Regulation of AMPK activity and mitochondrial homeostasis by type 10 soluble adenylyl cyclase

Inaugural-Dissertation

to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)
submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by
VIGNESH JAYARAJAN
(from Tamilnadu)

Berlin, 2018

The present work was done under the supervision of PD Dr. Yury Ladilov at the Institute of Gender in Medicine, Centre for Cardiovascular Research, Charité University of Medicine, Berlin from July 2013 till December 2017.

1st Reviewer: **PD. Dr. Yury Ladilov**

2nd Reviewer: **Prof. Dr. Silke Rickert-Sperling**

Date of defense: **25.04.2018**

Acknowledgments

The entire period of my PhD study remained to be a constant learning experience filled with unique opportunities, great challenges and a lot of joy from achieving my professional goals. I would like to take the opportunity to thank all the individuals who showed their support during these 3½ years and made them enriching, fun and memorable.

I would like to extend my gratitude to Prof. Vera Regitz-Zagrosek for offering me this opportunity to explore my potential as a scientist in the Institute of Gender in Medicine, Charité – Universitätsmedizin, Berlin.

I would like to render my honest thankfulness to my examiner, Prof. Dr. Silke Rickert-Sperling, for her support and understanding without which submission of my thesis would have not been possible.

I would like to extend my deepest appreciation to my supervisor, PD Dr. Yury Ladilov for his guidance, constant support and patience. His mentoring has helped me to explore new paths, grow as a scientist and enjoy the learning experience.

I would like to pledge my sincere gratitude to Prof. Dr. Matthias F. Melzig for his constructive feedback on my thesis.

To my lab mates, past and present, thanks for creating an amazing and fun-filled lab environment. Over the years all these people have become my second family. A special thanks to technical assistants for their constant accessibility, support and valuable advice. Thanks to the scientific community at the Centre of Cardiovascular Research for always being very helpful and kind and also for sharing their valuable time and knowledge during seminars.

To Marie Skłodowska-Curie foundation, for funding the first 2½ years of my PhD as an Early Stage Researcher within the RADOX consortium. Special thanks to Dr. Carola Schubert for providing utmost support in all administrative duties.

I would like to thank my family for their love and support and also for their understanding of my long absence from home. Without their encouragement and support this would have not been possible. I dedicate my work to my supportive, brilliant and loving wife, Vidhya, a person with positive vibes that keeps me motivated. Thanks for their belief in me.

Table of Contents

1 Abbreviations

ACC	acetyl-CoA carboxylase
ADP	adenosine di-phosphate
AIS	auto-inhibitory sequence
AMPK	AMP-activated protein kinase
ATP	adenosine tri-phosphate
CAMKK2	calmodulin kinase kinase 2
cAMP	3’-5’-cyclic adenosine monophosphate
COX	cytochrome c oxidase
CREB	cAMP response element binding protein
DRP1	dynamin-like protein 1
EPAC	exchange protein activated by cAMP
ERR	estrogen-related receptor
ETC	electron transport chain
FRET	föster resonance energy transfer
IMS	inter-membrane space
LKB1	liver kinase B1
MELAS	mitochondrial encephalomyopathy
MFF	mitochondrial fission factor
NTPase	nucleoside triphosphatase
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PDE	phosphodiesterase
PGC1α	peroxisome proliferator-activated receptor‑γ co-activator 1α
PKA	protein kinase A
PKC	protein kinase C
PP	protein phosphatase
PPAR	peroxisome proliferator-activated receptor
ROS	reactive oxygen cycle
sAC	soluble adenylyl cyclase

TCA	tricarboxylic acid cycle
TFAM	mitochondrial transcription factor A
tmAC	transmembrane adenylyl cyclase
ULK1	unc-51 like kinase 1

2 Introduction

Metabolic diseases, such as diabetes and obesity, are commonly associated with cardiovascular disorders. Numerous modern therapeutic interventions to treat metabolic diseases are directed towards modulation of the activity of AMP-activated protein kinase (AMPK) (Yamauchi et al., 2002, Fryer et al., 2002, Hawley et al., 2002, Zhou et al., 2001), a key energy sensor maintaining the energy balance within the cells. Thus, a thorough investigation of AMPK function regulation, its upstream signaling and its downstream targets is important to improve the outcome of the therapies based on the AMPK activity modulation.

2.1 Structure of AMPK and regulation of its activity

2.1.1 Structure of AMPK

AMPK is a heterotrimeric complex made up of one catalytic α-subunit and two regulatory subunits, β and γ. In humans, there are two subunits of α (α1 and α2) and β (β1 and β2) and three γ subunits (γ1, γ2 and γ3) (Stapleton et al., 1996, Thornton et al., 1998, Cheung et al., 2000). Each AMPK complex is made up of at least one of each α, β and γ-subunits with all possible combinations, thus potentially forming 12 distinct AMPK complexes (Ross et al., 2016b) (**Fig. A**).

The α-subunit possesses a highly conserved Ser/Thr kinase domain near the N-terminus in the activation loop and an important amino acid residue, Thr172, which can be phosphorylated by an upstream kinase (Hawley et al., 1996). In addition to regulation through phosphorylation, AMPKα-subunit activity can also be self-regulated by the auto-inhibitory sequence (AIS) located at the C-terminal of the α-subunit (**Fig. A**). In overexpression studies, AIS-null AMPK demonstrated more than 10-fold increase in the activity as compared to wild-type AMPK (Crute et al., 1998, Pang et al., 2007). The β-subunit of AMPK does not have any catalytic activity. The β-subunit interacts with α- and γ-subunits and acts as a scaffold in assembling heterotrimeric complex of AMPK. In addition, β-subunit contains a carbohydrate-binding domain that allows AMPK to interact with glycogen (Hudson et al., 2003). Such association of AMPK with glycogen inhibits its activity (McBride et al., 2009). Recently, it has been suggested that myristoylation of β-subunit is important for the membrane association and activation of AMPK (Oakhill et al., 2010a).

The γ-subunit contains four tandem cystathionine β-synthase (CBS) domains that enable AMPK to respond to changes in the ratio of ATP to AMP (Xiao et al., 2007). Binding of AMP and ADP to the γ-subunit promotes AMPK activity (Hardie et al., 2011, Gowans et al., 2013, Ross et al., 2016a) (**Fig. A**).

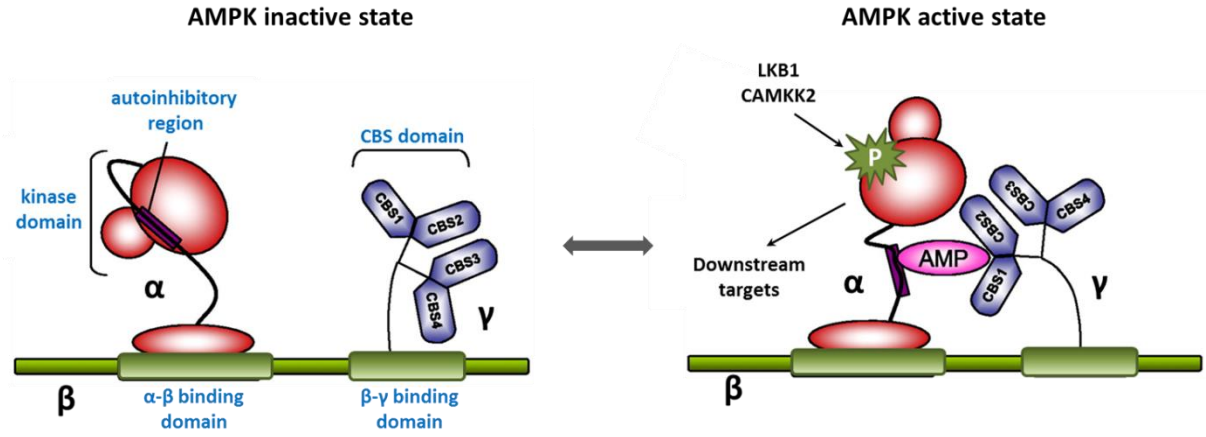

Fig. A. AMPK structure and activation.
Schematic representation of the AMPK structure showing the α-, β- and γ-subunits and their respective domains. The β-subunit acts like a platform to which the α- and γ-subunits anchor by binding to the conserved domains. The upstream kinases, such as LKB1 and CAMKK2, phosphorylate AMPKα-subunit at Thr172. Allosteric activation happens by binding of AMP to the CBS domains in γ-subunit. The figure is modified from Ignoul and Eggermont (2005).

2.1.2 Regulation of AMPK activity

The two main mechanisms increasing AMPK activity are (i) phosphorylation at Thr172 in the catalytic α-subunit by the upstream kinases and (ii) an increase in the ratio of AMP to ATP reflecting energy demand (**Fig. A**). Two main kinases have been found to phosphorylate AMPK at Thr172, i.e., the tumor-suppressor liver kinase B1 (LKB1) and the calcium-sensitive calmodulin kinase kinase 2 (CAMKK2) (Hawley et al., 2003, Woods et al., 2003, Shaw et al., 2004, Hurley et al., 2005, Hawley et al., 2005). Unlike AMPK, the activity of LKB1 is not dependent on phosphorylation of its activation loop (Oakhill et al., 2010b). However, LKB1 requires binding of the scaffold protein, mouse protein 25α (MO25α) (Oakhill et al., 2010a) and stabilization protein, Ste20-related adaptor (STRAD) (Oakhill et al., 2010a, Baas et al.,

2003) for its activity. Together, they form a trimeric LKB1–STRAD–MO25α complex that is constitutively active and has been shown to mediate AMPK Thr172 phosphorylation.

CAMKK2 can directly phosphorylate AMPK on Thr172 in response to the cellular calcium elevation (Hurley et al., 2005, Hawley et al., 2005, Woods et al., 2005), thus providing a connection between calcium signaling and regulation of energy metabolism by AMPK (Marcelo et al., 2016). Upstream of CAMKK2, a cAMP-regulated guanine nucleotide exchange factors (EPAC) plays an important role, where stimulation of EPAC leads to calcium release from the endoplasmic reticulum by promoting Rap2–phospholipase C–inositol triphosphate signaling (Schmidt et al., 2001). Increased calcium release, in turn, activated CAMKK2 and led to the phosphorylation of AMPK at Thr172 (Park et al., 2012).

The second important mechanism of AMPK stimulation is the increase in the AMP/ATP or ADP/ATP ratios, i.e., under energy imbalance. Indeed, most of the energy-demanding cellular processes are driven by the hydrolysis of ATP to ADP or AMP leading to the increase in AMP/ATP or ADP/ATP ratio. Hence cells can sense their energy state by monitoring the changes in AMP/ATP and ADP/ATP ratios. Binding of AMP, and also ADP with less affinity than AMP, to AMPK at the CBS domain of the γ-subunit enhances its activity possibly by two mechanisms. First, by an allosteric mechanism, i.e., a conformational change in the structure, allowing better binding of upstream kinases to AMPK (Hawley et al., 2003). Second, AMP binding to AMPK protects AMPK from phosphatases by the allosteric mechanism and, thereby, inhibits dephosphorylation at Thr172 (Davies et al., 1995b).

Mitochondrial inhibition and several physiological conditions, including exercise and nutrient starvation, activate AMPK *in vivo*, most probably by increasing the ratio of AMP to ATP. Many small-molecule activators of AMPK have been developed, which stimulate AMPK activity by binding to the interface of the α- and β-subunits independent of the changes in AMP to ATP ratio, like A769662, 991, PF739 and MK8722 (Cool et al., 2006, Xiao et al., 2013, Cokorinos et al., 2017, Myers et al., 2017). AMP mimetic AICAR is one of the well-studied small molecules as an allosteric activator of AMPK (Narkar et al., 2008). AICAR mimics the multiple effects of AMP on AMPK causing an allosteric activation of AMPK.

In addition to the two main mechanisms that stimulate AMPK activity as described above, TGF-β-activated kinase-1 (TAK1), an MAP kinase kinase kinase, has also been shown to activate AMPK. Knockout of TAK1 in mouse embryonic fibroblasts led to poor AMPK activation

in response to oligomycin or metformin treatments (Momcilovic et al., 2006). Another study using cell-free assay has demonstrated that TAK1 directly phosphorylates AMPK at Thr172 (Xie et al., 2006).

Apart from AMPK activation, several kinases mediate the antagonizing effect on AMPK activity. For example, protein kinase B has been shown to directly phosphorylate the α-subunit of AMPK at Ser485 in rat heart preventing LKB1-mediated phosphorylation of AMPK at Thr172 (Horman et al., 2006). Similar effects have been demonstrated in rat beta cell line (INS-1) and mouse embryonic fibroblasts, where phosphorylation at Ser485 and subsequent inhibition of AMPK activity were mediated by protein kinase A (PKA) (Hurley et al., 2006b). Recently it has been shown in mouse embryonic fibroblasts and 293T cells that glycogen synthase kinase 3 can phosphorylate the α-subunit of AMPK at Thr481 and Ser477, which induces a structural change making Thr172 more accessible to protein phosphatases (PPs) and hence promoting Thr172 dephosphorylation (Suzuki et al., 2013). Protein phosphatases (PPs) also play an important role in regulating AMPK activity. Although the exact mechanisms that modulate the action of PPs remain poorly understood, both PP2A and PP2C were shown to dephosphorylate Thr172 on AMPK (Davies et al., 1995a, Kudo et al., 1996, Sanders et al., 2007).

2.2 Role of AMPK in cell metabolism

Under metabolic stress, AMPK restores energy balance by inhibiting anabolism and by promoting catabolism. Several biosynthetic pathways are inhibited by AMPK under conditions of energy deficit. AMPK phosphorylates and inhibits acetyl-CoA carboxylases ACC1 and ACC2, which are important for *de novo* lipid synthesis. In addition, AMPK inhibits *de novo* synthesis of glucose (gluconeogenesis) by phosphorylating cyclic-AMP-regulated transcriptional co-activator 2 (CRTC2) and class II histone deacetylase (HDAC) and thereby promoting nuclear exclusion of these proteins, which are important for the transcription of gluconeogenic genes (Koo et al., 2005, Mihaylova et al., 2011). Processes involved in the hyperplasic or hypertrophic cell growth are some of the important ATP consumers in the cells. Accordingly, these processes are suppressed by AMPK via mTOR inhibition. By direct phosphorylation, AMPK activates TSC2, the negative regulator of mTORC1 (Inoki et al., 2003), and inhibits RAPTOR, a subunit of mTORC1 needed for its activity (Gwinn et al., 2008). AMPK also directly

phosphorylates and activates eEF2K, a negative regulator of protein translation, and regulates protein synthesis (Leprivier et al., 2013). Interestingly, eEF2K is a downstream target of the mTOR pathway (Faller et al., 2015), in addition strengthening the several crosstalk and connections between AMPK and mTOR signaling pathways controlling metabolism and cell growth. Altogether, AMPK limits ATP consumption by inhibiting the processes of protein, glucose and lipid synthesis during cellular energy stress.

For replenishing ATP stores, AMPK also actively pushes the cell to break down macromolecules to generate energy. These processes include improved glucose uptake and utilization, recycling of cellular macromolecules by autophagy and mobilization of lipid stores. Indeed, AMPK enhances glucose uptake by phosphorylating TBC domain family member 1 (TBC1D1) that controls the translocation of glucose transporter GLUT4 and also thioredoxin-interacting protein (TXNIP) that controls GLUT1 translocation in the cells (Wu et al., 2013, Hardie, 2013). AMPK also affects the activity of a rate-limiting enzyme in glycolysis (PFK1) by directly phosphorylating PFKFB3 (6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3). By doing this, AMPK increases the glycolytic flux under energy deprivation (Bando et al., 2005). Apart from promoting glucose utilization, AMPK also stimulates the cells to use their lipid stores. Particularly, AMPK stimulates the release of free fatty acids from triglyceride stores by phosphorylating at Ser406 and inducing adipose triglyceride lipase (ATGL) (Ahmadian et al., 2011). The released free fatty acids are then used for β-oxidation after being imported into mitochondria. Interestingly, AMPK also controls the import of free fatty acids into mitochondria. Mitochondria depend on carnitine palmitoyltransferase (CPT1) to import free fatty acids and the activity of CPT1 can be potentially inhibited by malonyl-CoA generated by ACC1 and ACC2. As mentioned earlier, AMPK phosphorylates and inhibits ACC and thereby promotes the activity of CPT1 and mitochondrial import of free fatty acids (McGarry et al., 1978, Saggerson, 2008).

2.3 Importance of AMPK in mitochondrial homeostasis

A mounting body of evidence details the necessity of AMPK in the regulation of several aspects of mitochondrial life cycle (**Fig. B**). These aspects include control of mitochondrial mass through stimulation of mitochondrial biogenesis, regulation of the shape of the

mitochondrial network in cells and mitochondrial quality control through regulation of mitochondrial clearance or mitophagy.

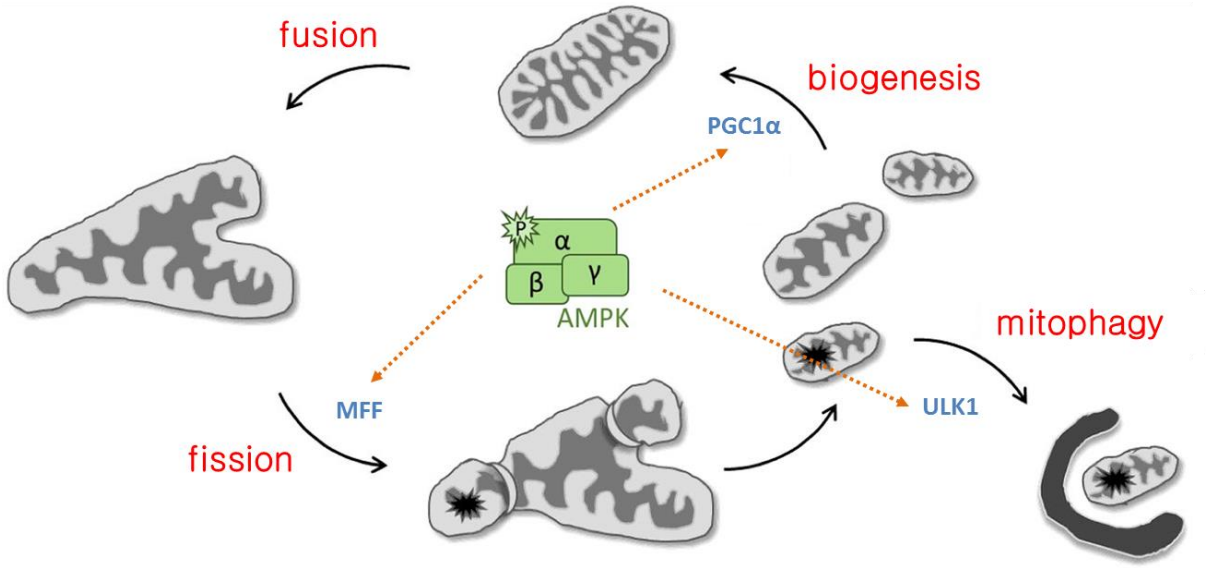

Fig. B. AMPK regulates mitochondrial homeostasis.
AMPK regulates mitochondrial homeostasis at different steps by directly phosphorylating several key players. AMPK phosphorylates mitochondrial fission factor (MFF) and promotes mitochondrial fission, an important initial step in mitophagy, i.e., elimination of damaged mitochondria via autophagy. AMPK phosphorylates ULK1, the upstream kinase involved in the formation of autophagosome. To restore mitochondrial mass, AMPK also promotes mitochondrial biogenesis by activating PGC1α, which enhances the transcription of genes required for mitochondrial biogenesis. Dotted arrows indicate direct phosphorylation by AMPK. Image modified from Thornton (2017).

2.3.1 Role of AMPK in mitochondrial biogenesis

Mitochondrial biogenesis takes place to produce more ATP in response to increased cellular energy expenditure. Growth and division of already existing mitochondria are the two important processes in mitochondrial biogenesis. Growth is achieved by increasing the mitochondrial mass via adding a new material to the already existing mitochondrial network. In humans, mitochondrial DNA is just 16.6 kb in length with 37 genes encoding 13 proteins and 24 RNAs. All these 13 proteins are involved in the formation of OXPHOS complexes essential for mitochondrial respiration and ATP synthesis. Remaining mitochondrial

membrane proteins and all matrix proteins (in total, >1000 proteins) are encoded by the nuclear genome (Mishra and Chan, 2014).

One of the best studied experimental and physiological conditions that result in increased mitochondrial mass is exercise. Muscle activity and exercise strongly promote mitochondrial biogenesis pathways in order to increase the oxidative capacity of muscle fibers (Jornayvaz and Shulman, 2010). Of importance, exercise strongly activates AMPK (Jornayvaz and Shulman, 2010), thus, suggesting AMPK as a signaling link in the exercise-induced mitochondrial biogenesis. In addition, upregulation of mitochondrial biogenesis in skeletal muscle was observed when a constitutively active AMPKγ3 subunit was overexpressed in mice (Garcia-Roves et al., 2008). Another study observed that induction of mitochondria biogenesis in muscles has been hindered in mice expressing a dominant-negative mutant of AMPK (loss-of-function experiments) under energy stress (Zong et al., 2002). Similarly, defective mitochondrial function and biogenesis was reported in mice having muscle-specific knockout of AMPKα-subunits (Lantier et al., 2014). AMPK has also been shown to control mitochondrial content in various cell and tissue types, including adipocytes (Mottillo et al., 2016), myoblasts (Koka et al., 2014, Gerhart-Hines et al., 2011, Park et al., 2012), macrophages (Galic et al., 2011) and hepatocytes (Hasenour et al., 2014). Taken together, these findings reiterate the function of AMPK as a key regulator of mitochondrial biogenesis.

AMPK regulates mitochondrial biogenesis with the help of several downstream effectors. Numerous nuclear-encoded mitochondrial proteins are under the transcriptional control of the family of transcription factors called peroxisome proliferator-activated receptor γ coactivator 1 (PGC1) (Puigserver et al., 1998, Wu et al., 1999) (**Fig. B**). These transcription factors establish their function with other transcription factors, like peroxisome proliferator-activated receptors (PPARs) and estrogen-related receptors (ERRs) (Eichner and Giguère, 2011). Overexpression of PGC1α in muscle is sufficient to promote mitochondrial biogenesis, demonstrating PGC1α as a master regulator of mitochondrial biogenesis (Lin et al., 2002). It has been reported that expression of PGC1α increased in response to the overexpression of the constitutively active form AMPKγ3 subunit (Garcia-Roves et al., 2008) and PGC1α is required to mediate the transcriptional upregulation of most of the genes involved in oxidative phosphorylation upon AMPK activation (Jäger et al., 2007). PGC1α gets activated when phosphorylated on Ser538 and Thr177 by AMPK (Jäger et al., 2007). In addition to

phosphorylation, PGC1α can be activated via deacetylation by NAD^+-dependent deacetylase sirtuin 1 (Park et al., 2012, Purushotham et al., 2009). AMPK supports NAD^+ synthesis, and therefore Sirt1 activity, by promoting the transcription of nicotinamide phosphoribosyl transferase (Nampt), a rate-limiting enzyme in *de novo* NAD^+ synthesis (Brandauer et al., 2013).

2.3.2 Role of AMPK in mitochondrial fission

Mitochondria build a dynamic, highly mobile and interconnected network by constantly undergoing fusion and fission events (Mishra and Chan, 2016). Changes in mitochondrial morphology have been linked to the changes in mitochondrial function and fate. Particularly, it has been suggested that mitochondrial elongation or fusion improves ATP production under stress conditions (Tondera et al., 2009). Also, such elongated form helps mitochondria to exchange biomaterials and lipids molecules (Rambold et al., 2015) during nutrient starvation and to evade mitophagy (Gomes et al., 2011, Rambold et al., 2011). Mitochondrial fragmentation is also an important event in mitochondrial dynamics and is required for the recycling of damaged mitochondria via mitophagy (Eiyama et al., 2013). Interestingly, insults that induce mitochondrial fragmentation also potentially activate AMPK. For example, inhibitors of the respiratory chain complex I (rotenone) and complex III (antimycin A) readily induced AMPK activation and mitochondrial fragmentation. By performing several overexpression of dominant-negative AMPKα-subunit and AMPK knockout experiments, it has been shown that AMPK is important for mitochondrial fragmentation (Toyama et al., 2016). AMPK promotes mitochondrial fragmentation by phosphorylating MFF at Ser155 and Ser173 residues (Toyama et al., 2016, Ducommun et al., 2015) (**Fig. B**). MFF is localized on the mitochondrial outer membrane and acts as a key receptor of dynamin-like protein (DRP1), which initiates mitochondrial fission by constricting mitochondria at the site of recruitment (Otera et al., 2010). MFF needs to be phosphorylated at Ser155 and Ser173 in order to recruit DRP1 and this phosphorylation of MFF requires AMPK activation (Toyama et al., 2016). Thus, the shape of mitochondrial network, i.e., mitochondrial fission, is regulated by AMPK via MFF–DRP1 axis.

2.3.3 Regulation of autophagy and mitophagy by AMPK

Autophagy is a process to recycle cellular components like organelles, macromolecules and pathogens inside a specialized machinery called autophagolysosomes (Bento et al., 2016). The

autophagy process is highly complex and controlled by several multiprotein complexes that are involved right from the initiation to the completion of the autophagy. AMPK has been reported to regulate several steps of this process. First, the AMPK-mediated inhibition of mTOR activity liberates the autophagy initiating Unc-51 like kinase-1 (ULK1) from the mTOR-mediated inhibitory phosphorylation (Kim et al., 2011b). Of note, ULK1 undergoes phosphorylation by AMPK on at least four sites (Ser467, Ser555, Thr574 and Ser637) and, thereby, initiation of autophagy (Egan et al., 2011, Kim et al., 2011a) (**Fig. B**). Of note, in ULK1-knockout cells neither metabolic stress nor starvation failed to induce autophagy (Egan et al., 2011). The study also reported the increased accumulation of defective mitochondria due to lack of mitophagy in cells expressing mutated form of ULK1 that cannot be phosphorylated by AMPK, showing the necessity of AMPK-mediated ULK1 phosphorylation in autophagy (Egan et al., 2011).

