifier de nouveaux partenaires d'interaction de la CK cytosolique du cerveau (BCK) et de l'AMPK dans le cerveau humain. Différents candidats d'interaction furent identifiés, dont des protéines membranaires associées aux vésicules (VAMP) interagissant avec les deux kinases. L'interaction AMPK-VAMP fut confirmée par co-immunoprecipitation à partir de vésicules synaptiques, mais ne menait pas à la phosphorylation de VAMP, suggérant que VAMP recrute AMPK pour la régulation de processus d'endo- et d'exocytose. Une seconde stratégie combinant un essai d'interactions biophysique, basé sur la résonance plasmonique de surface (SPR), avec des essais de phosphorylation *in vitro* permit la sélection de cibles AMPK isoforme spécifique. Une de ces cibles fut la fumarate hydratase, dont la phosphorylation préférentielle par l'AMPK221 provoque une augmentation de l'efficacité enzymatique *in vitro*. Finalement, une classe de candidats d'interaction, les glutathion S-transferases GSTM1 et -P1, fut caractérisée en détail par un panel de méthodes d'interactomique (SPR, double hybride, co-immunoprécipitation). Cette étude les identifie comme interacteurs fiables à haute affinité ainsi que nouveaux substrats de l'AMPK. Dans le cas de GSTP1 la phosphorylation par AMPK provoque une augmentation de son activité enzymatique suggérant un rôle direct de la signalisation par AMPK dans la défense contre le stress oxydatif.