While, autophagy generally denotes recycling and degradation of any unwanted cellular constituents, mitophagy refers to the autophagy process that specifically involves degradation of mitochondria. Dysregulation of mitophagy has been associated with neurodegenerative diseases (Cha et al., 2015, Nah et al., 2015), improper heart development (Gong et al., 2015) and aging (Sun et al., 2015). Several studies have also shown the importance of AMPK and ULK1 in mitophagy (Itakura et al., 2012, Zhu et al., 2013, Honda et al., 2014, Wu et al., 2014). Particularly, the importance of mitophagy mediated by AMPK was reported in several contexts like during fasting and aging in muscles (Bujak et al., 2015), for browning of adipose tissue (Mottillo et al., 2016), for prevention of hepatosteatosis in hepatocytes (Inokuchi-Shimizu et al., 2014) and for promoting antimicrobial defense in macrophages (Yang et al., 2014). For an efficient mitophagy, mitochondrial fission is an essential initial step (Shirihai et al., 2015, Twig et al., 2008, Pryde et al., 2016), and studies report AMPK to be one of the important players in promoting mitochondrial fission (detailed in Section 1.3.2). Using fibroblasts isolated from mitochondrial encephalomyopathy (MELAS) patients, Garrido-Maraver et al. (2015) have reported that the deleterious effect of increased ROS formation due to impaired mitochondrial respiratory chain function and protein synthesis was improved by activating AMPK that promoted mitophagic degradation of dysfunctional mitochondria and mitochondrial biogenesis. Taken together, AMPK plays a

prominent role in removing damaged mitochondria by mitophagy and restoring mitochondrial mass with new mitochondria by promoting mitochondrial biogenesis.

Though stimulated AMPK activity has been extensively studied under several physiological and pathological situations, conditions and pathways that control basal AMPK activity have been poorly understood. Emerging data shed light on the significance of 3'–5'-cyclic adenosine monophosphate (cAMP) signaling in modulating basal AMPK activity (Djouder et al., 2010, Gerhart-Hines et al., 2011, Park et al., 2012, Koka et al., 2014). Hence, it is important to have a proper understanding about the sources of cAMP production and its role in regulation of AMPK activity.

2.4 cAMP signaling

Though cAMP was identified more than half a century ago to be an important cellular second messenger, several intracellular signaling domains and pathways of cAMP are yet to be elucidated. cAMP signaling is evolutionarily conserved and can be found in all species from microorganisms to mammals. cAMP signaling has been reported to mediate several cellular functions, such as cell growth (Stork and Schmitt, 2002) and death (Appukuttan et al., 2012a), cell motility (Zimmerman et al., 2015), transcription regulation (Chowanadisai et al., 2010), mitochondrial homeostasis (Di Benedetto et al., 2013, Acin-Perez et al., 2009, Valsecchi et al., 2013, De Rasmo et al., 2015) and metabolism (Catterall, 2015).

Mammalian cell possesses two main sources of cAMP: classical transmembrane (tmAC) and most recently identified soluble adenylyl cyclase (sAC) (**Fig. C**). sAC differs from the membrane-bound tmAC in a unique way, that is, sAC is not membrane bound and is the major source of cAMP in different intracellular compartments (Zippin et al., 2003). In addition, there are nine forms of tmAC encoded by nine different genes, whereas sAC has different splice variants encoded by a single gene (Chen et al., 2014).

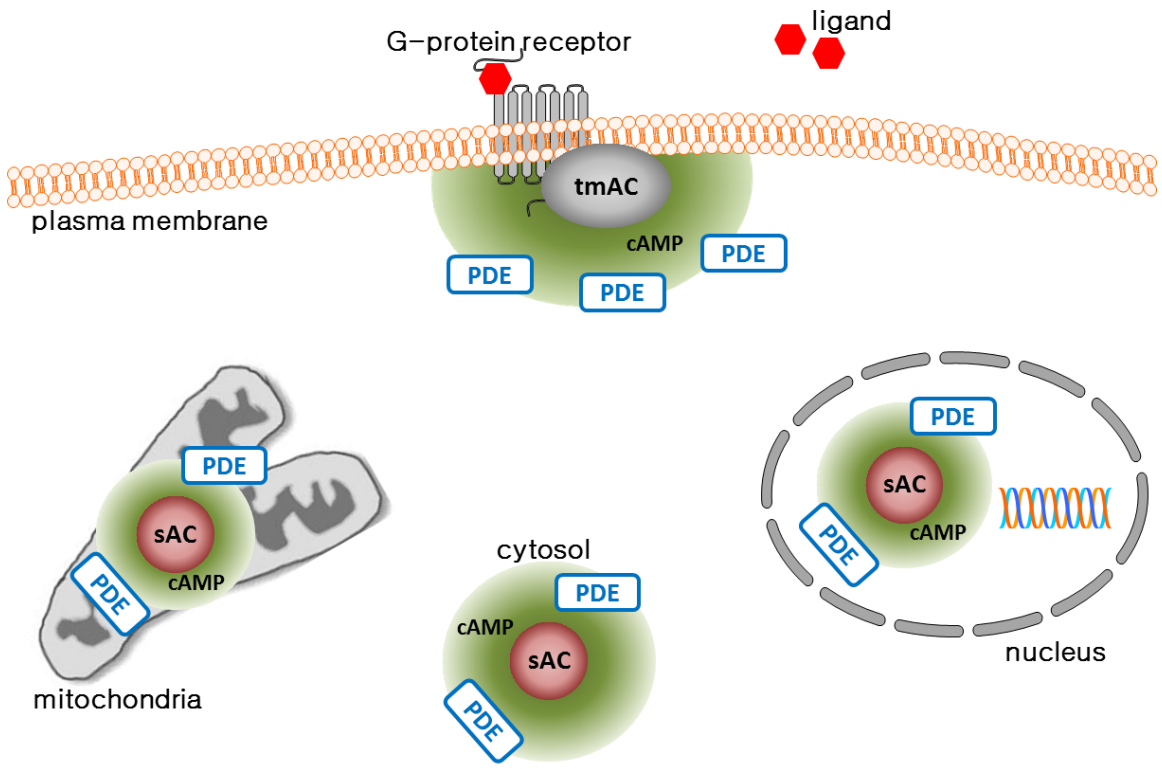

Fig. C. Sources of cAMP.
Transmembrane adenylyl cyclase (tmAC) produces cAMP upon ligand-induced activation of G-protein-coupled receptors. Phosphodiesterases (PDE) actively hydrolyze cAMP and hence prevent cAMP diffusion throughout the cells. sAC found in different cellular compartments acts as a major source of intracellular cAMP.

All nine tmACs are coupled to G-protein receptors and hence hormones and neurotransmitters (ligands) can either stimulate or inhibit the activity of tmACs based on whether they are coupled to stimulatory (G_s) or inhibitory (G_i) G-protein receptors (Federman et al., 1992) (**Fig. C**). In addition, kinases such as PKA, PKC and CAMKK2 can phosphorylate and regulate the activity of tmACs (Gancedo, 2013). Upon stimulation by extracellular signals, tmAC produces huge amount of cAMP from ATP that activates cAMP effector proteins such as PKA, EPAC, cyclic nucleotide-gated ion channels (Kaupp and Seifert, 2002) and Popeye domain containing proteins (Froese et al., 2012). Due to the hydrophilic nature of cAMP, it can rapidly diffuse through the cytosol leading to an uncontrolled signaling cascade. Though cytosolic viscosity slows the diffusion of cAMP (Feinstein et al., 2012), the uncontrolled propagation of cAMP signaling is mainly suppressed by the presence of PDEs that hydrolyze cAMP to AMP (Agarwal et al., 2016) (**Fig. C**). Thus, tmAC-dependent cAMP pool is restricted

to sub-plasmalemmal compartment. Nevertheless, under certain conditions, like stimulation with thyroid hormones or insulinotropic receptor, tmAC could produce cAMP intracellularly due to internalization (Ismail et al., 2016, Calebiro et al., 2009).

sAC is a major source of intracellular cAMP. First identified in the testis, sAC expression has been confirmed in almost all tissue and cell types investigated so far (Chen et al., 2000, Buck et al., 1999). sAC, in a similar fashion to tmAC, has a tandem arrangement of two catalytic domains C1 and C2 forming a pseudo-heterodimer. In contrast to tmAC, presence of sAC is not restricted to plasma membrane, but has been reported in several subcellular compartments such as cytosol, nucleus, mitochondria, centriole and lysosomes (Zippin et al., 2003, Rahman et al., 2016) (**Fig. C**). sAC, insensitive to G-protein stimulations, is constitutively active and can be stimulated by divalent cations like calcium and magnesium (Litvin et al., 2003, Zippin et al., 2013). Of note, sAC is a unique evolutionary conserved bicarbonate sensor with enzymatic activity (Steegborn, 2014).

2.5 Biochemistry of sAC

2.5.1 Isoforms of sAC

ADCY10 gene comprises 33 exons that encode the full-length form of sAC (sACfl) (Farrell et al., 2008). It has been reported that sAC mRNA undergoes extensive alternative splicing giving rise to smaller splice variants in addition to the 187 kDa sACfl. The predominant splice variant is a 50 kDa truncated isoform of sAC (sACt) that comprises only N-terminal part of the full length peptide (Buck et al., 1999), including catalytic domains C1 and C2 connected by a linker peptide (**Fig. D**). C2 domain plays an important role in specific binding of ATP as a substrate, which can then be converted to cAMP by C1 domain. *In vitro* studies with purified sAC lacking complete C1 domain showed no cyclase activity (Chen et al., 2014) The C-terminal part of the sACfl has an autoinhibitory domain that functions together with the ATP-binding nucleoside triphosphatase domain to inhibit sACfl activity (Leipe et al., 2004) (**Fig. D**). In addition, presence of a heme-binding domain that binds gaseous activators, such as nitric oxide and carbon monoxide, have been reported in the C-terminal region of sACfl, but not sACt (Middelhaufe et al., 2012). sACt shows higher level of activity in comparison to sACfl, where the activity of the sACfl is suppressed by the autoinhibitory domain at the C-terminal region

(Chaloupka et al., 2006). In addition to sACt, several other splicing variants were identified in human somatic tissue. These somatic sAC isoforms predominantly possess only C2 domain (**Fig. D**) and require homo- or hetero-dimerization with a yet unknown protein for the C1 activity (Kamenetsky et al., 2006, Geng et al., 2005, Chen et al., 2014).

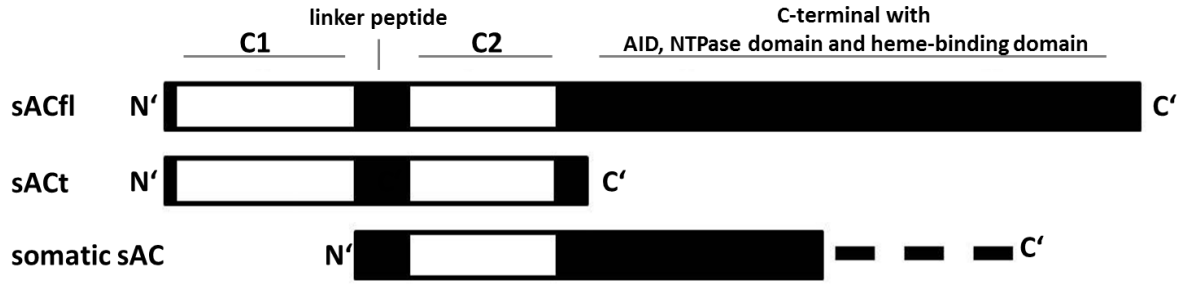

Fig. D. Isoforms of sAC.
Schematic overview of different isoforms of sAC, including full length (sACfl), truncated (sACt) and somatic (somatic sAC). Catalytic domains C1 and C2 are located at the N-terminal (N') region and are connected by a linker peptide. C-terminal (C') region of sACfl includes an autoinhibitory domain (AID), a nucleoside triphosphatase (NTPase) domain and a heme-binding domain.

2.5.2 Regulation of sAC

A unique property of sAC is that it can get activated by bicarbonate, making sAC the only protein with enzymatic activity that could be regulated by changes in bicarbonate concentration. Bicarbonate directly binds and activates sAC in a pH-independent manner. By performing *in vitro* studies with purified sAC, it has been shown that bicarbonate-induced activation of sAC remained constant irrespective of the changes in pH (7–8.5) and this change in pH itself had no effect on sAC activity under bicarbonate-free conditions (Chen et al., 2000). The EC_{50} for the bicarbonate stimulation of mammalian sAC is in the range 10–25 mmol/l, appropriate for sensing the physiological bicarbonate levels of 2–25 mmol/l (Chen et al., 2000). It has been shown that bicarbonate binds between Lys95 and Arg176 residues and increases the V_{max} of sAC (Kleinboelting et al., 2014, Litvin et al., 2003).

For sAC to be active, binding of two divalent cations to the catalytic domains of the enzyme is required, which helps to bind ATP and to convert the bound ATP to cAMP. Though *in vitro*

studies using purified proteins demonstrate that sAC is most active in the presence of manganese, it is unknown whether the intracellular concentration of manganese could stimulate sAC activity (Chen et al., 2000). In addition to divalent cations, sAC activity can also be influenced by the changes in cellular ATP concentrations, where reduced ATP concentration results to the declined activity of sAC (Zippin et al., 2013).

As mentioned earlier, sAC is insensitive to the G-protein receptor signaling. Though there are several residues that can be potentially phosphorylated, such as Tyr268 in the linker region between C1 and C2 domains and the residues Thr1132 and Tyr1134 in the C-terminal region, the relevance of these phosphorylation sites in regulating sAC activity or mobilization remains unknown (Hornbeck et al., 2012).

2.5.3 Intracellular localization of sAC

sAC has been identified in different cellular compartments, such as cytosol, nucleus, mitochondria, centrioles and lysosomes (Zippin et al., 2003, Corredor et al., 2012, Monterisi and Zaccolo, 2017, Zippin et al., 2004, Rahman et al., 2016). sAC-generated cAMP signaling is involved in the regulation of multiple cellular functions. The signaling gets even more precise as sAC along with cAMP effector proteins (PKA, EPAC) and cAMP degrading enzymes (PDEs) forms cAMP micro-domains to strictly regulate cAMP signaling in various cellular compartments (Agarwal et al., 2016, Carnegie et al., 2009). In addition, a dynamic nature of sAC, i.e, translocation between subcellular compartments, was reported under certain stimuli. Particularly, sAC translocates from cytosol to mitochondrial under various stresses inducing mitochondrial pathway of apoptotic (Kumar et al., 2009, Appukuttan et al., 2012a, Appukuttan et al., 2013) Furthermore, sAC shuttles between the nucleus and the cytosol and is predominantly localized in the nucleus during active proliferation state of keratinocytes, (Zippin et al., 2010)

2.5.4 Pharmacological regulation of sAC

As sAC is being involved in several pathologies and physiological processes (Zippin et al., 2010, Flacke et al., 2013a, Appukuttan et al., 2013, Appukuttan et al., 2012a), development of potent small molecules to either inhibit or activate sAC activity becomes inevitable. Although potential sAC activators have yet to be developed, few inhibitors have already been discovered.

2-hydroxy estradiol (2-HE) and 4-hydroxy estradiol (4-HE) are derivatives of estradiol and have been shown to inhibit purified mammalian sAC with an IC_{50} of 2–8 µmol/l. Unfortunately, 2-HE and 4-HE can also inhibit some types of tmAC with comparable potency (Steegborn et al., 2005b). It was postulated that 2-HE and 4-HE bind to the pocket adjacent to the enzyme's active site, which is common in both sAC and tmAC (Steegborn et al., 2005a). Another compound KH7 ((E)-2-(1H-Benzo[d]imidazol-2-ylthio)-N'-(5-bromo-2-hydroxybenzylidene) propanehydrazide) has been identified as a specific sAC inhibitor with an IC_{50} of 3 µmol/l (Hess et al., 2005). KH7 shows good membrane permeability and has no significant effect on the activation of tmACs up to 100 µmol/l (Bitterman et al., 2013). KH7 has been widely used as a pharmacological tool in most of studies (Flacke et al., 2013a, Kumar et al., 2014, Bitterman et al., 2013). However, KH7 has been shown to induce some side effects, such as mitochondrial and cellular toxicity (Ramos-Espiritu et al., 2016). Most recently, LRE1 has been identified as an improved and highly specific inhibitor of sAC. LRE1 competes for the bicarbonate binding site to inhibit sAC activity and shows no side effects like KH7 (Ramos-Espiritu et al., 2016).

2.6 Functional role of sAC

2.6.1 Role of sAC in cell growth

Initial studies supporting the role of sAC in cell growth demonstrated that a bicarbonate-sensitive nuclear cAMP signaling via PKA was responsible for the activation of nuclear transcription factor CREB (Zippin et al., 2004, Zippin et al., 2010). It has also been shown that sAC translocates from cytosol to the nucleus when epithelial cells are induced to re-enter cell cycle. In addition, predominant nuclear localization of sAC has been observed in the keratinocytes from hyperproliferative skin diseases (Zippin et al., 2010). Similarly, sAC overexpression was observed in human prostate carcinoma cell lines LNCaP and PC3 in comparison to normal human prostate epithelial cell line PNT2 (Flacke et al., 2013a). Direct evidence for the involvement of sAC in cell proliferation was shown when both sAC knockdown and pharmacological inhibition significantly reduced the proliferation of LNCaP and PC3 cells by causing cell cycle arrest in G2 phase (Flacke et al., 2013a). Mechanistic analyses revealed that EPAC controls proliferation in several cell models, including prostate carcinoma cells (Misra and Pizzo, 2009, Misra and Pizzo, 2013). It has been shown that

treatment with specific EPAC activator induced cell proliferation in prostate carcinoma cells (Misra and Pizzo, 2009). EPAC activity has been shown to be strongly dependent on sAC activity in prostate carcinoma cells, where sAC inhibition significantly reduced the amount of GTP-bound Rap1 (Flacke et al., 2013a). Hence, sAC seems to control cell proliferation in cancer cells via activation of EPAC/Rap1 signaling.

In addition to cell proliferation, sAC has also been reported to affect non-proliferative cell growth. sAC overexpression has been reported to promote axonal growth in embryonic neurons (Wu et al., 2006). Correspondingly, stimulating sAC with bicarbonate increased axon growth in cultured retinal ganglion cells, whereas inhibiting sAC activity attenuated the growth of axon (Corredor et al., 2012). In addition, it has been shown that sAC plays an important role in chronic β1-adrenergic stimulation induced hypertrophic growth of adult rat ventricular cardiomyocytes, where both sAC knockdown and pharmacological inhibition abolished the hypertrophic response to β1-adrenergic stimulation (Schirmer et al., 2012). Together, available reports suggest the importance of sAC in both proliferative and non-proliferative cell growth.

2.6.2 Role of sAC in apoptosis

In rat coronary endothelial cells (Kumar et al., 2009) and rat cardiomyocytes (Appukuttan et al., 2012a), stress stimuli like ischemia and acidosis lead to the mitochondria-dependent apoptosis via sAC–PKA axis. Similarly, sAC–PKA axis is involved in the oxysterol-induced apoptotic cell death (Appukuttan et al., 2013). Independent of stress stimulus, sAC translocates from the cytosol to the mitochondrial outer membrane (Kumar et al., 2009, Appukuttan et al., 2012a, Appukuttan et al., 2013). This translocation is accompanied by mitochondrial binding of the pro-apoptotic protein Bax. Inhibition of sAC or PKA prevented the phosphorylation of Bax at Thr167 and, thereby, its translocation to mitochondria (Appukuttan et al., 2013, Appukuttan et al., 2012a). In a different stress model in smooth muscle cells, treatment with H_2O_2 demonstrated similar sAC translocation and PKA activation, but Bax translocation was not observed. Instead, PKA-dependent activation of PP1 promoted p38 inactivation by dephosphorylation. This loss of p38 activity decreased inactivation of pro-apoptotic protein Bad that promotes mitochondrial pathway of apoptosis (Kumar et al., 2014). All these data suggest the role of sAC in the mitochondrial pathway of apoptosis.

2.6.3 sAC in cell motility

sAC is the only source of cAMP that promotes motility in sperm. Among the initial events in the capacitation, calcium and bicarbonate activate sAC to produce cAMP and, thereby, induce forward sperm motility (Tresguerres et al., 2011). Furthermore, sAC-knockout mice shows a male infertility phenotype (Esposito et al., 2004). Recent reports have shown that sAC is also involved in the regulation of trans-endothelial migration of leukocytes. In leukocytes, CD99 and sAC are co-localized in a signaling complex with ezrin and PKA. Stimulation of CD99 promotes sAC–PKA pathway that activates trans-endothelial migration, facilitating the passage of leukocytes across the endothelium (Watson et al., 2015).

2.6.4 sAC in regulation of cellular pH

sAC plays an important role in the regulation of pH homeostasis (Chang and Oude-Elferink, 2014). In epididymis and in the collecting duct of the kidney, sAC–PKA signaling is involved in the translocation of the vacuolar proton ATPase (V-ATPase) to the acid-secreting surface to maintain pH homeostasis in those organs (Brown et al., 2012, Pastor-Soler et al., 2008). Recently it was shown that sAC promotes lysosomal acidification by modulating V-ATPase function. Lysosomes in sAC-knockout cells were shown to have higher pH with hindered function, which could be reversed by treatment with membrane-permeable cAMP analogue (Rahman et al., 2016).

2.6.5 Role of sAC in mitochondrial function

It has been proposed that mitochondria-localized sAC is the only source of cAMP in the matrix (Acin-Perez et al., 2009). Increasing evidences suggest that cAMP–PKA pathway in the mitochondrial matrix directly regulates TCA cycle and respiration and thereby affects metabolism in mammalian cells (Acin-Perez et al., 2009, Schwoch et al., 1990, Papa et al., 1999, Chen et al., 2004, Zhao et al., 2011, Grimsrud et al., 2012, Prabu et al., 2006, Fang et al., 2007, Rasmo et al., 2010). Phosphoproteomic analyses have revealed that several enzymes involved in the TCA cycle and OXPHOS complexes were phosphorylated at the PKA consensus sites (Zhao et al., 2011, Grimsrud et al., 2012). By stimulating sAC–cAMP axis in the matrix, PKA has been shown to phosphorylate COXIV resulting in enhanced COX activity that supports OXPHOS (Acin-Perez et al., 2009). It has also been shown that PKA phosphorylates Complex I subunits (NDUFS4, ESSS and MWFE) of electron transport chain (ETC), which increases Complex I activity and stability, and hence enhances respiration (Chen et al., 2004, De Rasmo

et al., 2015, Scacco et al., 2000, Technikova-Dobrova et al., 2001, De Rasmo et al., 2012). In addition, stability and activity of Complex V in isolated rat mitochondria required sAC–cAMP signaling (De Rasmo et al., 2016). Recently, in primary rat cardiomyocytes, stimulation of sAC with bicarbonate has been shown to produce cAMP, which in turn induced oxygen consumption and ATP production and also increased the mitochondrial membrane potential (Wang et al., 2016). Similarly, sAC-mediated changes in ATP production has been demonstrated in liver cells (Acin-Perez et al., 2009) and in the brain (Hebert-Chatelain et al., 2016). Of note, a similar mitochondria-localized cAMP pathway has been discovered in yeast, where sAC–cAMP–PKA axis is involved in the regulation of cytochrome *c* oxidase 5a activity (Hess et al., 2014). This study demonstrates, therefore, an evolutionary conserved role of the intra-mitochondrial sAC in modulating mitochondrial function.

As mentioned earlier, nuclear-localized sAC–PKA pathway lead to phosphorylation and activation of transcription factor CREB in the nucleus (Zippin et al., 2010). Since CREB plays a role in mitochondrial biogenesis (Ryu et al., 2005, Lee et al., 2005), it is tempting to speculate that activation of sAC-PKA-CREB signaling, i.e., under exercise due to elevation of cytosolic calcium and bicarbonate, the two main sAC activators, may promote mitochondrial biogenesis. In a similar manner, CREB found in mitochondrial matrix is also phosphorylated/regulated by matrix-localized sAC–cAMP–PKA signaling, where CREB bind to the cAMP response elements on the mitochondria DNA and promotes transcription (Lee et al., 2005, Ryu et al., 2005, Cammarota et al., 1999, De Rasmo et al., 2009). Thus, in addition to direct modulation of OXPHOS activity via PKA-dependent phosphorylation, intra- or extra-mitochondrial cAMP signaling also affects mitochondrial biogenesis.

3 Working hypothesis

Among several signaling pathways contributing to the AMPK activity, cAMP signaling emerges to play an important role. Indeed, cAMP signaling promotes AMPK activity in endothelial cells (Chen et al., 2013), muscle cells (Park et al., 2012) and adipocytes (Omar et al., 2009). These effects have been attributed, at least partly, to the activation of EPAC. In contrast, inhibitory effect of PKA-dependent phosphorylation of the α-subunit at S173, S485/491 or S497 has been demonstrated (Ferretti et al., 2016, Hurley et al., 2006a). In addition, it has been suggested that PKA stimulate AMPK via phosphorylation and activation of the upstream kinase LKB1 (Kimball et al., 2004). The contradictory effect of these studies may be due to differences in models or treatment patterns. Furthermore, the approach used in some studies failed in specificity, i.e., total cAMP was elevated by the inhibition of PDEs (Koka et al., 2014, Park et al., 2012) without considering the sources of cAMP. Of note, the role of the cAMP signaling has been exclusively attributed to the single cAMP source, i.e., to the activity of the G-protein-coupled transmembrane adenylyl cyclase (tmAC). The subsequent diffusion of cAMP from plasmalemma throughout the cytosol is prevented by phosphodiesterases that degrade cAMP. Apart from tmAC, a second source of cAMP, the type 10 soluble adenylyl cyclase (sAC), has been demonstrated for mammalian cells (Zippin et al., 2003). sAC has been shown to localize in various intracellular compartments. Particularly, cytosolic and nuclear sAC localization allows building the cAMP pool proximal to the main AMPK localization compartments. Whether sAC-dependent cAMP pool may modulate AMPK activity and, thereby, mitochondrial homeostasis remains unknown and was the aim of this present study.

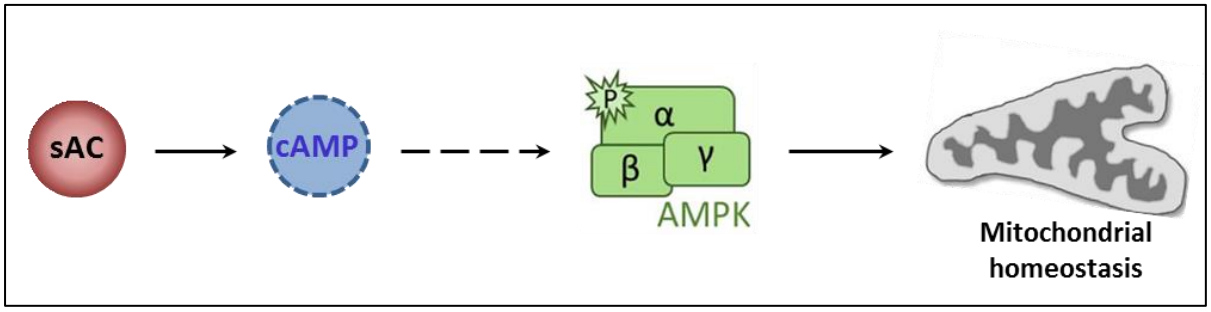

Fig. E. Schematic representation of the hypothesis.
The broken line indicates the hypothetic signaling link between sAC-dependent cAMP pool and AMPK.

To check the hypothesis, following aims were set:

1. To investigate the effect of sAC knockdown on AMPK activity in H9C2 cells, adult rat cardiomyocytes and coronary endothelial cells.

2. To explore the downstream cAMP targets (PKA or EPAC) that may mediate the effect of sAC knockdown on AMPK activity in H9C2 cells.

3. To examine the effect of sAC knockdown in H9C2 cells on mitochondrial homeostasis.

4. To test the effect of sAC overexpression on AMPK activity and cellular energy balance in H9C2 cells.

5. To study the effect of bicarbonate-mediated sAC stimulation on AMPK activity and cellular energy balance in H9C2 cells.

6. To examine alteration of sAC–AMPK axis in cardiac pathology induced by myocardial infarction in male and female mice.

4 Materials

4.1 Devices

Analytical balance	Kern 440-47N (Germany)
Aspiration system	Integra VACUSAFE (Germany)
Binocular microscope	Axiovert 25, Carl Zeiss (Germany)
Centrifuge	Heraeus Megafuge 1.0 R, Kendro (Germany)
CO_2-Incubator	Thermo Scientific, HERAcell 150i (Germany)
Electrophoretic transfer	Trans-Blot® Cell, Bio-Rad (Germany)
Electroporator	Amaxa™ Nucleofector™ II, Lonza (Germany)
ELISA-spectrophotometer	Benchmark Plus, Bio-Rad (Germany)
FACS machine	MACSQuant X, Miltenyi Biotech(Germany)
Fluorescence microscope	Zeiss (Germany)
Freezer 20°C	SANYO (Germany)
Fridge 4°C	Leibherr Comfort (Germany)
Imaging system	Bio-Rad ChemiDoc (Germany)
Luminometer	1420 Multilabel Counter Victor ^{3TM}, Perkin Elmer (USA)
pH-meter	Mettler Toledo (Germany)
Pipette controller	Accu-jet Pro® (Germany)
Rocking platform	WT16, Biometra (Germany)
StepOnePlus real-time PCR system	Applied Bioscience (Germany)
Sterile hood	Thermo Holten safe (Germany)
Table centrifuge	Eppendorf 5417 (Germany)
Thermomixer comfort	Eppendorf (Germany)
UV trans-illuminator	ThermoFischer Scientific (Germany)
Vortex	Genie 2, Scientific industry (USA)

4.2 Consumables

96-well plate	White or black plates, Corning™ (Germany)
Cell culture dishes	40, 60, 96 mm dishes Sarstedt (Germany)
Cell culture flasks	T75, T175 flasks Sarstedt (Germany)
Cell culture plates	6, 12, 24 well plates Sarstedt (Germany)
Cover slips	24 mm Marienfeld-Superior (USA)
Falcon tubes	15, 50 ml tubes Sarstedt (Germany)
Nitrocellulose blotting membrane	Healthcare Life Science (Germany)
Pipet tips	10, 100, 1000 µl Sarstedt (Germany)
Pipets	5, 10, 25, 50 ml pipets Corning (Germany)
Safe-lock tubes	0.5, 1.5, 2 ml tubes Eppendorf (Germany)

4.3 Chemicals

All the chemicals were from Invitrogen (USA), Merck (Germany), Sigma-Aldrich Chemie (Germany), Carl Roth (Germany), Serva (Germany), Gibco Life Technologies (Germany) and Promega (Germany).

4.4 Buffers for live imaging

4.4.1 Standard buffer

Salts	**mmol/l**
KCL	5
$Na_3PO_4 \cdot 12H_2O$	1
$MgSO_4$	1
HEPES	20
NaCl	110

4.4.2 Bicarbonate buffer

Salts	**mmol/l**
KCL	5
$Na_3PO_4 \cdot 12H_2O$	1
$MgSO_4$	1
HEPES	20
NaCl	68
$NaHCO_3$	42

Standard buffer was adjusted to have pH around 7.4 at 35°C and bicarbonate buffer gassed with 10% CO_2 before adjusting the pH to 7.4 at 35°C. Immediately before the experiment all buffers were supplemented with glucose (8 mmol/l), sodium pyruvate (1 mmol/l) and calcium chloride (2 mmol/l).

4.5 Plasmids used

s.No.	***Plasmids***	***Backbone/promoter***	***Source***
1	GFP	pTurbo/CMV	Acin-Perez et al. (2009)
2	sAC	pTurbo/CMV	Acin-Perez et al. (2009)
3	Scramble shRNA	pENTR4/U6	Watson et al. (2015)
4	sAC shRNA	pENTR4/U6	Watson et al. (2015)
5	Cytosolic AMPK FRET sensor	pcDNA3/CMV	Miyamoto et al. (2015)
6	Nuclear AMPK FRET sensor	pcDNA3/CMV	Miyamoto et al. (2015)
7	Cytosolic cAMP FRET sensor	pcDNA3/CMV	Lefkimmiatis et al. (2013)

4.6 Kits used

ATPLite Luminescence Assay System	PerkinElmer (USA)
Cytotoxicity Detection Kit	Roth (Germany)
Direct cAMP ELISA Kit	Enzo (Germany)
NucleoBond® Plasmid Maxi Kit	Machery Nagel (Germany)
Pierce 660 nm Protein Assay	Thermo Scientific (USA)
Quick-gDNA Miniprep Kit	Zymo Research (Germany)

4.7 Antibodies used

Primary antibody (dilution)	**Secondary antibody (dilution)**	**Band (kDa)**	**Company (cat. number)**
ACC (1:1000)	DaR (1:5000)	280	Cell Signalling (3676S)
AMPK (1:1000)	DaR (1:5000)	68	Cell Signalling (2332S)
GAPDH (1:10,000)	DaM (1:10,000)	36	MD Millipore (MAB374)
pACC (1:1000)	DaR (1:5000)	280	Cell Signalling (11818S)
pAMPK (1:1000)	DaR (1:5000)	68	Cell Signalling (2535S)
PGC1α (1:1000)	DaR (1:5000)	105	Abcam (ab54481)
R21 (sAC, 1:1000)	DaM (1:5000)	50	CEP Biotech (R21.002)
Sirt3 (1:1000)	DaR (1:5000)	28	Cell Signalling (5490S)
SOD2 (1:1000)	DaR (1:5000)	25	MD Millipore (6-984)
β-Actin (1:5000)	DaR (1:10,000)	43	Santa Cruz (sc-1616-R)
TFAM (1:1000)	DaR (1:5000)	24	Acris (AP26439SU-N)
α-Tubulin (1:10,000)	DaM (1:20,000)	ca. 50	Sigma (T9026)
von Willebrand Factor (1:500)	FITC-labelled DaR (1:1000)	270	Sigma (F3520)

5 Methods

5.1 Cell culture and treatments

Cardiac rat embryonic myoblasts (H9C2, ATCC CRL-1446) was purchased from American Type Culture Collection. Cells were expanded and frozen in aliquots within 4 weeks of purchase. For the experiments, cells were thawed and cultured for no more than 4 further passages and were not allowed to grow more than 60% confluence. Cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Sigma, #D2902), supplemented with 10% fetal bovine serum (FBS), 21 mmol/l sodium bicarbonate and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a 5% CO_2 incubator at 37°C. For experiments with different bicarbonate stimulations, cells were cultured in DMEM containing either no bicarbonate (0% CO_2) or with 21 mmol/l (5% CO_2), or 42 mmol/l bicarbonate (10% CO_2) and supplemented with 15 mmol/l HEPES and 3% FBS. NaCl was used to adjust osmolarity and pH was set to 7.4 with sodium hydroxide and hydrochloric acid.

5.2 Isolation and culturing of cardiomyocytes and coronary endothelial cells

Ventricular cardiomyocytes were isolated from male Wistar rats by perfusion of hearts with collagenase type II (300 U/ml) and cultured, as previously described (Appukuttan et al., 2012a). For this purpose, adult male rats were euthanized using deep isoflurane (5%) anesthesia, hearts were rapidly excised, washed with ice-cold 0.9% NaCl and connected to the perfusion system. Anesthesia depth was monitored by limb withdrawal using toe pinching. To separate cardiomyocytes from non-cardiac cells, cardiomyocytes were sedimented by low force and short centrifugation (5 g, 1 min, four times) and finally without centrifugation in medium containing 4% BSA. To prevent growth of non-myocytes, medium was supplemented with 10 µmol/l cytosine-β-D-arabinofuranoside. After 1 h of plating, cells were washed with culture medium (2% fetal calf serum) to remove nonattached cells. A high purity of cardiomyocyte culture (>93%) was confirmed by light microscopy. Experiments were performed on the third day after preparation.

Coronary endothelial cells were obtained as a by-product of cardiomyocytes isolation as described previously (Kumar et al., 2009). Myocytes-free cell suspension was plated for 1 h in DMEM, supplemented with 20% FBS for the coronary endothelial cells to get attached to the plate. The purity of the cell culture (>98%) was confirmed by immunochemical staining with

antibodies against von Willebrand factor. Experiments were performed with monolayers reaching 90% confluence.

5.3 sAC overexpression and knockdown

For sAC overexpression experiments, cells were transfected with either GFP or sAC encoding plasmids. In short, 400000 cells were resuspended in 100 µl of electroporation buffer (5 mmol/l KCl, 15 mmol/l $MgCl_2$, 50 mmol/l mannitol, 86.4 mmol/l Na_2HPO_4, 33.6 mmol/l NaH_2PO_4, pH=7.2) containing 8 µg plasmids and were electroporated using T-020 program for H9C2 cells and S-005 for coronary endothelial cells in Amaxa Nucleofector II Device. After electroporation, cells were seeded, resuspended in warm culture medium and were distributed equally into 3 wells of a 6-well plate. All downstream analyses were carried out 24 h after transfection. For sAC knockdown experiments, cells were transfected either with scramble or with sAC shRNA encoding plasmids. Electroporation procedure was the same as for sAC overexpression. All downstream analyses were carried out 3 days after transfection. sAC shRNA (5'-GGGGTACCAAAAAA**GTGGAAAGTGGAACGAAAGCA**TCTCTTGAA**TGCTTTCGTTC CACTTTCCAC***AAACAAGGCTTTTCTCCAAG*-3') was expressed by the U6 promoter and a randomized sequence (scramble) based on sAC shRNA sequence was used as a control. Hairpin loop in the sequence is underlined. Adenoviral infection was used to knockdown sAC in adult rat primary cardiomyocytes. In short, adenoviral particles carrying sAC shRNA and scramble shRNA construct were prepared using AdEasy Adenoviral Vector System (Agilent Technologies). The recombinant virus particles were multiplied in HEK293 cells and recovered by several freeze–thaw cycles. sAC knockdown in cardiomyocytes was achieved 3 days after infection with 10^6 viral particles per milliliter culture medium (Appukuttan et al., 2012a).

5.4 Western blotting

Cells were lysed in Laemmli buffer and proteins were quantified using Pierce 660 nM Assay Kit. Equal amounts of total proteins were separated on SDS-polyacrylamide gels and were transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA in TBST for 1 h at room temperature and then was incubated overnight with the primary antibody, as listed in Section 4. Next day, the membrane was washed with TBST for 30 min and then was incubated for 1 h at room temperature with HRP-conjugated secondary antibody. After 30

min washing with TBST, the membrane was covered with an enhanced chemoluminescence solution (100 mM Tris-HCl, pH 8.5; 0.225 mM coumaric acid; 1.25 mM luminol; 0.009% (v/v) H_2O_2) for 2 min. Specific bands were visualized using Bio-Rad chemiluminescence system. To confirm equal protein loading, membrane was stripped and immunodetected for actin, tubulin or GAPDH. In case of animal studies, where expression of housekeeping proteins, such as actin, tubulin or GAPDH was affected by myocardial infarction, ponceau staining was used as the loading control.

5.5 cAMP quantification

The analysis of the total cellular cAMP content was performed using a cAMP ELISA Kit (Enzo, Germany). Shortly, cells were washed with PBS and lysed using the ice-cold lysis buffer provided with the kit. Lysates were loaded into the wells of the GxR IgG-coated 96-well plate along with alkaline phosphatase conjugated cAMP and rabbit polyclonal antibody to cAMP and incubated at room temperature for 2 h. A series of cAMP standards were used for calibration. GxR IgG binds in a competitive manner to either cAMP from the lysate or to the alkaline phosphatase conjugated cAMP. After incubation, the plate was washed, leaving only bound cAMP. Further addition of the substrate solution generated a yellow color when catalyzed by the alkaline phosphatase. The yellow color was read at 405 nm in a microplate reader. The amount of signal is indirectly proportional to the amount of cAMP in the lysates. The measured absorbance at 405 nm was used to calculate the concentration of the cAMP by applying a calibration curve. Values were presented as a ratio of cAMP to total protein content.

5.6 Analysis of lactate dehydrogenase in culture medium

Lactate dehydrogenase (LDH) activity in the cell culture medium was used as an indicator for cell toxicity and was determined applying the Cytotoxicity Detection Kit (Roche Applied Science). After each experiment, culture medium was centrifuged at 500 *g* for 5 min at 4°C and the supernatant was used for LDH analysis applying ELISA reader. A set of LDH standards were measured along with the samples and used for calibration. LDH activity was expressed in mU/ml.

5.7 Measurement of mitochondrial mass

Relative amounts of nuclear DNA and mitochondrial DNA (mtDNA) were determined by quantitative real-time PCR. The ratio of mtDNA to nuclear DNA reflects the mitochondrial content in a cell. Total genomic DNA was isolated using Quick-gDNA Miniprep Kit (Zymo Research). Quantitative PCR was performed by using the following primers (mtDNA specific cytochrome b primers, forward 5′-CCACATCTGCCGAGACGTAA-3′, reverse 5′-TAGTCCTCGTCCCACATGGA-3′; and nuclear DNA specific β-globin primers, forward 5′-AAGTACCACTAAGCCCCCTTTC-3′, reverse 5′-GGGAACACAAAAGACCTCTTCTGG-3′) and SYBR Green PCR Kit in a StepOnePlus real-time PCR system (Applied Biosystem). mtDNA content was normalized to nuclear DNA content and values were expressed as relative units (r.u.).

MitoTracker MitoGreen dye (Invitrogen) accumulates in mitochondria irrespective of mitochondrial membrane potential. Difference in the MitoGreen fluorescence in the cells can be used as an indication of difference in the mitochondrial mass (Pendergrass et al., 2004). Briefly, cells were loaded with MitoGreen (100 nmol/l) at 37°C for 20 min. Cells were then washed, trypsinized and resuspended in ice-cold PBS and were subjected to FACS analysis. Cells were excited at 490 nm and the emission fluorescence was collected at 535 nm. At least 10,000 events were recorded per sample and values were expressed as arbitrary units (a.u.).

5.8 Determination of mitochondrial membrane potential

Mitochondrial membrane potential was determined by measuring the ratio between membrane potential-dependent TMRM to membrane potential-independent MitoGreen (Thermo Fisher Scientific, Germany) staining. Since changes in mitochondrial mass influence TMRM loading, MitoGreen staining was used to avoid the influence of changes in mitochondrial mass. For this purpose, H9C2 cells were loaded with TMRM (50 nmol/l) and MitoGreen (100 nmol/l) at 37°C for 20 min. Cells were then washed, trypsinized and resuspended in ice-cold PBS and were subjected to FACS analysis. Cells were excited at 490 nm and the emission fluorescence was collected at 535 nm for MitoGreen and 590 nm for TMRM. Mitochondrial membrane potential was expressed as the fluorescence ratio of TMRM to MitoGreen fluorescence. At least 10,000 events were recorded per sample.

5.9 ATP measurements

Total cellular ATP was measured using ATPLite Luminescence Assay System (PerkinElmer). Briefly, cells were washed with PBS and lysed using the ice-cold lysis buffer (ATPLite Kit). Cell lysates were transferred to a white-walled 96-well plate. Similarly, ATP standards were serially diluted in ATPLite lysis buffer and were transferred to the same white-walled 96-well plate for calibration. Lyophilized ATPLite substrate was solubilized with ATPLite substrate buffer and was added to the wells containing either cell lysates or ATP standards. The plate was incubated for 2 min at dark and the bioluminescence was measured using the Victor Luminometer (PerkinElmer) and the amount of ATP in the lysates was calculated using the calibration curve. Values were presented as a ratio of ATP to total protein content.

5.10 Detection of cellular and mitochondrial reactive oxygen species

To examine total cellular reactive oxygen species (ROS) formation, cells were loaded with DCF (2',7'-dichlorodihydrofluorescein diacetate, succinimidyl ester, 10 µmol/l, Invitrogen), a nonfluorescent dye, which is converted into the highly fluorescent DCF in the presence of free radicals. DCF fluorescence was analyzed by excitation at 488 nm and emission at 530 nm applying ELISA reader. Values were presented as a ratio of DCF fluorescence to protein. Similarly, formation of mitochondrial ROS was examined by loading cells with MitoSox (10 µmol/l, Invitrogen), a fluorogenic dye that accumulates in mitochondria. It is rapidly oxidized by superoxide and produces red fluorescence. MitoSox fluorescence was analyzed by excitation at 510 nm and emission at 580 nm applying ELISA reader. Values were presented as a ratio of DCF fluorescence or MitoSox fluorescence to total protein.

5.11 FRET experiments and live imaging

For FRET-based live cell imaging, cells were transfected with plasmids encoding the corresponding sensors. Transfection was performed by electroporation as mentioned earlier. After electroporation, cells were resuspended in warm culture medium and were distributed equally into 3 wells of a 6-well plate containing 24 mm diameter glass coverslips. Twenty-four hours after transfection, FBS in the culture medium was reduced to 5% and cells were cultured for another 24 h.

FRET-based live cell imaging was performed in temperature controlled (35oC) gas-tight or open chamber. For experiments with different inhibitors and activators, cells were maintained in the standard buffer (see Section 4.4.1 for buffer composition) and the live imaging was performed in an open chamber, so that the chemical compounds can be added directly into the chamber. For experiments with bicarbonate stimulation, cells were initially superfused with the standard buffer in a gas-tight chamber. After 10 min stabilization, superfusion was changed from the standard buffer to the bicarbonate buffer (see Section 4.4.2 for buffer composition). Bicarbonate buffer was pre-gassed and was also gassed during the experiment with air containing 10% CO_2. cAMP sensors were excited at 430 nm and the emission light was acquired at 470 nm and 535 nm, where 470/535 nm ratio showed the changes in cAMP concentration over time. AMPK sensors were excited at 430 nm and the emission light was acquired at 475 nm and 535 nm, where 535/475 nm ratio showed the changes in AMPK activity over time. Images were acquired for every 10 s with an invert microscope (oil immersion objective 40×, Zeiss, Germany) and imaging system (Visitron, Germany). The analysis of the FRET signal was performed by VisiView software (Visitron, Germany). Emission signals obtained in the cell-free region (background) were subtracted from corresponding emission signals obtained within the region of interest and presented as ratio of 470/535 nm for cAMP concentration and 535/475 nm for AMPK activity.

5.12 Statistical analysis

The data are given as mean ± SEM, where mean indicates biological replicates. Comparisons of the mean between two groups were performed using unpaired two-tailed *t*-tests assuming equal variance. For more than two groups, two-way ANOVA followed by Bonferroni post-test was used. Statistical significance was accepted when $P < 0.05$.

6 Results

6.1 Transfection protocol optimization

Since the majority of experiments performed in this study require expression of certain proteins or shRNA, the electroporation-based transfection protocol with plasmids was first optimized using GFP-encoding plasmids in H9C2 cells. Among different concentrations of GFP-encoding plasmids tested (**Fig. 1A, C**), 8 µg plasmids per 400,000 cells gave a maximal efficacy, i.e., 76.6 ± 0.3% (mean ± SD, *n*=2) after 48 h of transfection. This transfection protocol led to a slight elevation of cell death as demonstrated by trypan blue staining and LDH assay (**Fig. 1D, E**).

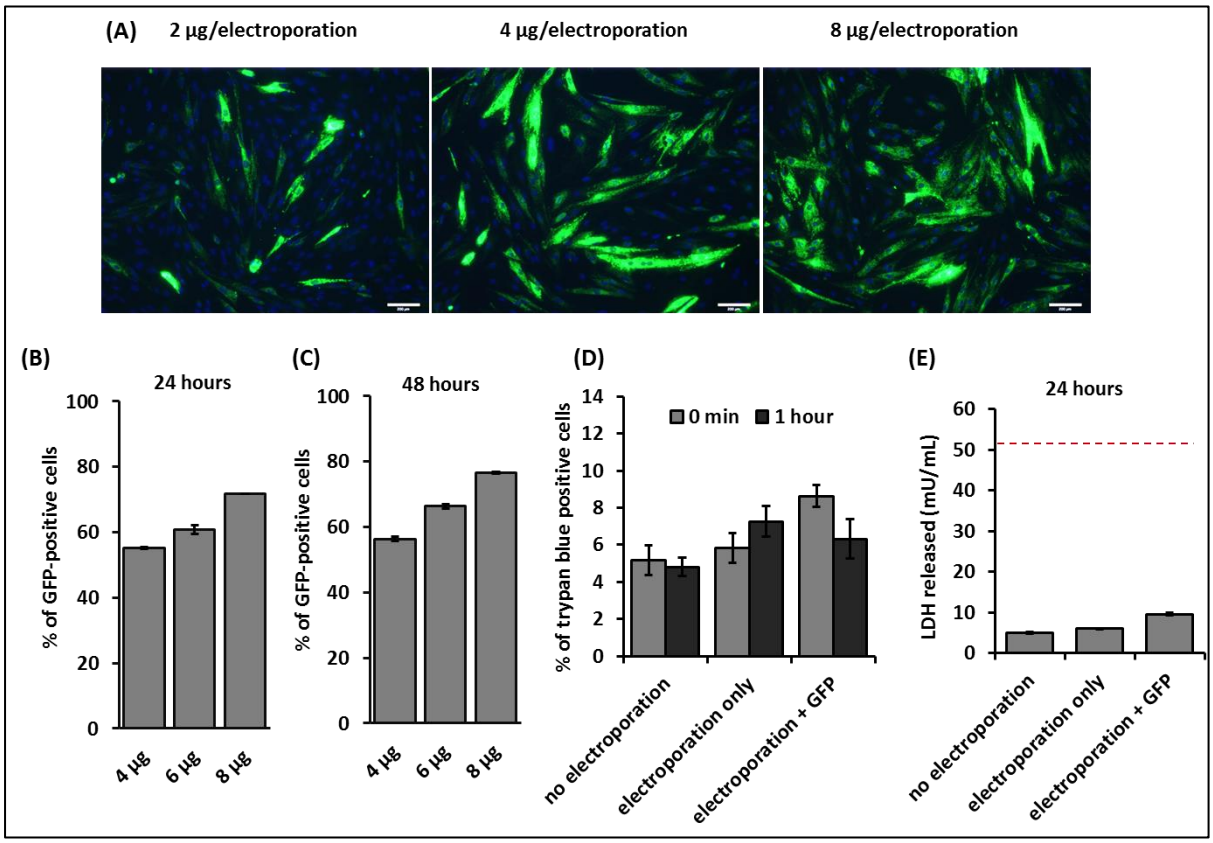

Fig. 1. Optimization of transfection protocol in H9C2 cells.

(A) Fluorescent microscopy images of H9C2 cells overexpressing GFP (green) with indicated amount of plasmid used per electroporation. Nuclei were stained with Hoechst 33342 (blue). (B and C) FACS analysis of GFP-positive cells 24 h and 48 h after transfection with indicated amount of plasmid used per electroporation. (D) Percentage of trypan blue positive cells at indicated time points after electroporation alone or with GFP-encoding plasmids. (E) Quantification of LDH released into the cell culture medium 24 h after transfection. The red dotted line indicates maximal LDH released after treatment with 0.1% triton X-100. In B–E, values are expressed as mean ± SD; *n*=2.

6.2 sAC controls basal AMPK activity

6.2.1 sAC knockdown affects basal AMPK activity in H9C2 cells

To check whether sAC influences basal AMPK activity, sAC knockdown has been performed in H9C2 cells via transfection with sAC-targeted shRNA. Significant reduction of sAC expression and total cellular cAMP content was observed 3 days after transfection (**Fig. 2A, B**). These effects were accompanied by reduced phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC), indicating a decline in AMPK activity (**Fig. 2C, D**). ACC is the direct downstream target of AMPK and the phosphorylation level of ACC is used as a marker of AMPK activity in the cell (Yan et al., 2015).

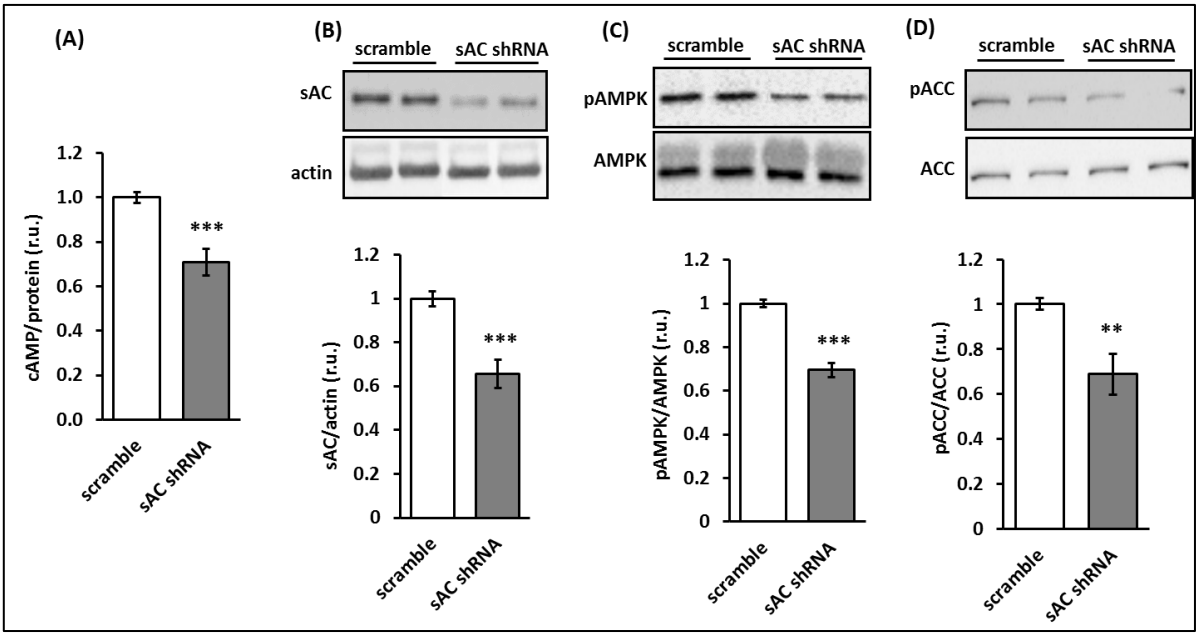

Fig. 2. sAC knockdown reduces basal AMPK activity in H9C2 cells.
Analysis of total cellular cAMP (A) as well as western blot analyses of sAC (B), pAMPK (C) and pACC (D) were performed in H9C2 cells 3 days after transfection with plasmids encoding either scramble or sAC-targeted shRNA. Values are expressed as mean ± SEM; *n*=12 for cAMP and *n*=6 for sAC, pAMPK and pACC. ***P*<0.01 and ****P*<0.001 *vs* scramble.

6.2.2 sAC knockdown reduces basal AMPK activity in primary cells

To examine whether the sAC knockdown-induced downregulation of AMPK activity in H9C2 cells can be reproduced in other cell types, sAC knockdown was performed in ventricular cardiomyocytes and coronary endothelial cells isolated from adult male rats. Knockdown of

sAC in cardiomyocytes has been previously optimized using adenoviral vector carrying sAC-targeted shRNA (Appukuttan et al., 2012a). sAC knockdown in cardiomyocytes 3 days after transfection resulted in about 80% reduction in sAC protein level (experiments performed in cooperation with Dr. A. Appukuttan, Ruhr-University Bochum (Appukuttan et al., 2012a)) and led to the significant reduction in AMPK activity as demonstrated by reduced phosphorylation of AMPK and ACC (**Fig. 3A–C**).

The effect of sAC knockdown was further tested in rat coronary endothelial cells. The purity of the cell isolation and culture (>98%) were confirmed by immunochemical staining with antibodies against von Willebrand factor (**Fig. 3D**). A similar electroporation-based protocol was used for sAC knockdown in endothelial cells as described for H9C2 cells (detailed in Section 5.2). In consistent with H9C2 cells and adult cardiomyocytes, sAC knockdown significantly reduced AMPK phosphorylation in endothelial cells (**Fig. 3E, F**). Thus, sAC knockdown leads to the downregulation of AMPK activity in a cell type independent manner.

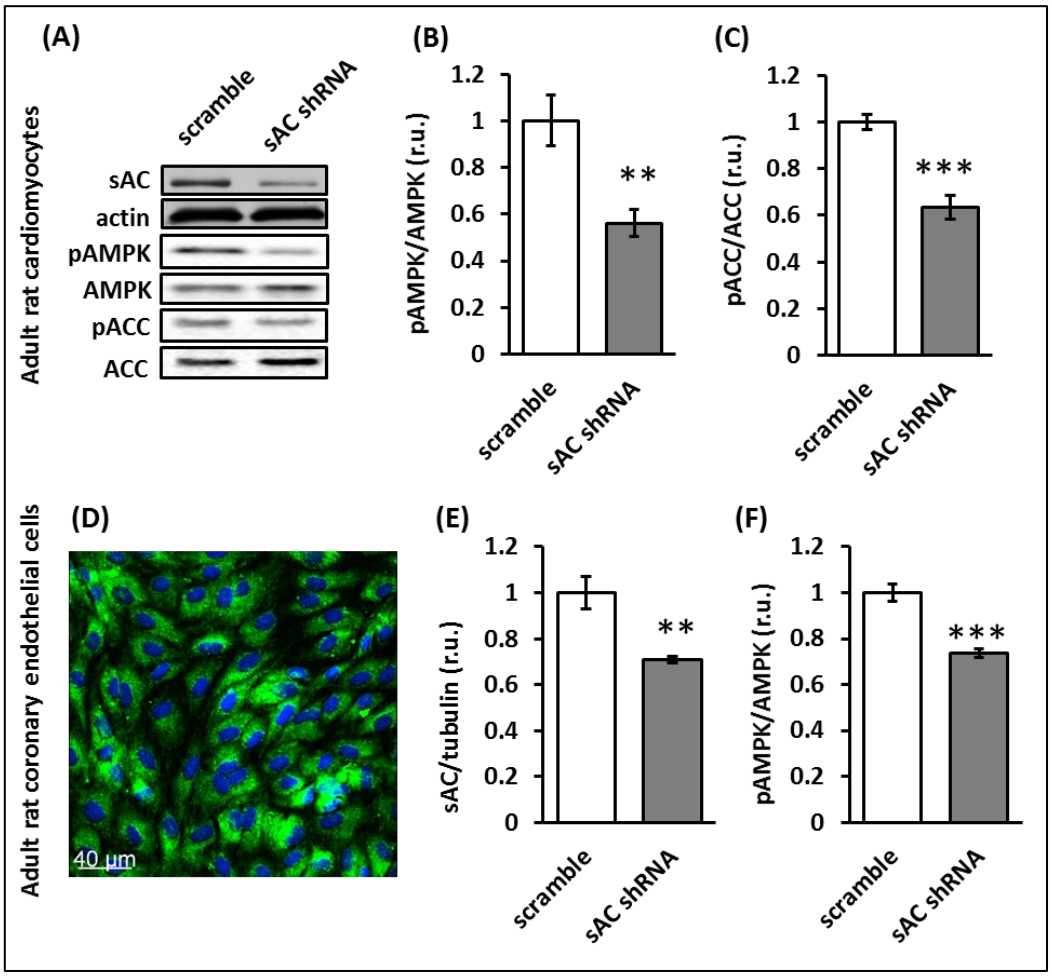

Fig. 3. sAC knockdown reduces basal AMPK activity in adult rat cardiomyocytes and coronary endothelial cells.

Western blots analyses of sAC, pAMPK and pACC were performed in adult rat cardiomyocytes (A–C) 3 days after transfection with DNA encoding either scramble or sAC-targeted shRNA. (D) Representative immunofluorescence image of coronary endothelial cells stained with endothelial cell-specific marker von Willebrand factor (green) and counterstained with DAPI (blue) for nuclei. Western blots analyses of sAC and pAMPK were performed in rat coronary endothelial cells (E–F) 3 days after transfection with DNA encoding either scramble or sAC-targeted shRNA. Values are expressed as mean ± SEM; n=8 for adult rat cardiomyocytes and n=4 for coronary endothelial cells. **P<0.01 and ***P<0.001 *vs* scramble.

6.3 EPAC mediates sAC-dependent regulation of AMPK activity

Since the sAC knockdown was accompanied by significant reduction of the cellular cAMP, we aimed to find out which downstream targets of cAMP, i.e., PKA or EPAC, are involved in the regulation of AMPK activity by sAC. For this purpose, sAC knockdown cells were treated either with PKA or EPAC agonist. Treatment with specific PKA agonist had no effect on the AMPK phosphorylation in sAC knockdown cells, whereas it reduced AMPK phosphorylation in control cells (**Fig 4A, B**). In contrast, EPAC agonist rescued the effect of sAC knockdown (**Fig. 4C, D**). Thus, sAC–EPAC axis is involved in regulation of the basal AMPK activity.

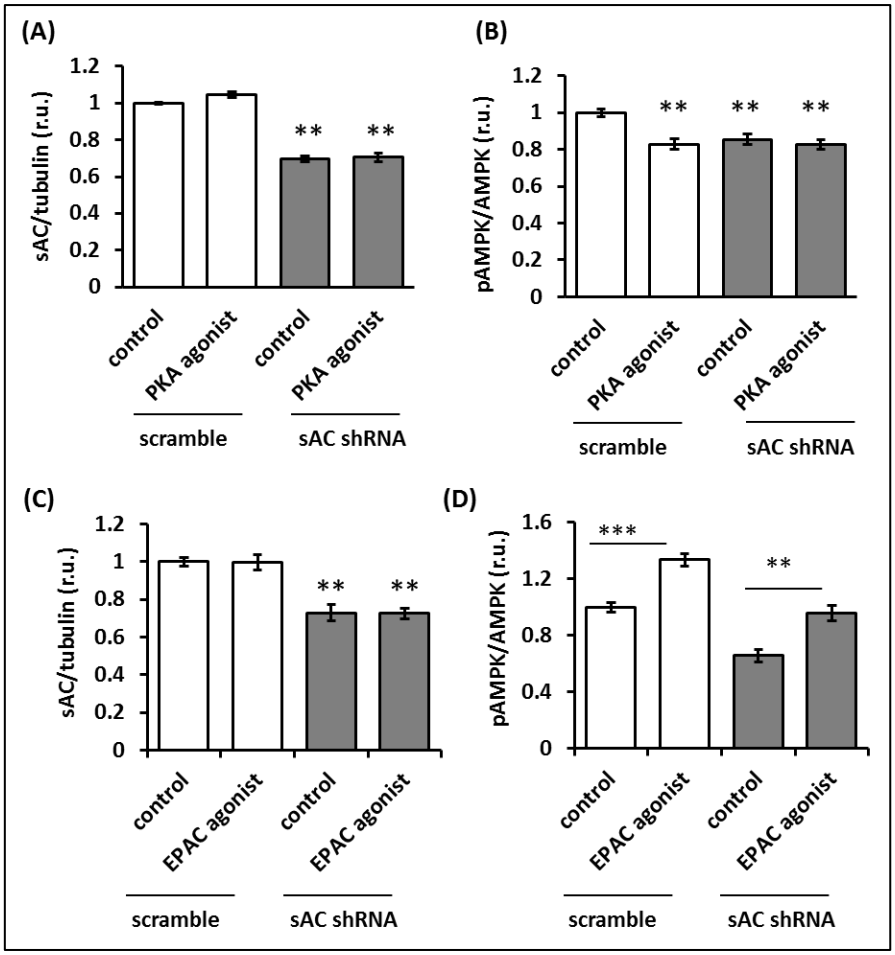

Fig. 4. EPAC mediates sAC-dependent regulation of AMPK activity in H9C2 cells.
Western blot analyses of sAC and pAMPK were performed in H9C2 cells 3 days after transfection with plasmids encoding either scramble or sAC-targeted shRNA. Before lysis, cells were treated either with PKA agonist, 200 µmol/l 6-Bnz-cAMP (A–B), or with EPAC agonist, 200 µmol/l 8-CPT-2Me-cAMP (C–D), for 1 h. Values are expressed as mean ± SEM; *n*=4 for PKA agonist and *n*=6 for EPAC agonist. ***P*<0.01 and ****P*<0.001 *vs* scramble control.

6.4 Role of sAC–AMPK axis in mitochondrial homeostasis

6.4.1 Downregulation of sAC–AMPK axis affects mitochondrial health and cellular redox and energy balances

Since AMPK activity is a key regulator of mitochondrial homeostasis (Herzig and Shaw, 2017), we proved the effects of sAC knockdown on mitochondrial parameters. Downregulation of sAC expression was accompanied by mitochondrial depolarization and increased basal mitochondrial ROS formation (**Fig. 5A, B**). Consistently, these effects were accompanied by the increased total cellular ROS formation and reduced total ATP content (**Fig. 5C, D**).

Similarly, sAC knockdown increased basal mitochondrial ROS formation in primary endothelial cells (**Fig. 5E**).

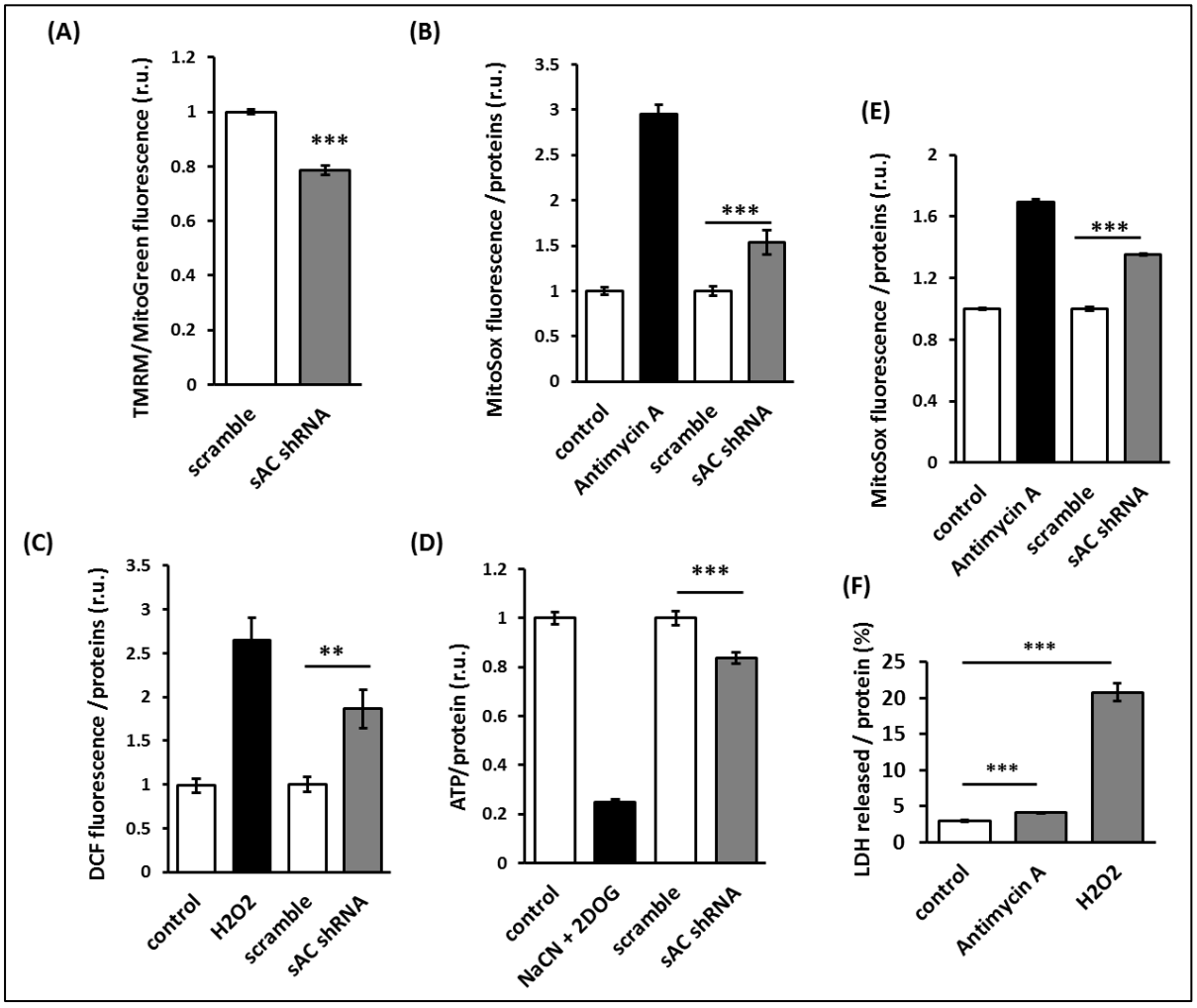

Fig. 5. Effect of sAC knockdown on mitochondrial membrane potential, ROS formation and cellular ATP.

Analyses of mitochondrial membrane potential (A, *n*=6), mitochondrial ROS (B, *n*=6), total cellular ROS (C, *n*=7) and total cellular ATP (D, *n*=7) in H9C2 cells and mitochondrial ROS in coronary endothelial cells (E, *n*=3) were performed either in untransfected cells (control) or in cells 3 days after transfection with plasmids encoding either scramble or sAC-targeted shRNA (sAC shRNA). Treatments with 10 µmol/l antimycin A and 1 mmol/l H_2O_2 for 1 h were used as positive controls and the cytotoxicity of the treatments with antimycin A and H_2O_2 was determined by LDH assay (F, *n*=3). Treatment with 1 mmol/l NaCN and 5 mmol/l 2-deoxy-D-glucose (NaCN + 2DOG) for 40 min was performed for metabolic inhibition. Values are expressed as mean ± SEM. ***P*<0.01 and ****P*<0.001 *vs* scramble.

6.4.2 Downregulation of sAC–AMPK axis affects mitochondrial clearance

The sAC knockdown-induced mitochondrial depolarization and ROS formation may be due to the disturbed mitochondrial clearance, or mitophagy, leading to the accumulation of damaged mitochondria. To prove this hypothesis, mitochondrial mass was examined in H9C2 cells. Indeed, mitochondrial mass was significantly increased after sAC knockdown as demonstrated by the elevation in MitoGreen staining, a membrane potential independent mitochondrial dye (Pendergrass et al., 2004) (**Fig. 6A**). The increase in mitochondrial mass was further confirmed by the elevation of mtDNA/nucDNA ratio (**Fig. 6B**). Similarly, sAC knockdown increased mitochondrial mass in primary endothelial cells (**Fig. 6C**).

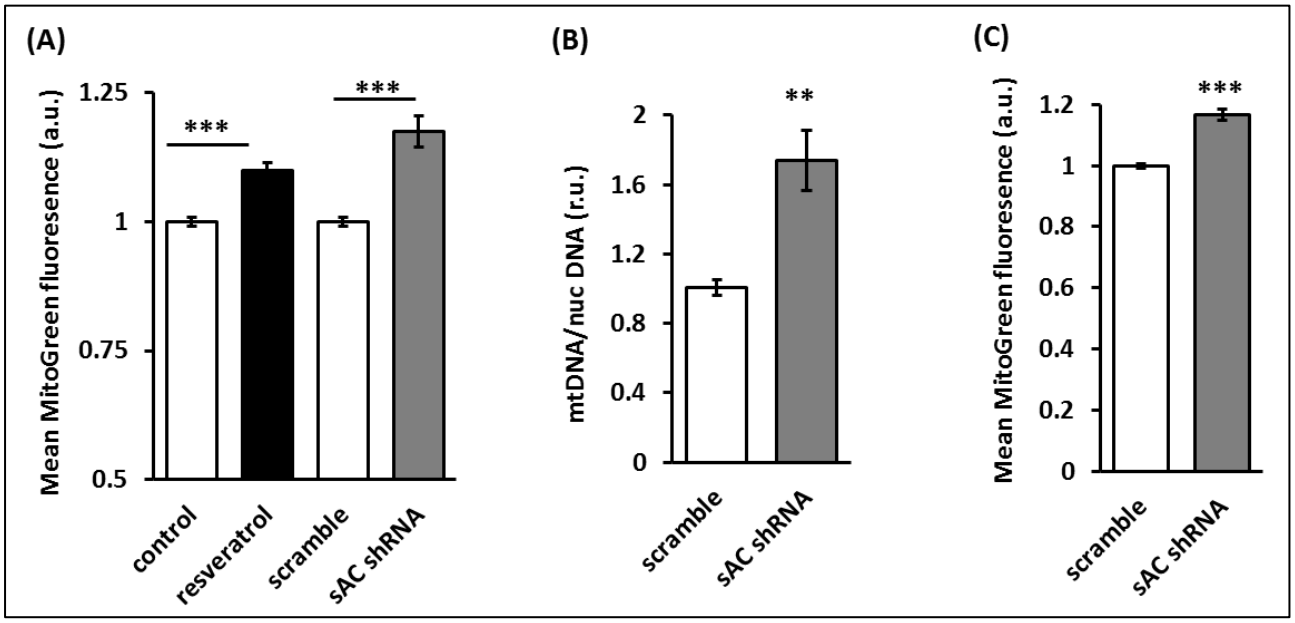

Fig. 6. sAC knockdown leads to the elevation of mitochondrial mass.

Analysis of mitochondrial mass as measured by MitoGreen staining (A, *n*=6) and by the ratio of mtDNA to nuclear DNA (nucDNA) (B, *n*=6) in h9C2 cells and by MitoGreen staining (C, *n*=4) in coronary endothelial cells was performed either in untransfected cells (control) or in cells 3 days after transfection with plasmids encoding either scramble or sAC-targeted shRNA. Treatment with 50 µmol/l resveratrol for 24 h was used as a positive control (Park et al., 2012). Values are expressed as mean ± SEM. ***P*<0.01 and ****P*<0.001 *vs* scramble.

6.4.3 Downregulation of sAC–AMPK axis affects mitochondrial biogenesis

To exclude the potential effect of mitochondrial biogenesis on the elevation of mitochondrial mass induced by sAC knockdown, expression analysis of two key regulators of mitochondrial biogenesis, i.e., nuclear PGC1α and mitochondrial TFAM, was performed. We found that

expression of PGC1α and TFAM was significantly reduced by sAC knockdown (**Fig. 7A–C**). Of note, the expression of two key mitochondrial enzymes controlling mitochondrial function and ROS formation, i.e., Sirt3 and SOD2, was also significantly reduced under sAC knockdown (**Fig. 7A, D and E**). Thus, downregulation of sAC significantly reduced mitochondrial biogenesis and clearance resulting in the accumulation of damaged mitochondria and disturbed cellular energy and redox balance.

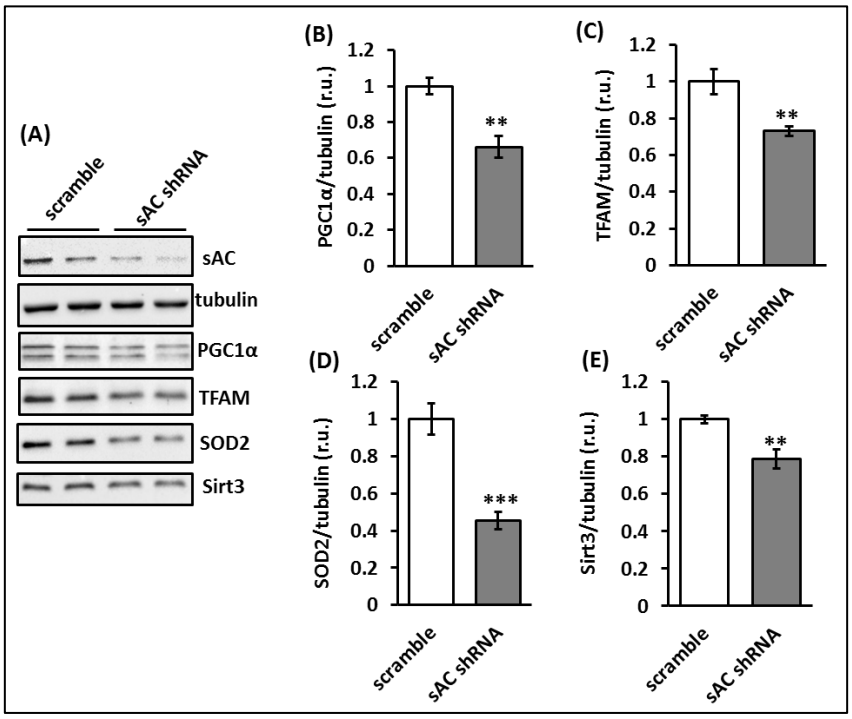

Fig. 7. sAC knockdown suppresses mitochondrial biogenesis in H9C2 cells.
Western blot analyses of PGC1α, TFAM, SOD2 and Sirt3 were performed 3 days after transfection with plasmids encoding either scramble or sAC-targeted shRNA. Values are expressed as mean ± SEM; *n*=5. ***P*<0.01 and ****P*<0.001 *vs* scramble.

6.5 Overexpression of sAC promotes AMPK activity

Several reports demonstrated that upregulation of cAMP signaling affects AMPK activity, though data are controversial and AMPK activation as well as inhibition has been reported (Hurley et al., 2006b). Thus, to prove whether upregulation of sAC expression affects activity of AMPK, an overexpression of the active 50 kDa sAC isoform (Appukuttan et al., 2014) has been performed in H9C2 cells. GFP-encoded plasmids with the same construct were used as control.

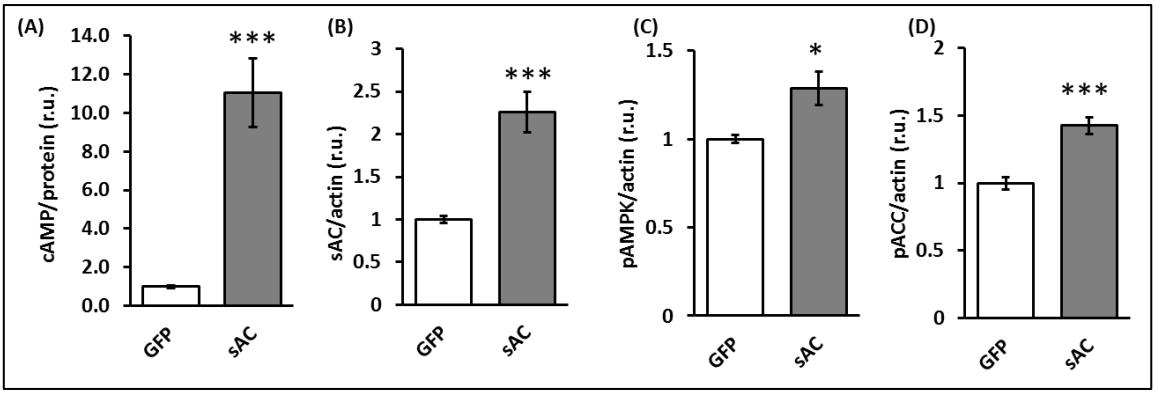

Fig. 8. Overexpression of sAC promotes AMPK activity in H9C2 cells.
Analysis of total cellular cAMP (A) as well as western blot analyses of sAC (B), pAMPK (C) and pACC (D) were performed 24 h after transfection with plasmids encoding either GFP or 50 kDa sAC isoform. Values are expressed as mean ± SEM; *n*=4. **P*<0.05 and ****P*<0.001 *vs* GFP.

sAC overexpression significantly elevated cellular cAMP concentration (**Fig. 8A, B**) and increased AMPK and ACC phosphorylation (**Fig. 8C, D**). Noteworthy, these effects were accompanied by the significant increase in cellular ATP content, whereas no effect was found on the mitochondrial mass after sAC overexpression (**Fig. 9A, B**).

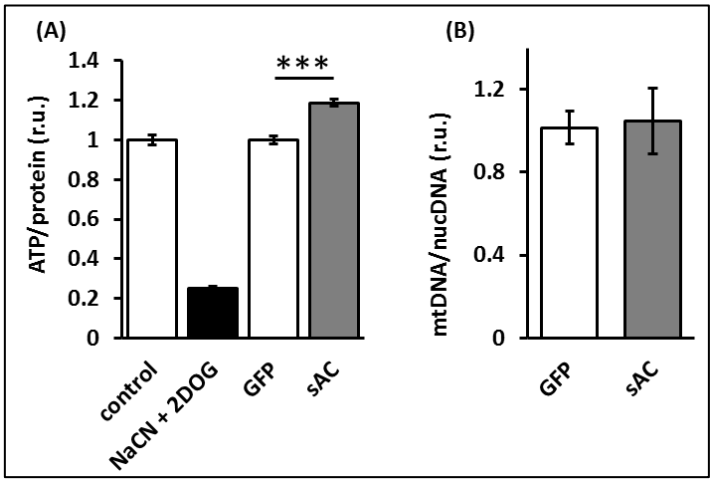

Fig. 9. sAC overexpression supports energy balance in H9C2 cells.
Analyses of total cellular ATP (A) and mitochondrial mass (B) were performed 24 h after transfection with plasmids encoding either GFP or 50 kDa sAC isoform. Treatment with 1 mmol/l NaCN and 5 mmol/l 2-deoxy-D-glucose (NaCN + 2DOG) for 40 min was performed for metabolic inhibition. Values are expressed as mean ± SEM; *n*=4. ****P*<0.001 *vs* GFP.

6.6 Stimulation of sAC with bicarbonate

6.6.1 Stimulation of sAC promotes AMPK activity

As a different approach for the upregulation of sAC signaling (other than sAC overexpression), sAC activity was stimulated by treatment with bicarbonate, a natural sAC activator (Steegborn, 2014). We found that bicarbonate dose-dependently increased phosphorylation of AMPK and ACC (**Fig. 10A**) and was stable for at least 24 h (**Fig. 10B**). Since the effect of bicarbonate could be prevented by sAC knockdown (**Fig. 10C**), we suggested a causal role of sAC in bicarbonate-induced AMPK activation.

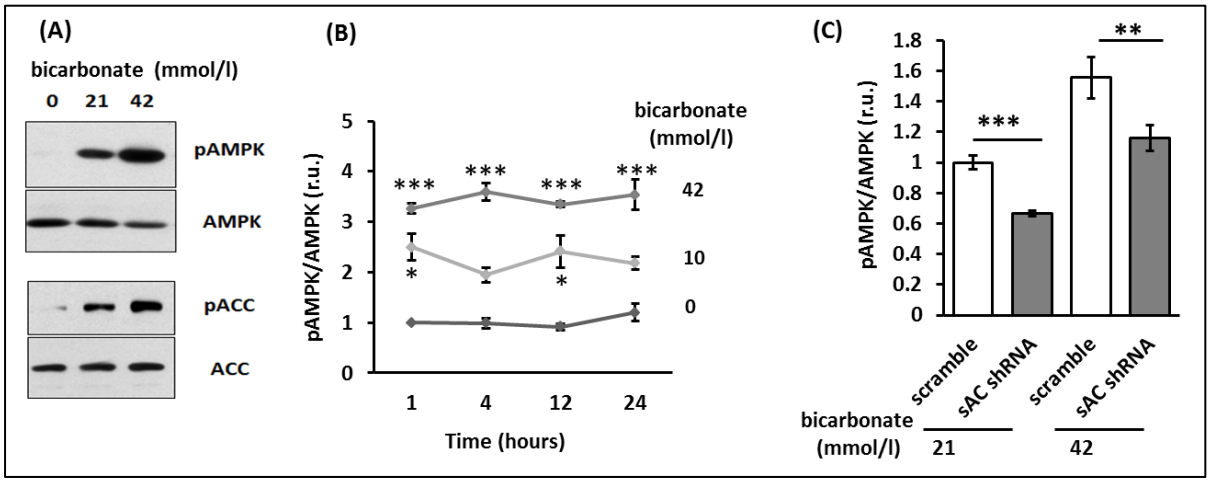

Fig. 10. Stimulation of sAC promotes AMPK activity in H9C2 cells.

Western blot analyses of pAMPK and pACC were performed 1 h after bicarbonate stimulation (A) or at different time points as indicated (B). (C) Western blot analysis of pAMPK was performed 3 days after transfection with plasmids encoding either scramble or sAC-targeted shRNA. Before lysis, cells were stimulated with 42 mmol/l bicarbonate for 1 h. For B and C, values are expressed as mean ± SEM; n=4. *P<0.05, **P<0.01 and ***P<0.001 *vs* 0 mmol/l bicarbonate group of corresponding time points in B and *vs* scramble in C.

6.6.2 Stimulation of sAC promotes AMPK activity in cytosol

To strengthen the findings, in addition to the western blot analysis, a FRET-based live imaging of cAMP concentration and AMPK activity was performed in H9C2 cells treated with bicarbonate. To check the responsiveness of untargeted (mainly localized in cytosol) and nuclear-targeted FRET-based AMPK activity sensors, a metabolic stress model, i.e., treatment with 1 mmol/l NaCN and 5 mmol/l 2-deoxy-D-glucose (NaCN + 2DOG), was used (Wright et al., 2003, Miyamoto et al., 2015). It has been shown that under metabolic stress AMPK gets

activated and translocates to the nucleus (Kodiha et al., 2007). **Fig. 11A and B** shows that the sensors are responsive to the changes in the activity of AMPK in both cytosol and nucleus. In addition, treatment with specific AMPK activator A769662 leading to the increase of FRET-ratio further confirms the validity of the AMPK activity assay (**Fig. 11C**).

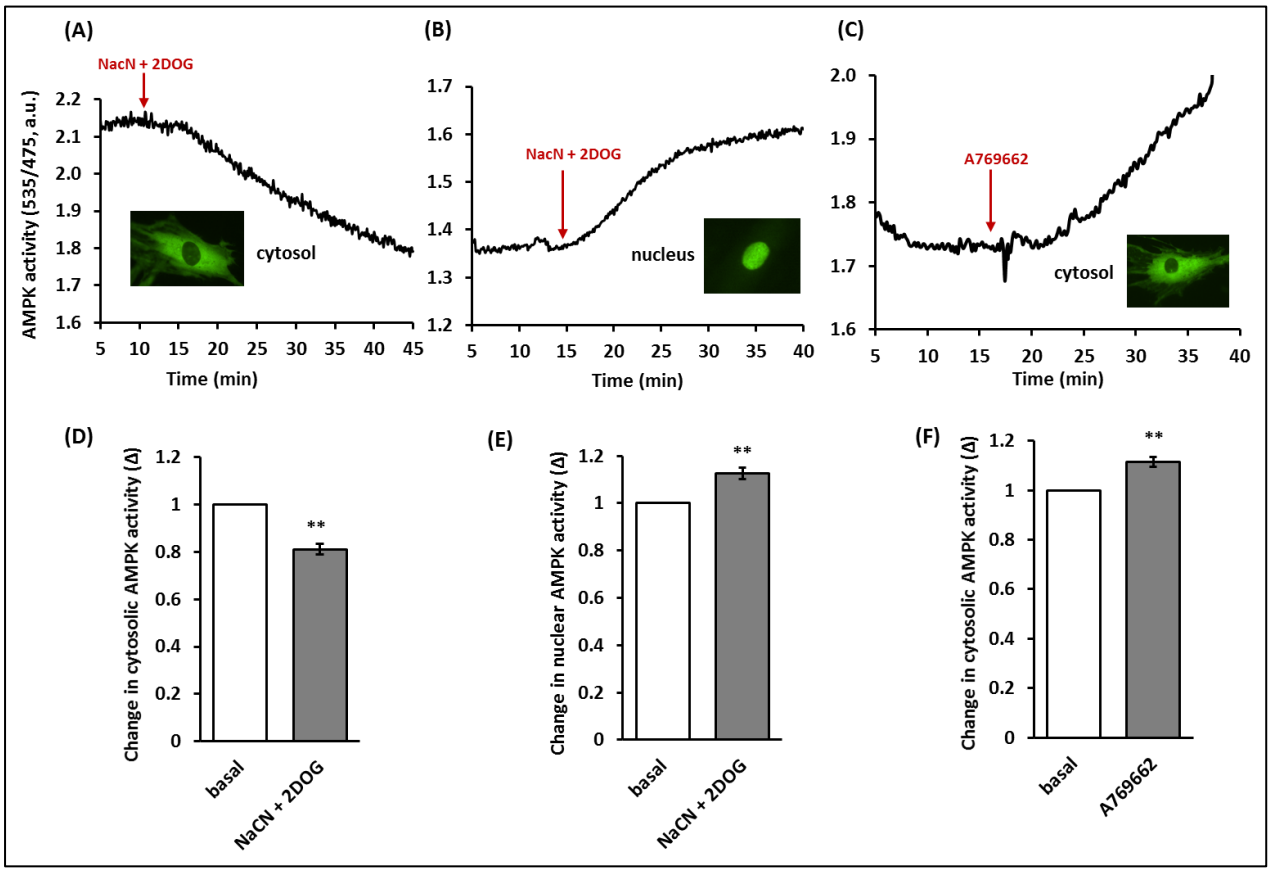

Fig. 11. Characterization of FRET-based AMPK activity sensors in H9C2 cells.

Representative kinetics of AMPK activity observed in cytosol (A) and in nucleus (B) after treatment with 1 mmol/l NaCN and 5 mmol/l 2-deoxy-D-glucose (NaCN + 2DOG), or in cytosol (C) after treatment with 100 µmol/l A769662, an AMPK activator. Inserts show the expression of AMPK-FRET sensors in cytosol or nucleus. D – E shows the quantification of the kinetics shown in graphs A – C. Values are expressed as mean ± SEM; n=3. $^{**}P$<0.01 *vs* the basal activity.

Using the advantage of FRET technology, cells were transfected with untargeted EPAC-based cAMP probe (Lefkimmiatis et al., 2013) to monitor the changes in cytosolic cAMP concentration upon bicarbonate stimulation. As shown in **Fig. 12A**, bicarbonate stimulation increased the cAMP concentration, which could be prevented by preincubation with LRE1, a specific sAC inhibitor (Ramos-Espiritu et al., 2016). Similarly, AMPK activity was markedly

elevated under bicarbonate treatment and this effect was attenuated by sAC inhibition with LRE1 (**Fig. 12B**). These findings further argue for the sAC-dependent increase of the cytosolic cAMP concentration and AMPK activity under bicarbonate treatment.

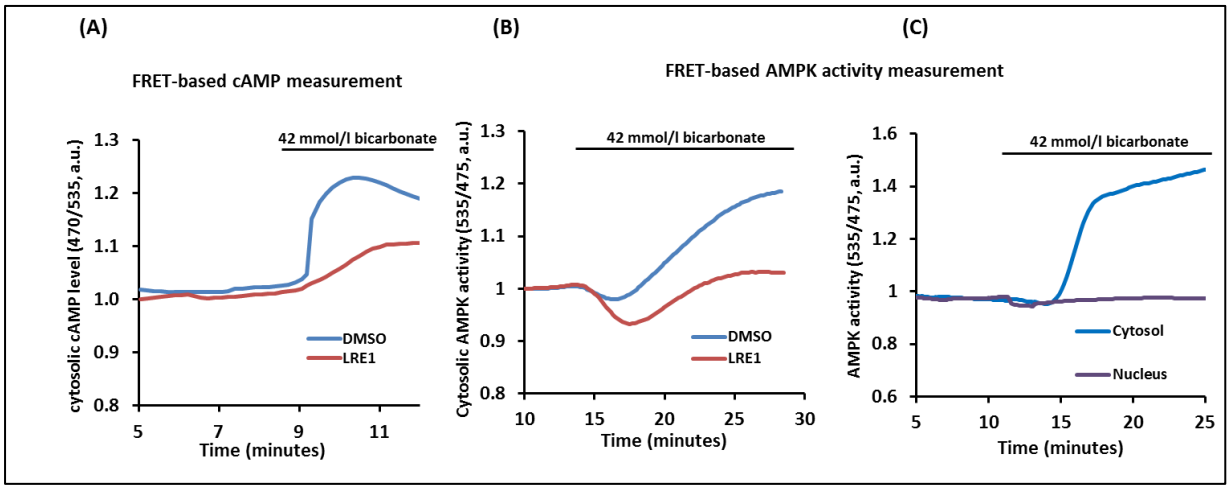

Fig. 12. Stimulation of sAC promotes AMPK activity in cytosol of H9C2 cells.

Representative kinetics of cytosolic cAMP level (A) and AMPK activity (B) as well as AMPK activity in cytosol and nucleus (C) upon stimulation with bicarbonate. To inhibit sAC, cells were preincubated for 15 min with specific sAC inhibitor LRE1 (100 µmol/l). Data are representative of at least three (A and B) or four (C) individual experiments with similar results.

To find out which subcellular AMPK compartments were affected by sAC-dependent cAMP pool, H9C2 cells were transfected either with untargeted AMPK activity sensor, i.e., cytosolically localized sensor, or with sensors targeted to nucleus as previously described (Miyamoto et al., 2015). We found that treatment with bicarbonate led to the rapid elevation of AMPK activity in cytosol, but not in nucleus (**Fig. 12B, C**). Similar to sAC overexpression, stimulation of sAC with bicarbonate significantly increased cellular ATP content without affecting mitochondrial mass (**Fig. 13A, B**). Altogether, these finding show that stimulation of sAC with bicarbonate leads specifically to the activation of cytosolic AMPK, resulting in improved cellular energy balance.

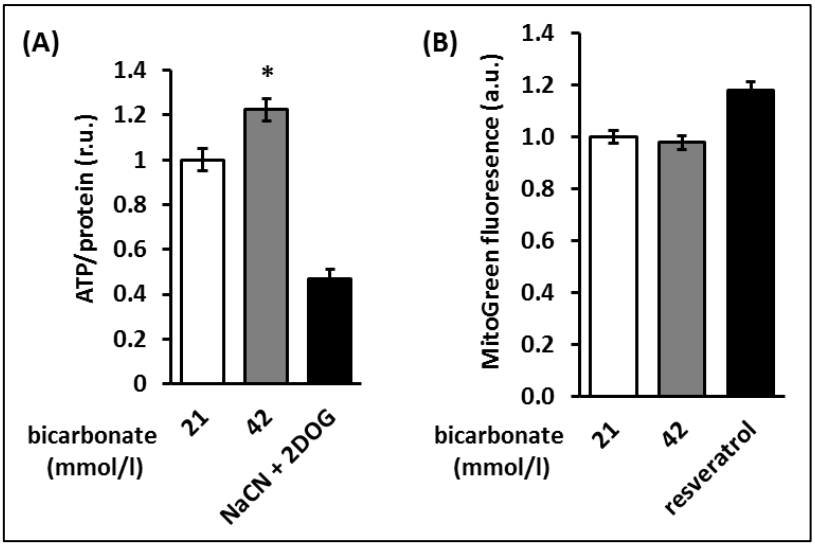

Fig. 13. Stimulation of sAC supports cellular energy balance.

Analyses of total cellular ATP (4 h treatment, A) and mitochondrial mass (24 h treatment, B) were performed after incubating the H9C2 cells in growth medium with 21 or 42 mmol/l bicarbonate. Treatment with 1 mmol/l NaCN and 5 mmol/l 2-deoxy-D-glucose (NaCN + 2DOG) for 40 min was performed for metabolic inhibition. Treatment with 50 µmol/l resveratrol for 24 h was used as a positive control. Values are expressed as mean ± SEM, *n*=4 for ATP and mean ± SD, *n*=2 for mitochondrial mass. **P*<0.05 *vs* 21 mmol/l bicarbonate group.

6.6.3 Stimulation of sAC, but not tmAC, promotes AMPK activity

Apart from sAC, tmAC is also a major source of cAMP in the cell, though restricted to sub-plasmalemmal compartments (Steegborn, 2014). Several reports suggested that tmAC activation leads to PKA-dependent phosphorylation of AMPK at inactivating site Ser173 (Hurley et al., 2006b). To examine whether stimulation of the tmAC affects AMPK activity, treatment with tmAC activator forskolin was performed and FRET-based cytosolic cAMP content and AMPK activity were analyzed. Similar to the bicarbonate stimulation of sAC, stimulation of tmAC with forskolin increased cytosolic cAMP content (**Fig. 14A**). Surprisingly, forskolin treatment had no effect on AMPK activity (**Fig. 14B**). Blocking cAMP degradation by inhibiting PDEs with pan-inhibitor IBMX during forskolin treatment also failed to promote AMPK activity in cytosol (**Fig. 14A, B**). Altogether, these findings suggest the role of sAC, but not tmAC, in cAMP-dependent activation of AMPK.

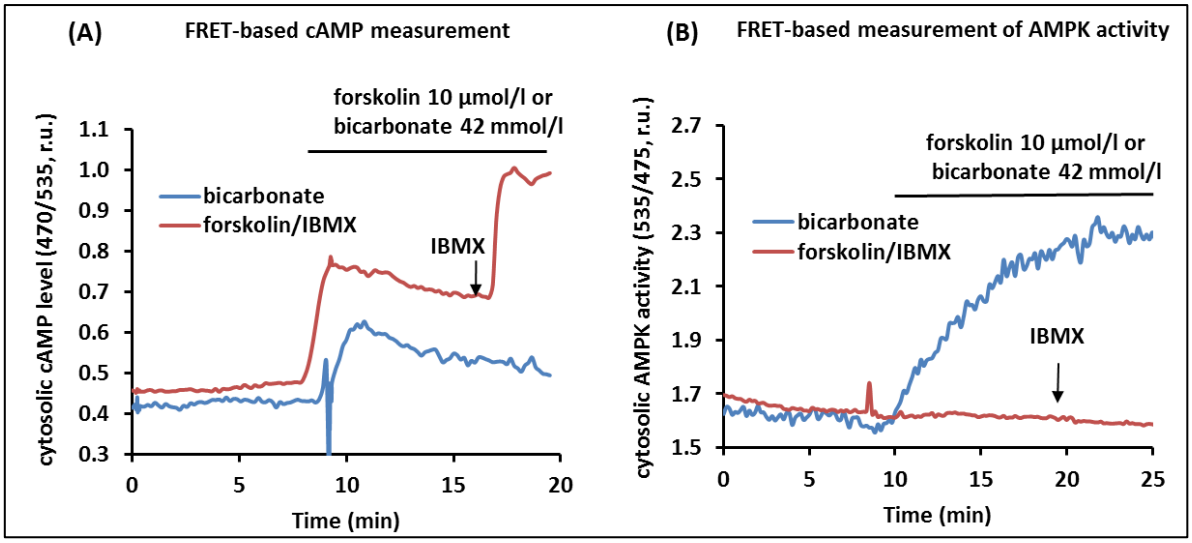

Fig. 14. Stimulation of sAC, but not tmAC, promotes AMPK activity in H9C2 cells.

Representative kinetics of cAMP level (A) and AMPK activity (B) in cytosol upon bicarbonate (blue line) or forskolin stimulation (red line). Black line indicates the treatment period. Treatment with 200 µmol/l IBMX (a pan-inhibitor of PDEs) was performed to prevent cAMP degradation. Data are representative of four to five individual experiments with similar results.

6.7 Alterations of sAC–AMPK axis in cardiac pathology

A recent study demonstrated that heart failure induced by pressure overload in male rats leads to pronounced downregulation of sAC in cardiac tissue (Wang et al., 2016). To prove whether cardiac pathologies are associated with dysregulation of sAC–AMPK axis, myocardial infarction (MI) model induced by permanent ligation of the left anterior descending artery has been applied in male and female mice. All animal experiments as well as functional and morphological analyses were performed as previously described (Schuster et al., 2016). For this thesis, the lysates of remote cardiac area, septum, were further used for western blot analysis.

Sham animals underwent the same surgical procedure, but the ligature was not tied. Applying cardiac tissue samples obtained in this study, the follow-up analyses were performed.

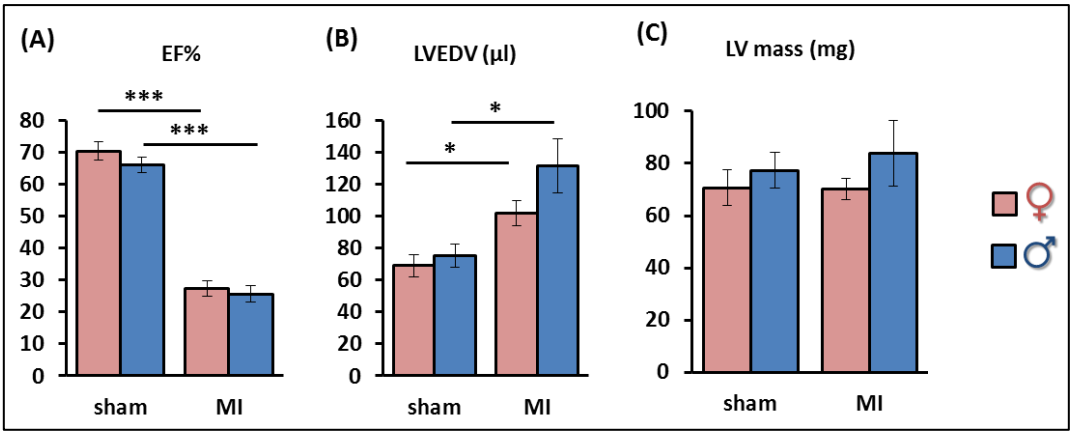

Fig. 15. Effect of MI on left ventricular function and morphology.

High-resolution echocardiographic measurement of the left ventricular ejection fraction (EF%) (A), left ventricular end-diastolic volume (LVEDV) (B) and left ventricular mass (LV mass) (C) was performed 2 weeks after permanent coronary artery ligation (MI) in male (♂) and female (♀) mice. Values are expressed as mean ± SEM; *n*=4–5 for sham and *n*=8–10 for MI groups. **P*<0.05 and ****P*<0.001 *vs* corresponding sham group.

MI led to the pronounced reduction of ejection fraction (**Fig. 15A**) and significant elevation of left ventricular end-diastolic volume (**Fig. 15B**) in a sex-independent manner, though no significant alterations have been found in left ventricular mass (**Fig. 15C**). The complete pathophysiological information can be retrieved from the work of Schuster et al. (2016).

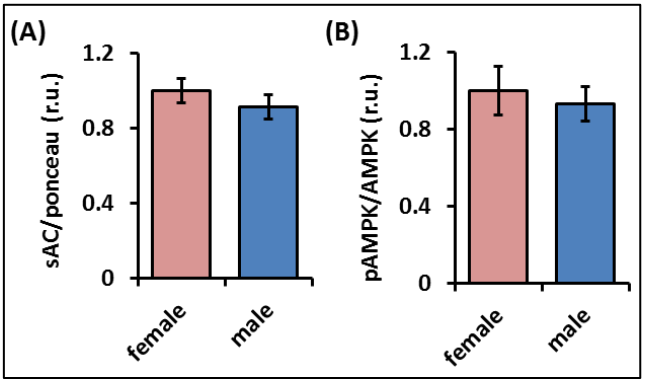

Fig. 16. Male and female sham animals have similar cardiac sAC and pAMPK expression.

Western blot analyses of sAC and pAMPK were performed in the septum samples of male (♂) and female (♀) mice 2 weeks after sham operation (A and B). Values are expressed as mean ± SEM; *n*=4–5. sAC expression was normalized to the ponceau signal as described in Section 5.4.

By performing western blot analysis of sAC and pAMPK in the septum samples of these animals, we found no difference in the expression of both proteins between male and female

in control, sham operated groups (**Fig. 16A, B**). MI led to the significant reduction of AMPK phosphorylation in male mice, whereas it was preserved in female mice (**Fig. 17A, B**). Interestingly, the female sex specific protection of AMPK activity under MI was accompanied by the significant elevation of sAC expression (**Fig. 17C, D**). No effect of MI on sAC expression was found in male mice. Thus, expression sAC and pAMPK is affected by MI in a sex-dependent manner.

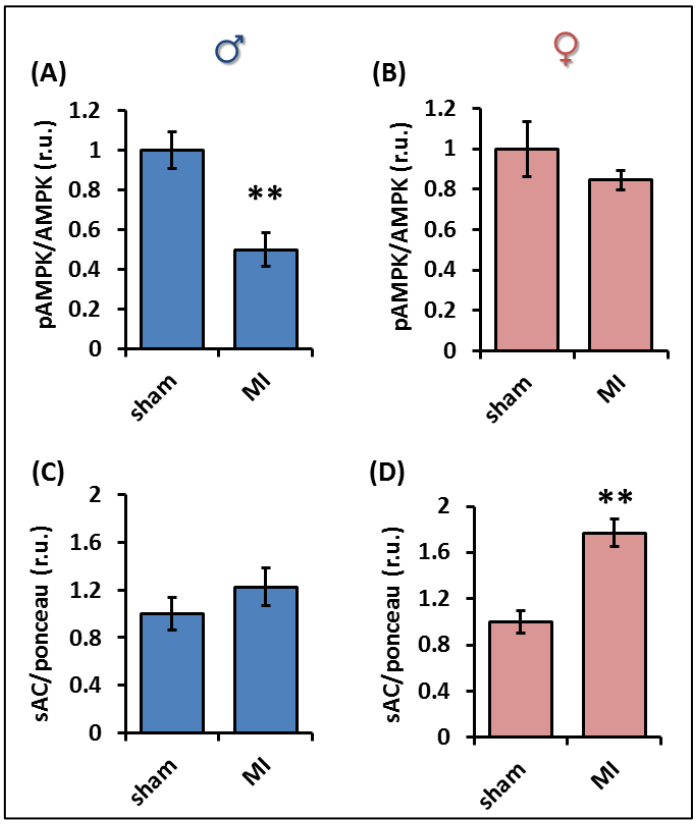

Fig. 17. MI affects sAC expression and AMPK phosphorylation in a sex-dependent manner. Western blot analyses of pAMPK (A and B) and sAC (C and D) were performed with the septum samples of male (♂) and female (♀) mice 2 weeks after sham operation or coronary artery ligation (MI). Values are expressed as mean ± SEM; *n*=4–5 for sham and *n*=9–10 for MI groups. ***P*<0.01 *vs* sham.

7 Discussion

7.1 Aims and main findings

The aim of this study was to prove the role of sAC in regulating AMPK activity and its functional significance for mitochondrial homeostasis.

The main findings are as follows:

1. Downregulation of sAC expression in H9C2 cells as well as in primary adult rat cardiomyocytes and coronary endothelial cells significantly reduced the basal AMPK activity.
2. Treatments with EPAC, but not PKA, activator rescued the effect of sAC downregulation on AMPK activity demonstrating its causal role in the sAC–AMPK axis.
3. Downregulation of sAC markedly disturbed mitochondrial and cellular homeostasis as shown by:
    a) elevation of mitochondrial and total cellular ROS formation;
    b) mitochondrial depolarization;
    c) suppressed mitochondrial biogenesis;
    d) disturbed mitophagy;
    e) reduced cellular ATP content.
4. sAC overexpression or stimulation with bicarbonate increased AMPK activity and supported cellular energy balance.
5. In contrast to sAC, stimulation of tmAC did not affect AMPK activity.
6. Analysis of the sAC–AMPK signaling in mouse model of myocardial infarction revealed an increased sAC expression in female, but not in male injured hearts, which was associated with improved pAMPK to AMPK ratio in female hearts.

7.2 sAC–AMPK axis characterization

AMPK is a key sensor of the cellular energy balance and has been shown to be strongly activated by AMP and ADP binding to γ-subunit or by phosphorylation at Thr172 of the α-subunit. Furthermore, several signaling pathways, like ROS and TAK1 (Herzig and Shaw, 2017), regulate AMPK activity. The seminal study of Park et al. (2012) also emphasized the stimulating action of cAMP signaling on AMPK activity and mitochondrial health. However, the approach used by the authors failed in specificity, i.e., total cAMP was elevated by

inhibition of PDEs. Of note, other studies applying more specific approach, i.e., stimulation of G_s-protein-coupled receptors, demonstrated an opposite, i.e., inhibitory, effect of cAMP elevation (Hurley et al., 2006b, Djouder et al., 2010, Damm et al., 2012). In these studies, specifically a sub-plasmalemmal cAMP pool was affected by the stimulation of tmAC. Thus, it seems that AMPK activity regulation by cAMP signaling is compartment-dependent. The current model of cAMP signaling describes two main cAMP compartments built by two distinct types of cAMP synthesizing enzymes, i.e., tmAC and sAC (Zippin et al., 2003). The tmAC synthetizes cAMP in response to the stimulation of G_s-protein-coupled receptors. However, this cAMP pool is mainly restricted to plasmalemma due to the activity of PDEs (Chen et al., 2000). In contrast, sAC demonstrates a basal activity and builds cAMP pools in various intracellular compartments, like nucleus and cytosol, i.e., in close proximity to the main AMPK compartments. Thus, sAC is an ideal candidate for regulating the AMPK activity by cAMP and particularly under basal conditions. Indeed, by performing sAC knockdown in H9C2 cells, a significant downregulation of AMPK activity was observed in this study (**Fig. 2**). This effect seems to be not restricted to the particular cell type and was also observed in primary cardiomyocytes as well as in coronary endothelial cells (**Fig. 3**). Therefore, sAC plays an essential role in supporting the basal AMPK activity independent of the cell type.

To analyze the potential downstream target of sAC involved in the basal AMPK activity regulation, we examined the role of PKA and EPAC. Previous reports suggested their controversial role in regulating AMPK activity, i.e., stimulating effect of EPAC (Park et al., 2012) and inhibitory effect of PKA (Hurley et al., 2006b). In accordance with these reports, we found that activation of EPAC, but not PKA, rescued AMPK activity in sAC-knockdown cells. Consistently, in control cells treatment with EPAC agonist promoted AMPK activity, whereas PKA agonist suppressed it. Thus, sAC–EPAC axis positively regulates AMPK activity under basal conditions (**Fig. 4**).

Due to the significance of AMPK in regulating cell metabolism and mitochondrial homeostasis as well as association of AMPK downregulation with aging and several diseases (Herzig and Shaw, 2017), intensive efforts have been undertaken to develop tools leading to the upregulation of AMPK activity, like AMPK agonists, resveratrol or PDE inhibitors (Cool et al., 2006, Xiao et al., 2013, Cokorinos et al., 2017, Myers et al., 2017, Narkar et al., 2008, Park et al., 2012). Therefore, we were interested to examine whether upregulation/stimulation of sAC promotes AMPK activity. Since bicarbonate is a natural sAC stimulator (Chen et al., 2000),

we treated cells with bicarbonate and observed a stable, at least for 24 h, and dose-dependent upregulation of AMPK activity. This effect of bicarbonate is likely due to the activation of sAC, because (i) bicarbonate leads to the elevation of cAMP (Chen et al., 2000) (**Fig. 12A**) and (ii) sAC knockdown or pharmacological sAC inhibition prevented the bicarbonate effect on AMPK activity (**Figs. 10C and 12B**). Noteworthy, bicarbonate specifically elevated AMPK activity in cytosol, but not in nucleus. This finding shows that AMPK activated in cytosol does not translocate to nucleus, like it was observed under metabolic inhibition (Kodiha et al., 2007) (**Fig. 11A and B**). Similar to the sAC stimulation, sAC overexpression significantly promoted AMPK activity (**Fig. 8**). In accordance with our findings, the previous report of Jaitovich et al. (2015) demonstrated that hypercapnia, i.e., a state of the increased intracellular bicarbonate concentration due to the activity of carbonic anhydrases (Chen et al., 2008), leads to AMPK activation in skeletal muscle, though no mechanisms have been analyzed.

In contrast to sAC, activation of tmAC had no effect on AMPK activity as demonstrated by FRET-based live imaging. The inhibitory effect of tmAC stimulation on AMPK activity has been found previously in several cell types and was attributed to the PKA-dependent phosphorylation of the AMPKα-subunit at S173, S485/491 or S497 (Ferretti et al., 2016, Djouder et al., 2010), which prevents other kinases from phosphorylating AMPK at Thr172. Altogether, the findings of the study argue for the positive regulation of AMPK activity, either basal or stimulated, specifically by sAC-dependent cAMP pool, whereas tmAC, when stimulated, suppressed AMPK activity (Djouder et al., 2010).

7.3 sAC–AMPK axis regulates mitochondrial homeostasis

It is well established that AMPK is a key regulator of mitochondrial homeostasis (Herzig and Shaw, 2017). Particularly, AMPK promotes the activity and expression of PGC1α and ERRα, transcription regulators required for the expression of nuclear-encoded mitochondrial proteins (Garcia-Roves et al., 2008, Jäger et al., 2007, Hu et al., 2011). On the other hand, AMPK promotes the removal of damaged mitochondria by mitophagy, e.g., via phosphorylation and activation of MFF and ULK1 (Egan et al., 2011, Toyama et al., 2016). Therefore, we were interested to know whether sAC-dependent regulation of AMPK activity could be translated to the mitochondrial biology. Indeed, we found that sAC knockdown significantly reduced mitochondrial biogenesis (**Fig. 7**), which was accompanied by

mitochondrial depolarization, increased mitochondrial ROS formation and reduced cellular ATP content, i.e., typical fingerprints of mitochondrial dysfunction (**Fig. 5**). Surprisingly, we found a significant increase of mitochondrial mass (**Fig. 6**). Together with suppressed biogenesis (**Fig. 7**), the elevation of mitochondrial mass is a strong indicator of disturbed mitochondrial clearance or mitophagy (Xiao et al., 2016). Thus, downregulation of sAC–AMPK axis leads to the disturbance of mitochondrial clearance followed by the accumulation of dysfunctional mitochondria, energy imbalance and oxidative stress. In contrast, both sAC overexpression and sAC stimulation (with bicarbonate) increased total cellular ATP content (**Figs. 9 and 13**), thus supporting the idea that sAC–AMPK axis is involved in mitochondrial homeostasis. In line with our findings, previous reports demonstrated that AMPK activation in the heart was important to prevent depletion of ATP during ischemia (Xing et al., 2003) and to promote glucose uptake and ATP recovery (Russell et al., 2004) and to reduce ROS formation (Zaha et al., 2016) during reperfusion. In addition, AMPK activation improved mitochondrial respiratory chain function and protein synthesis in the isolated fibroblasts from MELAS patients (Garrido-Maraver et al., 2015).

Altogether, the findings of the study demonstrate that a positive effect of sAC-specific cAMP pool on AMPK activity is translated to mitochondrial homeostasis and cellular energy and redox balance.

7.4 Translational aspect of the sAC–AMPK signaling

Accumulating data suggest the involvement of sAC in numerous pathologies accompanied by excessive cell death (Chagtoo et al., 2017, Appukuttan et al., 2012b) or growth (Flacke et al., 2013b, Misra and Pizzo, 2009, Misra and Pizzo, 2013, Wu et al., 2006, Corredor et al., 2012). Of note, a recent study of Wang et al. (2016) showed that advanced heart failure in rats is accompanied by a significant downregulation of sAC expression and increased sensitivity of mitochondria to calcium stress, and therefore demonstrating a potential role of sAC in cardiomyopathy. Whether this sAC downregulation contributes to the AMPK activity remains unknown. In this study, we tracked the alterations of sAC and pAMPK expression in the remote cardiac tissue in mouse that underwent MI (**Figs. 16 and 17**). Surprisingly, we found a significant upregulation of sAC expression in female, but not in male mice. Furthermore, MI led to the significant downregulation of AMPK in male, but not in female, hearts. Thus, one may suppose that preserved activity of AMPK in injured female hearts may be due to the

enhanced sAC expression. Of note, in contrast to the Wang's study (Wang et al., 2016) which demonstrates downregulation of sAC under TAC-induced cardiomyopathy in male rats, MI did not affect sAC expression in male mice, whereas increased it in female animals. This discrepancy is may be due to the differences in species (mouse *vs* rat) or in the model of cardiomyopathy (MI *vs* TAC).

The finding that sAC and pAMPK expression is altered under MI in a sex-dependent manner merits further consideration. Though, there are no data describing sex differences in sAC expression, several reports emphasized the role of estrogen in AMPK activity in heart physiology or pathology. Indeed, estrogen-mediated AMPK signaling activation has been attributed to the cardiomyocytes protection during angiotensin II-induced heart hypertrophy and injury in mice (Shen et al., 2014). Another study performed in ovariectomized rats showed that estrogen reduced cardiac apoptosis by increasing AMPK activation in aorta and heart (Bendale et al., 2013). Thus, estrogen positively influences AMPK activity. However, the underlying cellular mechanisms remain unknown. The present study suggests sAC as a potential signaling link between estrogen and AMPK, though the causal effect of sAC overexpression on AMPK activity in injured hearts still have to be confirmed.

8 Conclusions

This study reports that a sAC-specific cAMP pool positively affects AMPK activity and plays a pivotal role in mitochondrial homeostasis (**Fig. 18**). In brief, we show that sAC downregulation leads to the reduction of AMPK activity in cardiac H9C2 cells, primary adult rat cardiomyocytes and coronary endothelial cells, i.e., in a cell type independent manner. Treatment with EPAC agonist recovered AMPK activity upon sAC knockdown, whereas treatment with PKA agonist failed to rescue AMPK activity after sAC knockdown and even inhibited AMPK activity in control cells. Thus, EPAC, but not PKA, is a signaling link between sAC and AMPK. Downregulation of sAC–AMPK axis resulted in mitochondrial depolarization, increased total cellular and mitochondrial ROS formation and reduced cellular ATP content. Of note, mitochondrial mass was increased and mitochondrial biogenesis was reduced in sAC-knockdown cells. These findings suggest the disturbed mitochondrial clearance.

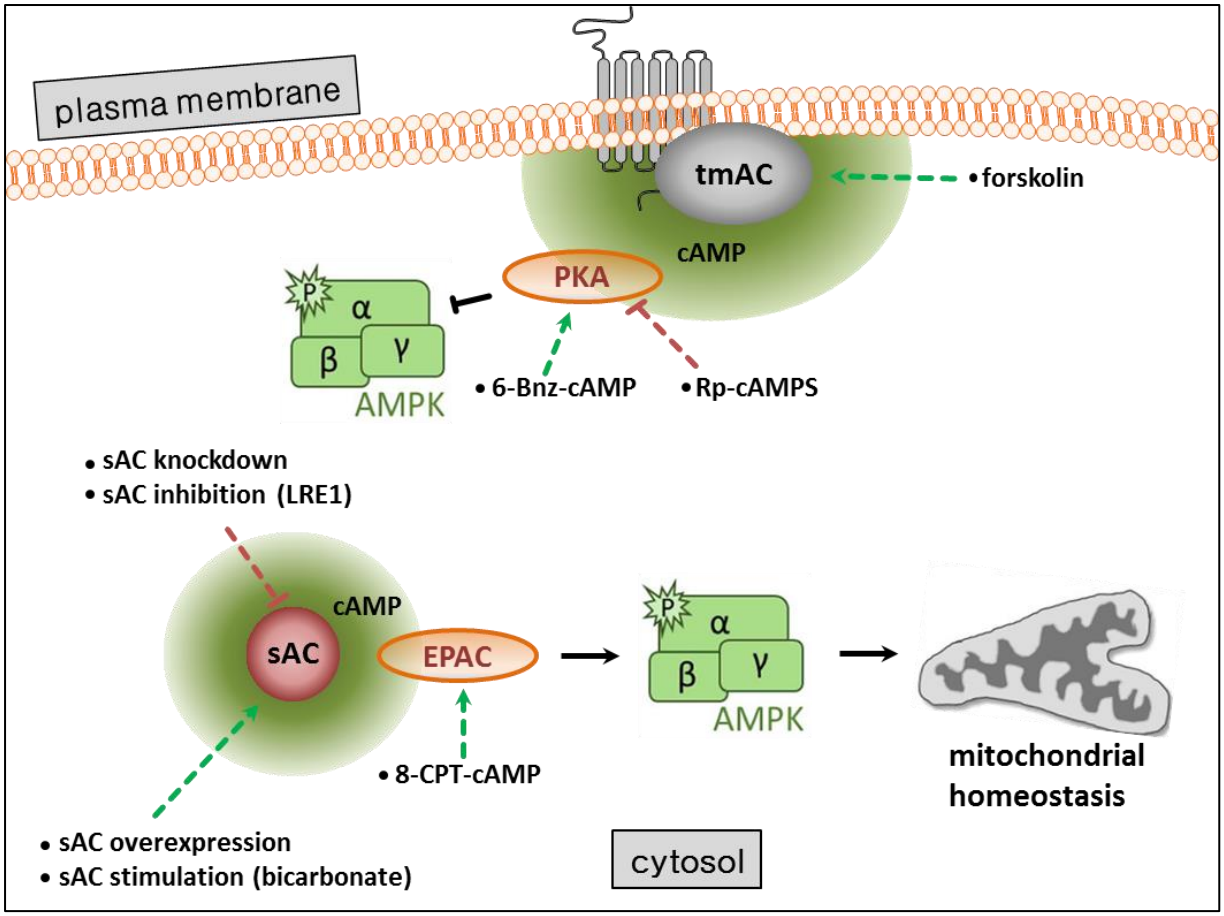

Fig. 18.Schematic representation of the study.
(•) indicates tools used to either stimulate (dotted-green arrow) or inhibit (dotted-red arrow) particular enzymes.

Upregulation of sAC activity by either sAC overexpression or stimulation with bicarbonate significantly increased AMPK activity and supported cellular energy status by increasing total cellular ATP. In contrast to sAC, stimulation of tmAC with forskolin had no effect on AMPK activity. These findings suggest a clear difference in the effect of the two main cAMP pools on AMPK activity, i.e., tmAC- and sAC-synthesized cAMP.

By analyzing the alteration of sAC expression and pAMPK level in the mouse model of MI, we found an increased sAC expression in female, but not in male injured hearts, which was associated with improved pAMPK to AMPK ratio in female hearts (**Fig. 19**). These findings suggest a sex difference in alteration of sAC-AMPK axis in cardiac pathology.

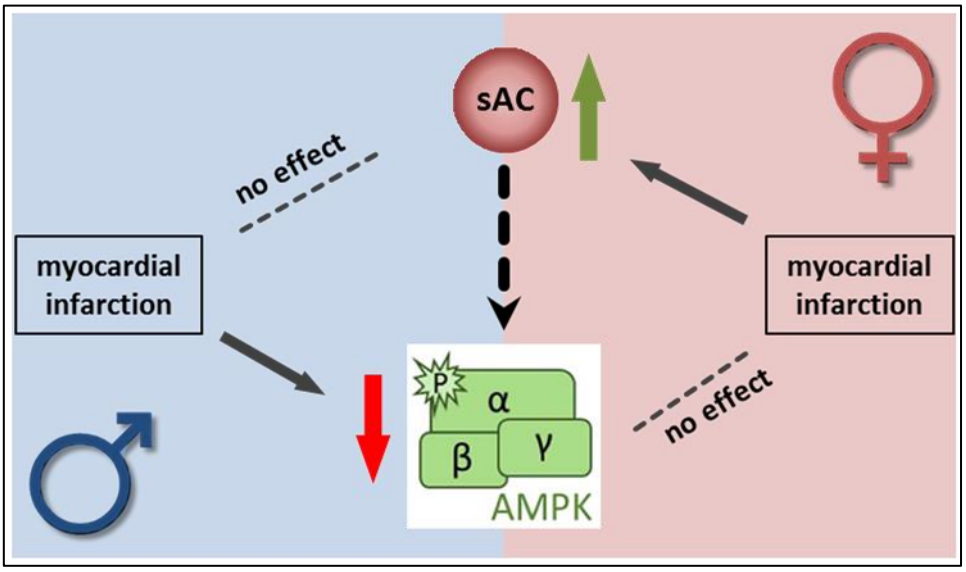

Fig. 19.Schematic representation of the sex differences observed in mice septum after MI. Green arrow denotes increase in the expression of sAC and red arrow denotes reduction in the phosphorylation of AMPK. Dotted arrow indicates hypothetical signaling. ♂ indicates male and ♀ indicates female.

AMPK, being a central regulator of several metabolic pathways and mitochondrial homeostasis, appears to be a promising target for treating obesity, insulin resistance, type 2 diabetes, nonalcoholic fatty liver disease and cardiovascular diseases. The findings of this study provide a novel understanding in the molecular basis of diseases associated with mitochondrial dysfunction and can contribute to the development of strategies to fight mitochondrial dysfunction by modulating sAC–AMPK axis.

9 Limitations of the study and outlook

First, the major part of the study was performed with immortalized rat cardiomyoblast H9C2 cells. To add more value and translational significance to the study, more experiments with primary cardiomyocytes or coronary endothelial cells should be performed. However, the main aim of the study was to elucidate the basic cell signaling and, hence, using a cellular system free of *in vivo* stimuli, such as hormones and metabolites, has an advantage. In addition, the study involved genetic manipulation of sAC and expression of FRET sensors. For these reasons, H9C2 cells seemed to be a suitable model..

Second, the causal role of AMPK in the effects of sAC up- and downregulation on the cellular energy balance and mitochondrial homeostasis still has to be confirmed. Indeed, the mitochondrial matrix-localized sAC plays a key role in the activity of the electron transport chain and, thereby, in ATP synthesis (Acin-Perez et al., 2009, Acin-Perez et al., 2011). Thus, one may suppose that sAC knockdown may also affect this intra-mitochondrial cAMP signaling, leading to the mitochondrial depolarization and ROS formation independent of AMPK downregulation. In addition, the recent report of Rahman et al. (2016) shows that sAC activity supports lysosomal function, suggesting that sAC downregulation may directly, i.e., independent of AMPK, disturb mitochondrial clearance leading to the accumulation of damaged mitochondria as has been found in this study.

Third, though a female sex specific alteration in sAC overexpression and AMPK activity has been observed under MI, whether a causal link between sAC and AMPK, in fact, exists in this or other forms of cardiomyopathy and whether sAC–AMPK axis plays a causal role in the cardiac pathology still have to be elucidated.

10 Summary

In this study, the effect of sAC on the AMPK activity and mitochondrial homeostasis was analyzed. sAC knockdown led to the depletion of cellular cAMP concentration accompanied by decreased AMPK activity in cardiac H9C2 cells, adult rat cardiomyocytes and coronary endothelial cells. EPAC, but not PKA, is a signaling link between sAC and AMPK. Functionally, sAC knockdown led to the mitochondrial depolarization, reduced cellular ATP content and increased mitochondrial ROS formation. Furthermore, sAC downregulation led to the disturbed mitochondrial clearance, as indicated by increased mitochondrial mass and reduced mitochondrial biogenesis. Consistently, sAC overexpression or stimulation with bicarbonate significantly increased AMPK activity and cellular ATP content, whereas stimulation of tmAC had no effect. By analyzing sAC–AMPK signaling in mouse model of myocardial infarction, we found an increased sAC expression in female, but not in male injured hearts, which was associated with reduced pAMPK to AMPK ratio. No changes in AMPK activity was observed in injured female hearts. The study reveals a novel sAC–AMPK signaling axis, which is cell type independent and that significantly contributes to the cellular energy and redox balance by supporting mitochondrial biogenesis and clearance. Activation of the axis improved cellular energy balance. The study provides an evidence for potential translational significance of the sAC–AMPK signaling in myocardial infarction in a sex-dependent manner.

Zusammenfassung

In der vorliegenden Studie wurde der Effekt von sAC auf die AMPK Aktivität und mitochondriale Homöostase untersucht. Der sAC-Knockdown führte zu einer Abnahme der zellulären cAMP-Konzentration, begleitet von einer verminderten AMPK-Aktivität in kardialen H9C2-Zellen, adulten Ratten Kardiomyozyten und koronaren Endothelzellen. EPAC, aber nicht PKA, stellt einen Link innerhalb der Signaltransduktion zwischen sAC und AMPK dar. Funktionell führte der sAC-Knockdown zu mitochondrialer Depolarisation, reduziertem zellulärem ATP-Gehalt und erhöhter mitochondrialer ROS-Bildung. Darüber hinaus führte die Runterregulierung von sAC- zu einer gestörten mitochondrialen Clearance, was durch eine erhöhte mitochondriale Masse und eine reduzierte mitochondriale Biogenese indiziert wurde. Konsistent dazu, erhöhte die sAC-Überexpression oder Stimulation mit Bicarbonat signifikant die AMPK-Aktivität und den zellulären ATP-Gehalt, wohingegen die Stimulation von tmAC keine Wirkung zeigte. Durch die Analyse von sAC-AMPK-Signalen im Mausmodell für Myokardinfarkte fanden wir eine erhöhte sAC-Expression bei weiblichen, aber nicht bei männlichen verletzten Herzen, was mit einem reduzierten pAMPK-zu-AMPK-Verhältnis einherging. Bei verletzten weiblichen Herzen wurden keine Veränderungen der AMPK-Aktivität beobachtet. Die Studie zeigt eine neue sAC-AMPK-Signalachse auf, die zelltypunabhängig ist und durch Unterstützung der mitochondrialen Biogenese und Clearance signifikant zur zellulären Energie- und Redoxbilanz beiträgt. Die Aktivierung dieser Achse verbessert die zelluläre Energiebilanz. Die Studie liefert einen Beleg für die mögliche translationale Signifikanz der sAC-AMPK-Signalgebung beim Myokardinfarkt in einer geschlechtsabhängigen Weise.

11 Bibliography

ACIN-PEREZ, R., RUSSWURM, M., GÜNNEWIG, K., GERTZ, M., ZOIDL, G., RAMOS, L., BUCK, J., LEVIN, L. R., RASSOW, J., MANFREDI, G. & STEEGBORN, C. 2011. A Phosphodiesterase 2A Isoform Localized to Mitochondria Regulates Respiration. *The Journal of Biological Chemistry,* 286**,** 30423-30432.

ACIN-PEREZ, R., SALAZAR, E., KAMENETSKY, M., BUCK, J., LEVIN, L. R. & MANFREDI, G. 2009. Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. *Cell metabolism,* 9**,** 265-276.

AGARWAL, S. R., CLANCY, C. E. & HARVEY, R. D. 2016. Mechanisms Restricting Diffusion of Intracellular cAMP. 6**,** 19577.

AHMADIAN, M., ABBOTT, M. J., TANG, T., HUDAK, C. S. S., KIM, Y., BRUSS, M., HELLERSTEIN, M. K., LEE, H.-Y., SAMUEL, V. T., SHULMAN, G. I., WANG, Y., DUNCAN, R. E., KANG, C. & SUL, H. S. 2011. Desnutrin/ATGL is Regulated by AMPK and is Required for a Brown Adipose Phenotype. *Cell metabolism,* 13**,** 739-748.

APPUKUTTAN, A., FLACKE, J.-P., FLACKE, H., POSADOWSKY, A., REUSCH, H. P. & LADILOV, Y. 2014. Inhibition of soluble adenylyl cyclase increases the radiosensitivity of prostate cancer cells. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1842**,** 2656-2663.

APPUKUTTAN, A., KASSECKERT, S. A., KUMAR, S., REUSCH, H. P. & LADILOV, Y. 2013. Oxysterol-induced apoptosis of smooth muscle cells is under the control of a soluble adenylyl cyclase. *Cardiovascular Research,* 99**,** 734-742.

APPUKUTTAN, A., KASSECKERT, S. A., MICOOGULLARI, M., FLACKE, J.-P., KUMAR, S., WOSTE, A., ABDALLAH, Y., POTT, L., REUSCH, H. P. & LADILOV, Y. 2012a. Type 10 adenylyl cyclase mediates mitochondrial Bax translocation and apoptosis of adult rat cardiomyocytes under simulated ischaemia/reperfusion. *Cardiovascular Research,* 93**,** 340-349.

APPUKUTTAN, A., KASSECKERT, S. A., MICOOGULLARI, M., FLACKE, J. P., KUMAR, S., WOSTE, A., ABDALLAH, Y., POTT, L., REUSCH, H. P. & LADILOV, Y. 2012b. Type 10 adenylyl cyclase mediates mitochondrial Bax translocation and apoptosis of adult rat cardiomyocytes under simulated ischaemia/reperfusion. *Cardiovasc Res,* 93**,** 340-9.

BAAS, A. F., BOUDEAU, J., SAPKOTA, G. P., SMIT, L., MEDEMA, R., MORRICE, N. A., ALESSI, D. R. & CLEVERS, H. C. 2003. Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD. *The EMBO Journal,* 22**,** 3062.

BANDO, H., ATSUMI, T., NISHIO, T., NIWA, H., MISHIMA, S., SHIMIZU, C., YOSHIOKA, N., BUCALA, R. & KOIKE, T. 2005. Phosphorylation of the 6-Phosphofructo-2-Kinase/Fructose 2,6-Bisphosphatase/PFKFB3 Family of Glycolytic Regulators in Human Cancer. *Clinical Cancer Research,* 11**,** 5784.

BENDALE, D. S., KARPE, P. A., CHHABRA, R., SHETE, S. P., SHAH, H. & TIKOO, K. 2013. 17-β Oestradiol prevents cardiovascular dysfunction in post-menopausal metabolic syndrome by affecting SIRT1/AMPK/H3 acetylation. *British Journal of Pharmacology,* 170**,** 779-795.

BENTO, C. F., RENNA, M., GHISLAT, G., PURI, C., ASHKENAZI, A., VICINANZA, M., MENZIES, F. M. & RUBINSZTEIN, D. C. 2016. Mammalian Autophagy: How Does It Work? *Annual Review of Biochemistry,* 85**,** 685-713.

BITTERMAN, J. L., RAMOS-ESPIRITU, L., DIAZ, A., LEVIN, L. R. & BUCK, J. 2013. Pharmacological Distinction between Soluble and Transmembrane Adenylyl Cyclases. *The Journal of Pharmacology and Experimental Therapeutics,* 347**,** 589-598.

BRANDAUER, J., VIENBERG, S. G., ANDERSEN, M. A., RINGHOLM, S., RISIS, S., LARSEN, P. S., KRISTENSEN, J. M., FRØSIG, C., LEICK, L., FENTZ, J., JØRGENSEN, S., KIENS, B., WOJTASZEWSKI, J. F. P., RICHTER, E. A., ZIERATH, J. R., GOODYEAR, L. J., PILEGAARD, H. & TREEBAK, J. T. 2013. AMP-activated protein kinase regulates nicotinamide phosphoribosyl transferase expression in skeletal muscle. *The Journal of Physiology,* 591**,** 5207-5220.

BROWN, D., BOULEY, R., PĂUNESCU, T. G., BRETON, S. & LU, H. A. J. 2012. New insights into the dynamic regulation of water and acid-base balance by renal epithelial cells. *American Journal of Physiology - Cell Physiology,* 302**,** C1421-C1433.

BUCK, J., SINCLAIR, M. L., SCHAPAL, L., CANN, M. J. & LEVIN, L. R. 1999. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 79-84.

BUJAK, A. L., CRANE, J. D., LALLY, J. S., FORD, R. J., KANG, S. J., REBALKA, I. A., GREEN, A. E., KEMP, B. E., HAWKE, T. J., SCHERTZER, J. D. & STEINBERG, G. R. 2015. AMPK Activation of Muscle Autophagy Prevents Fasting-Induced Hypoglycemia and Myopathy during Aging. *Cell metabolism,* 21**,** 883-890.

CALEBIRO, D., NIKOLAEV, V. O., GAGLIANI, M. C., DE FILIPPIS, T., DEES, C., TACCHETTI, C., PERSANI, L. & LOHSE, M. J. 2009. Persistent cAMP-Signals Triggered by Internalized G-Protein–Coupled Receptors. *PLoS Biology,* 7**,** e1000172.

CAMMAROTA, M., PARATCHA, G., BEVILAQUA, L. R. M., DE STEIN, M. L., LOPEZ, M., DE IRALDI, A. P., IZQUIERDO, I. & MEDINA, J. H. 1999. Cyclic AMP-Responsive Element Binding Protein in Brain Mitochondria. *Journal of Neurochemistry,* 72**,** 2272-2277.

CARNEGIE, G. K., MEANS, C. K. & SCOTT, J. D. 2009. A-Kinase Anchoring Proteins: From protein complexes to physiology and disease. *IUBMB life,* 61**,** 394-406.

CATTERALL, W. A. 2015. Regulation of Cardiac Calcium Channels in the Fight-or-Flight Response. *Current molecular pharmacology,* 8**,** 12-21.

CHA, M.-Y., KIM, D. K. & MOOK-JUNG, I. 2015. The role of mitochondrial DNA mutation on neurodegenerative diseases. *Experimental & Molecular Medicine,* 47**,** e150.

CHAGTOO, M., GEORGE, N., PATHAK, N., TIWARI, S., GODBOLE, M. M. & LADILOV, Y. 2017. Inhibition of Intracellular Type 10 Adenylyl Cyclase Protects Cortical Neurons Against Reperfusion-Induced Mitochondrial Injury and Apoptosis. *Mol Neurobiol*.

CHALOUPKA, J. A., BULLOCK, S. A., IOURGENKO, V., LEVIN, L. R. & BUCK, J. 2006. Autoinhibitory regulation of soluble adenylyl cyclase. *Molecular Reproduction and Development,* 73**,** 361-368.

CHANG, J.-C. & OUDE-ELFERINK, R. P. J. 2014. Role of the bicarbonate-responsive soluble adenylyl cyclase in pH sensing and metabolic regulation. *Frontiers in Physiology,* 5**,** 42.

CHEN, J., LECUONA, E., BRIVA, A., WELCH, L. C. & SZNAJDER, J. I. 2008. Carbonic Anhydrase II and Alveolar Fluid Reabsorption during Hypercapnia. *American Journal of Respiratory Cell and Molecular Biology,* 38**,** 32-37.

CHEN, M.-L., YI, L., JIN, X., LIANG, X.-Y., ZHOU, Y., ZHANG, T., XIE, Q., ZHOU, X., CHANG, H., FU, Y.-J., ZHU, J.-D., ZHANG, Q.-Y. & MI, M.-T. 2013. Resveratrol attenuates vascular endothelial inflammation by inducing autophagy through the cAMP signaling pathway. *Autophagy,* 9**,** 2033-2045.

CHEN, R., FEARNLEY, I. M., PEAK-CHEW, S. Y. & WALKER, J. E. 2004. The Phosphorylation of Subunits of Complex I from Bovine Heart Mitochondria. *Journal of Biological Chemistry,* 279**,** 26036-26045.

CHEN, X., BAUMLIN, N., BUCK, J., LEVIN, L. R., FREGIEN, N. & SALATHE, M. 2014. A Soluble Adenylyl Cyclase Form Targets to Axonemes and Rescues Beat Regulation in Soluble Adenylyl Cyclase Knockout Mice. *American Journal of Respiratory Cell and Molecular Biology,* 51**,** 750-760.

CHEN, Y., CANN, M. J., LITVIN, T. N., IOURGENKO, V., SINCLAIR, M. L., LEVIN, L. R. & BUCK, J. 2000. Soluble Adenylyl Cyclase as an Evolutionarily Conserved Bicarbonate Sensor. *Science,* 289**,** 625-628.

CHEUNG, P. C., SALT, I. P., DAVIES, S. P., HARDIE, D. G. & CARLING, D. 2000. Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. *Biochemical Journal,* 346**,** 659-669.

CHOWANADISAI, W., BAUERLY, K. A., TCHAPARIAN, E., WONG, A., CORTOPASSI, G. A. & RUCKER, R. B. 2010. Pyrroloquinoline Quinone Stimulates Mitochondrial Biogenesis through cAMP Response Element-binding Protein Phosphorylation and Increased PGC-1α Expression. *The Journal of Biological Chemistry,* 285**,** 142-152.

COKORINOS, E. C., DELMORE, J., REYES, A. R., ALBUQUERQUE, B., KJØBSTED, R., JØRGENSEN, N. O., TRAN, J.-L., JATKAR, A., CIALDEA, K., ESQUEJO, R. M., MEISSEN, J., CALABRESE, M. F., CORDES, J., MOCCIA, R., TESS, D., SALATTO, C. T., COSKRAN, T. M., OPSAHL, A. C., FLYNN, D., BLATNIK, M., LI, W., KINDT, E., FORETZ, M., VIOLLET, B., WARD, J., KURUMBAIL, R. G., KALGUTKAR, A. S., WOJTASZEWSKI, J. F. P., CAMERON, K. O. & MILLER, R. A. 2017. Activation of Skeletal Muscle AMPK Promotes Glucose Disposal

and Glucose Lowering in Non-human Primates and Mice. *Cell Metabolism,* 25**,** 1147-1159.e10.

COOL, B., ZINKER, B., CHIOU, W., KIFLE, L., CAO, N., PERHAM, M., DICKINSON, R., ADLER, A., GAGNE, G., IYENGAR, R., ZHAO, G., MARSH, K., KYM, P., JUNG, P., CAMP, H. S. & FREVERT, E. 2006. Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metabolism,* 3**,** 403-416.

CORREDOR, R. G., TRAKHTENBERG, E. F., PITA-THOMAS, W., JIN, X., HU, Y. & GOLDBERG, J. L. 2012. Soluble adenylyl cyclase activity is necessary for retinal ganglion cell survival and axon growth. *The Journal of Neuroscience,* 32**,** 7734-7744.

CRUTE, B. E., SEEFELD, K., GAMBLE, J., KEMP, B. E. & WITTERS, L. A. 1998. Functional Domains of the α1 Catalytic Subunit of the AMP-activated Protein Kinase. *Journal of Biological Chemistry,* 273**,** 35347-35354.

DAMM, E., BUECH, T. R. H., GUDERMANN, T. & BREIT, A. 2012. Melanocortin-Induced PKA Activation Inhibits AMPK Activity Via ERK-1/2 and LKB-1 in Hypothalamic GT1-7 Cells. *Molecular Endocrinology,* 26**,** 643-654.

DAVIES, S., HELPS, N., COHEN, P. & HARDIE, D. 1995a. 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2Cα and native bovine protein phosphatase-2Ac. *FEBS Letters,* 377**,** 421-425.

DAVIES, S. P., HELPS, N. R., COHEN, P. T. & HARDIE, D. G. 1995b. 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2Cα and native bovine protein phosphatase-2Ac. *FEBS Letters,* 377**,** 421-425.

DE RASMO, D., MICELLI, L., SANTERAMO, A., SIGNORILE, A., LATTANZIO, P. & PAPA, S. 2016. cAMP regulates the functional activity, coupling efficiency and structural organization of mammalian FOF1 ATP synthase. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1857**,** 350-358.

DE RASMO, D., SIGNORILE, A., LARIZZA, M., PACELLI, C., COCCO, T. & PAPA, S. 2012. Activation of the cAMP cascade in human fibroblast cultures rescues the activity of oxidatively damaged complex I. *Free Radical Biology and Medicine,* 52**,** 757-764.

DE RASMO, D., SIGNORILE, A., ROCA, E. & PAPA, S. 2009. cAMP response element-binding protein (CREB) is imported into mitochondria and promotes protein synthesis. *FEBS Journal,* 276**,** 4325-4333.

DE RASMO, D., SIGNORILE, A., SANTERAMO, A., LARIZZA, M., LATTANZIO, P., CAPITANIO, G. & PAPA, S. 2015. Intramitochondrial adenylyl cyclase controls the turnover of nuclear-encoded subunits and activity of mammalian complex I of the respiratory chain. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1853**,** 183-191.

DI BENEDETTO, G., SCALZOTTO, E., MONGILLO, M. & POZZAN, T. 2013. Mitochondrial Ca2+ Uptake Induces Cyclic AMP Generation in the Matrix and Modulates Organelle ATP Levels. *Cell Metabolism,* 17**,** 965-975.

DJOUDER, N., TUERK, R. D., SUTER, M., SALVIONI, P., THALI, R. F., SCHOLZ, R., VAAHTOMERI, K., AUCHLI, Y., RECHSTEINER, H., BRUNISHOLZ, R. A., VIOLLET, B., MÄKELÄ, T. P., WALLIMANN, T., NEUMANN, D. & KREK, W. 2010. PKA phosphorylates and inactivates AMPKα to promote efficient lipolysis. *The EMBO Journal,* 29**,** 469-481.

DUCOMMUN, S., DEAK, M., SUMPTON, D., FORD, R. J., NÚÑEZ GALINDO, A., KUSSMANN, M., VIOLLET, B., STEINBERG, G. R., FORETZ, M., DAYON, L., MORRICE, N. A. & SAKAMOTO, K. 2015. Motif affinity and mass spectrometry proteomic approach for the discovery of cellular AMPK targets: Identification of mitochondrial fission factor as a new AMPK substrate. *Cellular Signalling,* 27**,** 978-988.

EGAN, D. F., SHACKELFORD, D. B., MIHAYLOVA, M. M., GELINO, S. R., KOHNZ, R. A., MAIR, W., VASQUEZ, D. S., JOSHI, A., GWINN, D. M., TAYLOR, R., ASARA, J. M., FITZPATRICK, J., DILLIN, A., VIOLLET, B., KUNDU, M., HANSEN, M. & SHAW, R. J. 2011. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science (New York, N.Y.),* 331**,** 456-461.

EICHNER, L. J. & GIGUÈRE, V. 2011. Estrogen related receptors (ERRs): A new dawn in transcriptional control of mitochondrial gene networks. *Mitochondrion,* 11**,** 544-552.

EIYAMA, A., KONDO-OKAMOTO, N. & OKAMOTO, K. 2013. Mitochondrial degradation during starvation is selective and temporally distinct from bulk autophagy in yeast. *FEBS Letters,* 587**,** 1787-1792.

ESPOSITO, G., JAISWAL, B. S., XIE, F., KRAJNC-FRANKEN, M. A. M., ROBBEN, T. J. A. A., STRIK, A. M., KUIL, C., PHILIPSEN, R. L. A., VAN DUIN, M., CONTI, M. & GOSSEN, J. A. 2004. Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 2993-2998.

FALLER, W. J., JACKSON, T. J., KNIGHT, J. R. P., RIDGWAY, R. A., JAMIESON, T., KARIM, S. A., JONES, C., RADULESCU, S., HUELS, D. J., MYANT, K. B., DUDEK, K. M., CASEY, H. A., SCOPELLITI, A., CORDERO, J. B., VIDAL, M., PENDE, M., RYAZANOV, A. G., SONENBERG, N., MEYUHAS, O., HALL, M. N., BUSHELL, M., WILLIS, A. E. & SANSOM, O. J. 2015. mTORC1 mediated translational elongation limits intestinal tumour initiation and growth. *Nature,* 517**,** 497-500.

FANG, J.-K., PRABU, S. K., SEPURI, N. B., RAZA, H., ANANDATHEERTHAVARADA, H. K., GALATI, D., SPEAR, J. & AVADHANI, N. G. 2007. Site Specific Phosphorylation of Cytochrome c Oxidase Subunits I, IVi1 and Vb in Rabbit Hearts Subjected to Ischemia/Reperfusion. *FEBS letters,* 581**,** 1302-1310.

FARRELL, J., RAMOS, L., TRESGUERRES, M., KAMENETSKY, M., LEVIN, L. R. & BUCK, J. 2008. Somatic 'Soluble' Adenylyl Cyclase Isoforms Are Unaffected in Sacytm1Lex/Sacytm1Lex 'Knockout' Mice. *PLOS ONE,* 3**,** e3251.

FEDERMAN, A. D., CONKLIN, B. R., SCHRADER, K. A., REED, R. R. & BOURNE, H. R. 1992. Hormonal stimulation of adenylyl cyclase through Gi-protein [beta][gamma] subunits. *Nature,* 356**,** 159-161.

FEINSTEIN, W. P., ZHU, B., LEAVESLEY, S. J., SAYNER, S. L. & RICH, T. C. 2012. Assessment of cellular mechanisms contributing to cAMP compartmentalization in pulmonary microvascular endothelial cells. *American Journal of Physiology - Cell Physiology,* 302**,** C839.

FERRETTI, A. C., TONUCCI, F. M., HIDALGO, F., ALMADA, E., LAROCCA, M. C. & FAVRE, C. 2016. AMPK and PKA interaction in the regulation of survival of liver cancer cells subjected to glucose starvation. *Oncotarget,* 7**,** 17815-28.

FLACKE, J.-P., FLACKE, H., APPUKUTTAN, A., PALISAAR, R.-J., NOLDUS, J., ROBINSON, B. D., REUSCH, H. P., ZIPPIN, J. H. & LADILOV, Y. 2013a. Type 10 Soluble Adenylyl Cyclase Is Overexpressed in Prostate Carcinoma and Controls Proliferation of Prostate Cancer Cells. *The Journal of Biological Chemistry,* 288**,** 3126-3135.

FLACKE, J. P., FLACKE, H., APPUKUTTAN, A., PALISAAR, R. J., NOLDUS, J., ROBINSON, B. D., REUSCH, H. P., ZIPPIN, J. H. & LADILOV, Y. 2013b. Type 10 soluble adenylyl cyclase is overexpressed in prostate carcinoma and controls proliferation of prostate cancer cells. *J Biol Chem,* 288**,** 3126-35.

FROESE, A., BREHER, S. S., WALDEYER, C., SCHINDLER, R. F. R., NIKOLAEV, V. O., RINNÉ, S., WISCHMEYER, E., SCHLUETER, J., BECHER, J., SIMRICK, S., VAUTI, F., KUHTZ, J., MEISTER, P., KREISSL, S., TORLOPP, A., LIEBIG, S. K., LAAKMANN, S., MÜLLER, T. D., NEUMANN, J., STIEBER, J., LUDWIG, A., MAIER, S. K., DECHER, N., ARNOLD, H.-H., KIRCHHOF, P., FABRITZ, L. & BRAND, T. 2012. Popeye domain containing proteins are essential for stress-mediated modulation of cardiac pacemaking in mice. *The Journal of Clinical Investigation,* 122**,** 1119-1130.

FRYER, L. G. D., PARBU-PATEL, A. & CARLING, D. 2002. The Anti-diabetic Drugs Rosiglitazone and Metformin Stimulate AMP-activated Protein Kinase through Distinct Signaling Pathways. *Journal of Biological Chemistry,* 277**,** 25226-25232.

GALIC, S., FULLERTON, M. D., SCHERTZER, J. D., SIKKEMA, S., MARCINKO, K., WALKLEY, C. R., IZON, D., HONEYMAN, J., CHEN, Z.-P., VAN DENDEREN, B. J., KEMP, B. E. & STEINBERG, G. R. 2011. Hematopoietic AMPK β1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. *The Journal of Clinical Investigation,* 121**,** 4903-4915.

GANCEDO, J. M. 2013. Biological roles of cAMP: variations on a theme in the different kingdoms of life. *Biological Reviews,* 88**,** 645-668.

GARCIA-ROVES, P. M., OSLER, M. E., HOLMSTRÖM, M. H. & ZIERATH, J. R. 2008. Gain-of-function R225Q Mutation in AMP-activated Protein Kinase γ3 Subunit Increases Mitochondrial Biogenesis in Glycolytic Skeletal Muscle. *Journal of Biological Chemistry,* 283**,** 35724-35734.

GARRIDO-MARAVER, J., PAZ, M. V., CORDERO, M. D., BAUTISTA-LORITE, J., OROPESA-ÁVILA, M., DE LA MATA, M., PAVÓN, A. D., DE LAVERA, I., ALCOCER-GÓMEZ, E., GALÁN, F., YBOT GONZÁLEZ, P., COTÁN, D., JACKSON, S. & SÁNCHEZ-ALCÁZAR, J. A. 2015. Critical role of AMP-activated protein kinase in the balance between mitophagy and mitochondrial biogenesis in MELAS disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1852**,** 2535-2553.

GENG, W., WANG, Z., ZHANG, J., REED, B. Y., PAK, C. Y. C. & MOE, O. W. 2005. Cloning and characterization of the human soluble adenylyl cyclase. *American Journal of Physiology - Cell Physiology,* 288**,** C1305.

GERHART-HINES, Z., DOMINY, J. E., BLÄTTLER, S. M., JEDRYCHOWSKI, M. P., BANKS, A. S., LIM, J.-H., CHIM, H., GYGI, S. P. & PUIGSERVER, P. 2011. The cAMP/PKA pathway rapidly activates SIRT1 to promote fatty acid oxidation independently of changes in NAD(+). *Molecular Cell,* 44**,** 851-863.

GOMES, L. C., DI BENEDETTO, G. & SCORRANO, L. 2011. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. *Nature cell biology,* 13**,** 589-598.

GONG, G., SONG, M., CSORDAS, G., KELLY, D. P., MATKOVICH, S. J. & DORN, G. W. 2015. Parkin-mediated mitophagy directs perinatal cardiac metabolic maturation. *Science (New York, N.Y.),* 350**,** aad2459-aad2459.

GOWANS, GRAEME J., HAWLEY, SIMON A., ROSS, FIONA A. & HARDIE, D G. 2013. AMP Is a True Physiological Regulator of AMP-Activated Protein Kinase by Both Allosteric Activation and Enhancing Net Phosphorylation. *Cell Metabolism,* 18**,** 556-566.

GRIMSRUD, P. A., CARSON, J. J., HEBERT, A. S., HUBLER, S. L., NIEMI, N. M., BAILEY, D. J., JOCHEM, A., STAPLETON, D. S., KELLER, M. P., WESTPHALL, M. S., YANDELL, B. S., ATTIE, A. D., COON, J. J. & PAGLIARINI, D. J. 2012. A quantitative map of the liver mitochondrial phosphoproteome reveals post-translational control of ketogenesis. *Cell metabolism,* 16**,** 672-683.

GWINN, D. M., SHACKELFORD, D. B., EGAN, D. F., MIHAYLOVA, M. M., MERY, A., VASQUEZ, D. S., TURK, B. E. & SHAW, R. J. 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Molecular cell,* 30**,** 214-226.

HARDIE, D. G. 2013. AMPK: A Target for Drugs and Natural Products With Effects on Both Diabetes and Cancer. *Diabetes,* 62**,** 2164-2172.

HARDIE, D. G., CARLING, D. & GAMBLIN, S. J. 2011. AMP-activated protein kinase: also regulated by ADP? *Trends in Biochemical Sciences,* 36**,** 470-477.

HASENOUR, C. M., RIDLEY, D. E., HUGHEY, C. C., JAMES, F. D., DONAHUE, E. P., SHEARER, J., VIOLLET, B., FORETZ, M. & WASSERMAN, D. H. 2014. 5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) Effect on Glucose Production, but Not Energy Metabolism, Is Independent of Hepatic AMPK in Vivo. *Journal of Biological Chemistry,* 289**,** 5950-5959.

HAWLEY, S. A., BOUDEAU, J., REID, J. L., MUSTARD, K. J., UDD, L., MÄKELÄ, T. P., ALESSI, D. R. & HARDIE, D. G. 2003. Complexes between the LKB1 tumor suppressor, STRADα/β and MO25α/β are upstream kinases in the AMP-activated protein kinase cascade. *Journal of Biology,* 2**,** 28-28.

HAWLEY, S. A., DAVISON, M., WOODS, A., DAVIES, S. P., BERI, R. K., CARLING, D. & HARDIE, D. G. 1996. Characterization of the AMP-activated Protein Kinase Kinase from Rat Liver and Identification of Threonine 172 as the Major Site at Which It Phosphorylates AMP-activated Protein Kinase. *Journal of Biological Chemistry,* 271**,** 27879-27887.

HAWLEY, S. A., GADALLA, A. E., OLSEN, G. S. & HARDIE, D. G. 2002. The Antidiabetic Drug Metformin Activates the AMP-Activated Protein Kinase Cascade via an Adenine Nucleotide-Independent Mechanism. *Diabetes,* 51**,** 2420.

HAWLEY, S. A., PAN, D. A., MUSTARD, K. J., ROSS, L., BAIN, J., EDELMAN, A. M., FRENGUELLI, B. G. & HARDIE, D. G. 2005. Calmodulin-dependent protein kinase kinase-β is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metabolism,* 2**,** 9-19.

HEBERT-CHATELAIN, E., DESPREZ, T., SERRAT, R., BELLOCCHIO, L., SORIA-GOMEZ, E., BUSQUETS-GARCIA, A., PAGANO ZOTTOLA, A. C., DELAMARRE, A., CANNICH, A., VINCENT, P., VARILH, M., ROBIN, L. M., TERRAL, G., GARCÍA-FERNÁNDEZ, M. D., COLAVITA, M., MAZIER, W., DRAGO, F., PUENTE, N., REGUERO, L., ELEZGARAI, I., DUPUY, J.-W., COTA, D., LOPEZ-RODRIGUEZ, M.-L., BARREDA-GÓMEZ, G., MASSA, F., GRANDES, P., BÉNARD, G. & MARSICANO, G. 2016. A cannabinoid link between mitochondria and memory. *Nature,* 539**,** 555-559.

HERZIG, S. & SHAW, R. J. 2017. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nature Reviews Molecular Cell Biology***,** nrm.2017.95.

HESS, K. C., JONES, B. H., MARQUEZ, B., CHEN, Y., ORD, T. S., KAMENETSKY, M., MIYAMOTO, C., ZIPPIN, J. H., KOPF, G. S., SUAREZ, S. S., LEVIN, L. R., WILLIAMS, C. J., BUCK, J. & MOSS, S. B. 2005. The "Soluble" Adenylyl Cyclase in Sperm Mediates Multiple Signaling Events Required for Fertilization. *Developmental cell,* 9**,** 249-259.

HESS, K. C., LIU, J., MANFREDI, G., MÜHLSCHLEGEL, F. A., BUCK, J., LEVIN, L. R. & BARRIENTOS, A. 2014. A mitochondrial CO(2)-adenylyl cyclase-cAMP signalosome controls yeast normoxic cytochrome c oxidase activity. *The FASEB Journal,* 28**,** 4369-4380.

HONDA, S., ARAKAWA, S., NISHIDA, Y., YAMAGUCHI, H., ISHII, E. & SHIMIZU, S. 2014. Ulk1-mediated Atg5-independent macroautophagy mediates elimination of mitochondria from embryonic reticulocytes. 5**,** 4004.

HORMAN, S., VERTOMMEN, D., HEATH, R., NEUMANN, D., MOUTON, V., WOODS, A., SCHLATTNER, U., WALLIMANN, T., CARLING, D., HUE, L. & RIDER, M. H. 2006. Insulin Antagonizes Ischemia-induced Thr172 Phosphorylation of AMP-activated Protein Kinase α-Subunits in Heart via Hierarchical Phosphorylation of Ser485/491. *Journal of Biological Chemistry,* 281**,** 5335-5340.

HORNBECK, P. V., KORNHAUSER, J. M., TKACHEV, S., ZHANG, B., SKRZYPEK, E., MURRAY, B., LATHAM, V. & SULLIVAN, M. 2012. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Research,* 40**,** D261-D270.

HU, X., XU, X., LU, Z., ZHANG, P., FASSETT, J., ZHANG, Y., XIN, Y., HALL, J. L., VIOLLET, B., BACHE, R. J., HUANG, Y. & CHEN, Y. 2011. AMP Activated Protein Kinase-α2 Regulates Expression of Estrogen-Related Receptor-α, a Metabolic Transcription Factor Related to Heart Failure Development. *Hypertension,* 58**,** 696-703.

HUDSON, E. R., PAN, D. A., JAMES, J., LUCOCQ, J. M., HAWLEY, S. A., GREEN, K. A., BABA, O., TERASHIMA, T. & HARDIE, D. G. 2003. A Novel Domain in AMP-Activated Protein Kinase Causes Glycogen Storage Bodies Similar to Those Seen in Hereditary Cardiac Arrhythmias. *Current Biology,* 13**,** 861-866.

HURLEY, R. L., ANDERSON, K. A., FRANZONE, J. M., KEMP, B. E., MEANS, A. R. & WITTERS, L. A. 2005. The Ca2+/Calmodulin-dependent Protein Kinase Kinases Are AMP-activated Protein Kinase Kinases. *Journal of Biological Chemistry,* 280**,** 29060-29066.

HURLEY, R. L., BARRE, L. K., WOOD, S. D., ANDERSON, K. A., KEMP, B. E., MEANS, A. R. & WITTERS, L. A. 2006a. Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular cAMP. *J Biol Chem,* 281**,** 36662-72.

HURLEY, R. L., BARRÉ, L. K., WOOD, S. D., ANDERSON, K. A., KEMP, B. E., MEANS, A. R. & WITTERS, L. A. 2006b. Regulation of AMP-activated Protein Kinase by Multisite Phosphorylation in Response to Agents That Elevate Cellular cAMP. *Journal of Biological Chemistry,* 281**,** 36662-36672.

IGNOUL, S. & EGGERMONT, J. 2005. CBS domains: structure, function, and pathology in human proteins. *American Journal of Physiology - Cell Physiology,* 289**,** C1369.

INOKI, K., ZHU, T. & GUAN, K.-L. 2003. TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. *Cell,* 115**,** 577-590.

INOKUCHI-SHIMIZU, S., PARK, E. J., ROH, Y. S., YANG, L., ZHANG, B., SONG, J., LIANG, S., PIMIENTA, M., TANIGUCHI, K., WU, X., ASAHINA, K., LAGAKOS, W., MACKEY, M. R., AKIRA, S., ELLISMAN, M. H., SEARS, D. D., OLEFSKY, J. M., KARIN, M., BRENNER, D. A. & SEKI, E. 2014. TAK1-mediated autophagy and fatty acid oxidation prevent hepatosteatosis and tumorigenesis. *The Journal of Clinical Investigation,* 124**,** 3566-3578.

ISMAIL, S., GHERARDI, M.-J., FROESE, A., ZANOUN, M., GIGOUX, V., CLERC, P., GAITS-IACOVONI, F., STEYAERT, J., NIKOLAEV, V. O. & FOURMY, D. 2016. Internalized Receptor for Glucose-dependent Insulinotropic Peptide stimulates adenylyl cyclase on early endosomes. *Biochemical Pharmacology,* 120**,** 33-45.

ITAKURA, E., KISHI-ITAKURA, C., KOYAMA-HONDA, I. & MIZUSHIMA, N. 2012. Structures containing Atg9A and the ULK1 complex independently target depolarized

mitochondria at initial stages of Parkin-mediated mitophagy. *Journal of Cell Science,* 125**,** 1488.

JÄGER, S., HANDSCHIN, C., ST.-PIERRE, J. & SPIEGELMAN, B. M. 2007. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 12017-12022.

JAITOVICH, A., ANGULO, M., LECUONA, E., DADA, L. A., WELCH, L. C., CHENG, Y., GUSAROVA, G., CECO, E., LIU, C., SHIGEMURA, M., BARREIRO, E., PATTERSON, C., NADER, G. A. & SZNAJDER, J. I. 2015. High CO(2) Levels Cause Skeletal Muscle Atrophy via AMP-activated Kinase (AMPK), FoxO3a Protein, and Muscle-specific Ring Finger Protein 1 (MuRF1). *The Journal of Biological Chemistry,* 290**,** 9183-9194.

JORNAYVAZ, F. R. & SHULMAN, G. I. 2010. Regulation of mitochondrial biogenesis. *Essays in biochemistry,* 47**,** 10.1042/bse0470069.

KAMENETSKY, M., MIDDELHAUFE, S., BANK, E. M., LEVIN, L. R., BUCK, J. & STEEGBORN, C. 2006. Molecular Details of cAMP Generation in Mammalian Cells: A Tale of Two Systems. *Journal of molecular biology,* 362**,** 623-639.

KAUPP, U. B. & SEIFERT, R. 2002. Cyclic Nucleotide-Gated Ion Channels. *Physiological Reviews,* 82**,** 769.

KIM, J., KUNDU, M., VIOLLET, B. & GUAN, K.-L. 2011a. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature cell biology,* 13**,** 132-141.

KIM, J., KUNDU, M., VIOLLET, B. & GUAN, K.-L. 2011b. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. 13**,** 132.

KIMBALL, S. R., SIEGFRIED, B. A. & JEFFERSON, L. S. 2004. Glucagon Represses Signaling through the Mammalian Target of Rapamycin in Rat Liver by Activating AMP-activated Protein Kinase. *Journal of Biological Chemistry,* 279**,** 54103-54109.

KLEINBOELTING, S., DIAZ, A., MONIOT, S., VAN DEN HEUVEL, J., WEYAND, M., LEVIN, L. R., BUCK, J. & STEEGBORN, C. 2014. Crystal structures of human soluble adenylyl cyclase reveal mechanisms of catalysis and of its activation through bicarbonate. *Proceedings of the National Academy of Sciences,* 111**,** 3727-3732.

KODIHA, M., RASSI, J. G., BROWN, C. M. & STOCHAJ, U. 2007. Localization of AMP kinase is regulated by stress, cell density, and signaling through the MEK→ERK1/2 pathway. *American Journal of Physiology - Cell Physiology,* 293**,** C1427.

KOKA, S., ALURI, H. S., XI, L., LESNEFSKY, E. J. & KUKREJA, R. C. 2014. Chronic inhibition of phosphodiesterase 5 with tadalafil attenuates mitochondrial dysfunction in type 2 diabetic hearts: potential role of NO/SIRT1/PGC-1α signaling. *American Journal of Physiology - Heart and Circulatory Physiology,* 306**,** H1558-H1568.

KOO, S.-H., FLECHNER, L., QI, L., ZHANG, X., SCREATON, R. A., JEFFRIES, S., HEDRICK, S., XU, W., BOUSSOUAR, F., BRINDLE, P., TAKEMORI, H. & MONTMINY, M. 2005. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature,* 437**,** 1109-11.

KUDO, N., GILLESPIE, J. G., KUNG, L., WITTERS, L. A., SCHULZ, R., CLANACHAN, A. S. & LOPASCHUK, G. D. 1996. Characterization of 5'AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 1301**,** 67-75.

KUMAR, S., APPUKUTTAN, A., MAGHNOUJ, A., HAHN, S., PETER REUSCH, H. & LADILOV, Y. 2014. Suppression of soluble adenylyl cyclase protects smooth muscle cells against oxidative stress-induced apoptosis. *Apoptosis,* 19**,** 1069-1079.

KUMAR, S., KOSTIN, S., FLACKE, J.-P., REUSCH, H. P. & LADILOV, Y. 2009. Soluble Adenylyl Cyclase Controls Mitochondria-dependent Apoptosis in Coronary Endothelial Cells. *The Journal of Biological Chemistry,* 284**,** 14760-14768.

LANTIER, L., FENTZ, J., MOUNIER, R., LECLERC, J., TREEBAK, J. T., PEHMØLLER, C., SANZ, N., SAKAKIBARA, I., SAINT-AMAND, E., RIMBAUD, S., MAIRE, P., MARETTE, A., VENTURA-CLAPIER, R., FERRY, A., WOJTASZEWSKI, J. F. P., FORETZ, M. & VIOLLET, B. 2014. AMPK controls exercise endurance, mitochondrial oxidative capacity, and skeletal muscle integrity. *The FASEB Journal,* 28**,** 3211-3224.

LEE, J., KIM, C.-H., SIMON, D. K., AMINOVA, L. R., ANDREYEV, A. Y., KUSHNAREVA, Y. E., MURPHY, A. N., LONZE, B. E., KIM, K.-S., GINTY, D. D., FERRANTE, R. J., RYU, H. & RATAN, R. R. 2005. Mitochondrial Cyclic AMP Response Element-binding Protein (CREB) Mediates Mitochondrial Gene Expression and Neuronal Survival. *The Journal of biological chemistry,* 280**,** 40398-40401.

LEFKIMMIATIS, K., LERONNI, D. & HOFER, A. M. 2013. The inner and outer compartments of mitochondria are sites of distinct cAMP/PKA signaling dynamics. *The Journal of Cell Biology,* 202**,** 453-462.

LEIPE, D. D., KOONIN, E. V. & ARAVIND, L. 2004. STAND, a Class of P-Loop NTPases Including Animal and Plant Regulators of Programmed Cell Death: Multiple, Complex Domain Architectures, Unusual Phyletic Patterns, and Evolution by Horizontal Gene Transfer. *Journal of Molecular Biology,* 343**,** 1-28.

LEPRIVIER, G., REMKE, M., ROTBLAT, B., DUBUC, A., MATEO, A.-R. F., KOOL, M., AGNIHOTRI, S., EL-NAGGAR, A., YU, B., SOMASEKHARAN, S. P., FAUBERT, B., BRIDON, G., TOGNON, C. E., MATHERS, J., THOMAS, R., LI, A., BAROKAS, A., KWOK, B., BOWDEN, M., SMITH, S., WU, X., KORSHUNOV, A., HIELSCHER, T., NORTHCOTT, P. A., GALPIN, J. D., AHERN, C. A., WANG, Y., MCCABE, M. G., COLLINS, V. P., JONES, R. G., POLLAK, M., DELATTRE, O., GLEAVE, M. E., JAN, E., PFISTER, S. M., PROUD, C. G., DERRY, W. B., TAYLOR, M. D. & SORENSEN, P. H. 2013. The eEF2 Kinase Confers Resistance to Nutrient Deprivation by Blocking Translation Elongation. *Cell,* 153**,** 1064-1079.

LIN, J., WU, H., TARR, P. T., ZHANG, C.-Y., WU, Z., BOSS, O., MICHAEL, L. F., PUIGSERVER, P., ISOTANI, E., OLSON, E. N., LOWELL, B. B., BASSEL-DUBY, R. & SPIEGELMAN, B. M. 2002. Transcriptional co-activator PGC-1[alpha] drives the formation of slow-twitch muscle fibres. *Nature,* 418**,** 797-801.

LITVIN, T. N., KAMENETSKY, M., ZARIFYAN, A., BUCK, J. & LEVIN, L. R. 2003. Kinetic Properties of "Soluble" Adenylyl Cyclase: SYNERGISM BETWEEN CALCIUM AND BICARBONATE. *Journal of Biological Chemistry,* 278**,** 15922-15926.

MARCELO, K. L., MEANS, A. R. & YORK, B. 2016. The Ca(2+)/Calmodulin/CaMKK2 Axis: Nature's Metabolic CaMshaft. *Trends in endocrinology and metabolism: TEM,* 27**,** 706-718.

MCBRIDE, A., GHILAGABER, S., NIKOLAEV, A. & HARDIE, D. G. 2009. The Glycogen-Binding Domain on the AMPK β Subunit Allows the Kinase to Act as a Glycogen Sensor. *Cell Metabolism,* 9**,** 23-34.

MCGARRY, J. D., LEATHERMAN, G. F. & FOSTER, D. W. 1978. Carnitine palmitoyltransferase I. The site of inhibition of hepatic fatty acid oxidation by malonyl-CoA. *Journal of Biological Chemistry,* 253**,** 4128-4136.

MIDDELHAUFE, S., LEIPELT, M., LEVIN, LONNY R., BUCK, J. & STEEGBORN, C. 2012. Identification of a haem domain in human soluble adenylate cyclase. *Bioscience Reports,* 32**,** 491.

MIHAYLOVA, M. M., VASQUEZ, D. S., RAVNSKJAER, K., DENECHAUD, P.-D., YU, R. T., ALVAREZ, J. G., DOWNES, M., EVANS, R. M., MONTMINY, M. & SHAW, R. J. 2011. Class IIa Histone Deacetylases are Hormone-activated regulators of FOXO and Mammalian Glucose Homeostasis. *Cell,* 145**,** 607-621.

MISHRA, P. & CHAN, D. C. 2014. Mitochondrial dynamics and inheritance during cell division, development and disease. *Nat Rev Mol Cell Biol,* 15**,** 634-646.

MISHRA, P. & CHAN, D. C. 2016. Metabolic regulation of mitochondrial dynamics. *The Journal of Cell Biology,* 212**,** 379-387.

MISRA, U. K. & PIZZO, S. V. 2009. Epac1-Induced Cellular Proliferation in Prostate Cancer Cells Is Mediated by B-Raf/ERK and mTOR Signaling Cascades. *Journal of cellular biochemistry,* 108**,** 998-1011.

MISRA, U. K. & PIZZO, S. V. 2013. Evidence for a Pro-Proliferative Feedback Loop in Prostate Cancer: The Role of Epac1 and COX-2-Dependent Pathways. *PLoS ONE,* 8**,** e63150.

MIYAMOTO, T., RHO, E., SAMPLE, V., AKANO, H., MAGARI, M., UENO, T., GORSHKOV, K., CHEN, M., TOKUMITSU, H., ZHANG, J. & INOUE, T. 2015. Compartmentalized AMPK Signaling Illuminated by Genetically Encoded Molecular Sensors and Actuators. *Cell reports,* 11**,** 657-670.

MOMCILOVIC, M., HONG, S.-P. & CARLSON, M. 2006. Mammalian TAK1 Activates Snf1 Protein Kinase in Yeast and Phosphorylates AMP-activated Protein Kinase in Vitro. *Journal of Biological Chemistry,* 281**,** 25336-25343.

MONTERISI, S. & ZACCOLO, M. 2017. Components of the mitochondrial cAMP signalosome. *Biochemical Society Transactions,* 45**,** 269.

MOTTILLO, E. P., DESJARDINS, E. M., CRANE, J. D., SMITH, B. K., GREEN, A. E., DUCOMMUN, S., HENRIKSEN, T. I., REBALKA, I. A., RAZI, A., SAKAMOTO, K., SCHEELE, C., KEMP, B. E., HAWKE, T. J., ORTEGA, J., GRANNEMAN, J. G. & STEINBERG, G. R. 2016. Lack of Adipocyte AMPK Exacerbates Insulin Resistance and Hepatic Steatosis through Brown and Beige Adipose Tissue Function. *Cell metabolism,* 24**,** 118-129.

MYERS, R. W., GUAN, H.-P., EHRHART, J., PETROV, A., PRAHALADA, S., TOZZO, E., YANG, X., KURTZ, M. M., TRUJILLO, M., GONZALEZ TROTTER, D., FENG, D., XU, S., EIERMANN, G., HOLAHAN, M. A., RUBINS, D., CONARELLO, S., NIU, X., SOUZA, S. C., MILLER, C., LIU, J., LU, K., FENG, W., LI, Y., PAINTER, R. E., MILLIGAN, J. A., HE, H., LIU, F., OGAWA, A., WISNIEWSKI, D., ROHM, R. J., WANG, L., BUNZEL, M., QIAN, Y., ZHU, W., WANG, H., BENNET, B., LAFRANCO SCHEUCH, L., FERNANDEZ, G. E., LI, C., KLIMAS, M., ZHOU, G., VAN HEEK, M., BIFTU, T., WEBER, A., KELLEY, D. E., THORNBERRY, N., ERION, M. D., KEMP, D. M. & SEBHAT, I. K. 2017. Systemic pan-AMPK activator MK-8722 improves glucose homeostasis but induces cardiac hypertrophy. *Science,* 357**,** 507-511.

NAH, J., YUAN, J. & JUNG, Y.-K. 2015. Autophagy in Neurodegenerative Diseases: From Mechanism to Therapeutic Approach. *Molecules and Cells,* 38**,** 381-389.

NARKAR, V. A., DOWNES, M., YU, R. T., EMBLER, E., WANG, Y.-X., BANAYO, E., MIHAYLOVA, M. M., NELSON, M. C., ZOU, Y., JUGUILON, H., KANG, H., SHAW, R. & EVANS, R. M. 2008. AMPK and PPARδ agonists are exercise mimetics. *Cell,* 134**,** 405-415.

OAKHILL, J. S., CHEN, Z.-P., SCOTT, J. W., STEEL, R., CASTELLI, L. A., LING, N., MACAULAY, S. L. & KEMP, B. E. 2010a. β-Subunit myristoylation is the gatekeeper for initiating metabolic stress sensing by AMP-activated protein kinase (AMPK). *Proceedings of the National Academy of Sciences,* 107**,** 19237-19241.

OAKHILL, J. S., CHEN, Z.-P., SCOTT, J. W., STEEL, R., CASTELLI, L. A., LING, N., MACAULAY, S. L. & KEMP, B. E. 2010b. β-Subunit myristoylation is the gatekeeper for initiating metabolic stress sensing by AMP-activated protein kinase (AMPK). *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 19237-19241.

OMAR, B., ZMUDA-TRZEBIATOWSKA, E., MANGANIELLO, V., GORANSSON, O. & DEGERMAN, E. 2009. Regulation of AMP-activated protein kinase by cAMP in adipocytes: roles for phosphodiesterases, protein kinase B, protein kinase A, Epac and lipolysis. *Cell Signal,* 21**,** 760-6.

OTERA, H., WANG, C., CLELAND, M. M., SETOGUCHI, K., YOKOTA, S., YOULE, R. J. & MIHARA, K. 2010. Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. *The Journal of Cell Biology,* 191**,** 1141-1158.

PANG, T., XIONG, B., LI, J.-Y., QIU, B.-Y., JIN, G.-Z., SHEN, J.-K. & LI, J. 2007. Conserved α-Helix Acts as Autoinhibitory Sequence in AMP-activated Protein Kinase α Subunits. *Journal of Biological Chemistry,* 282**,** 495-506.

PAPA, S., SARDANELLI, A. M., SCACCO, S. & TECHNIKOVA-DOBROVA, Z. 1999. cAMP-dependent protein kinase and phosphoproteins in mammalian mitochondria. An extension of the cAMP-mediated intracellular signal transduction. *FEBS Letters,* 444**,** 245-249.

PARK, S.-J., AHMAD, F., PHILP, A., BAAR, K., WILLIAMS, T., LUO, H., KE, H., REHMANN, H., TAUSSIG, R., BROWN, A. L., KIM, M. K., BEAVEN, M. A., BURGIN, A. B., MANGANIELLO, V. & CHUNG, J. H. 2012. Resveratrol Ameliorates Aging-Related Metabolic Phenotypes by Inhibiting cAMP Phosphodiesterases. *Cell,* 148**,** 421-433.

PASTOR-SOLER, N. M., HALLOWS, K. R., SMOLAK, C., GONG, F., BROWN, D. & BRETON, S. 2008. Alkaline pH- and cAMP-induced V-ATPase membrane accumulation is mediated by protein kinase A in epididymal clear cells. *American journal of physiology. Cell physiology,* 294**,** C488-C494.

PENDERGRASS, W., WOLF, N. & POOT, M. 2004. Efficacy of MitoTracker Green™ and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. *Cytometry Part A,* 61A**,** 162-169.

PRABU, S. K., ANANDATHEERTHAVARADA, H. K., RAZA, H., SRINIVASAN, S., SPEAR, J. F. & AVADHANI, N. G. 2006. Protein Kinase A-mediated Phosphorylation Modulates Cytochrome c Oxidase Function and Augments Hypoxia and Myocardial Ischemia-related Injury. *The Journal of biological chemistry,* 281**,** 2061-2070.

PRYDE, K. R., SMITH, H. L., CHAU, K.-Y. & SCHAPIRA, A. H. V. 2016. PINK1 disables the anti-fission machinery to segregate damaged mitochondria for mitophagy. *The Journal of Cell Biology,* 213**,** 163.

PUIGSERVER, P., WU, Z., PARK, C. W., GRAVES, R., WRIGHT, M. & SPIEGELMAN, B. M. 1998. A Cold-Inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis. *Cell,* 92**,** 829-839.

PURUSHOTHAM, A., SCHUG, T. T., XU, Q., SURAPUREDDI, S., GUO, X. & LI, X. 2009. Hepatocyte-Specific Deletion of SIRT1 Alters Fatty Acid Metabolism and Results in Hepatic Steatosis and Inflammation. *Cell Metabolism,* 9**,** 327-338.

RAHMAN, N., RAMOS-ESPIRITU, L., MILNER, T. A., BUCK, J. & LEVIN, L. R. 2016. Soluble adenylyl cyclase is essential for proper lysosomal acidification. *The Journal of General Physiology,* 148**,** 325-339.

RAMBOLD, A. S., COHEN, S. & LIPPINCOTT-SCHWARTZ, J. 2015. Fatty acid trafficking in starved cells: regulation by lipid droplet lipolysis, autophagy and mitochondrial fusion dynamics. *Developmental cell,* 32**,** 678-692.

RAMBOLD, A. S., KOSTELECKY, B., ELIA, N. & LIPPINCOTT-SCHWARTZ, J. 2011. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 10190-10195.

RAMOS-ESPIRITU, L., KLEINBOELTING, S., NAVARRETE, F. A., ALVAU, A., VISCONTI, P. E., VALSECCHI, F., STARKOV, A., MANFREDI, G., BUCK, H., ADURA, C., ZIPPIN, J. H., VAN DEN HEUVEL, J., GLICKMAN, J. F., STEEGBORN, C., LEVIN, L. R. & BUCK, J. 2016. Discovery of LRE1 as a specific and allosteric inhibitor of soluble adenylyl cyclase. *Nature chemical biology,* 12**,** 838-844.

RASMO, D. D., PALMISANO, G., SCACCO, S., TECHNIKOVA-DOBROVA, Z., PANELLI, D., COCCO, T., SARDANELLI, A. M., GNONI, A., MICELLI, L., TRANI, A., LUCCIA, A. D. & PAPA, S. 2010. Phosphorylation pattern of the NDUFS4 subunit of complex I of the mammalian respiratory chain. *Mitochondrion,* 10**,** 464-471.

ROSS, FIONA A., JENSEN, THOMAS E. & HARDIE, D G. 2016a. Differential regulation by AMP and ADP of AMPK complexes containing different γ subunit isoforms. *Biochemical Journal,* 473**,** 189-199.

ROSS, F. A., MACKINTOSH, C. & HARDIE, D. G. 2016b. AMP-activated protein kinase: a cellular energy sensor that comes in 12 flavours. *The Febs Journal,* 283**,** 2987-3001.

RUSSELL, R. R., LI, J., COVEN, D. L., PYPAERT, M., ZECHNER, C., PALMERI, M., GIORDANO, F. J., MU, J., BIRNBAUM, M. J. & YOUNG, L. H. 2004. AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *Journal of Clinical Investigation,* 114**,** 495-503.

RYU, H., LEE, J., IMPEY, S., RATAN, R. R. & FERRANTE, R. J. 2005. Antioxidants modulate mitochondrial PKA and increase CREB binding to D-loop DNA of the mitochondrial genome in neurons. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 13915-13920.

SAGGERSON, D. 2008. Malonyl-CoA, a Key Signaling Molecule in Mammalian Cells. *Annual Review of Nutrition,* 28**,** 253-272.

SANDERS, MATTHEW J., GRONDIN, PASCAL O., HEGARTY, BRONWYN D., SNOWDEN, MICHAEL A. & CARLING, D. 2007. Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochemical Journal,* 403**,** 139-148.

SCACCO, S., VERGARI, R., SCARPULLA, R. C., TECHNIKOVA-DOBROVA, Z., SARDANELLI, A., LAMBO, R., LORUSSO, V. & PAPA, S. 2000. cAMP-dependent Phosphorylation of the Nuclear Encoded 18-kDa (IP) Subunit of Respiratory Complex I and Activation of the Complex in Serum-starved Mouse Fibroblast Cultures. *Journal of Biological Chemistry,* 275**,** 17578-17582.

SCHIRMER, APPUKUTTAN, POTT, JAQUET & LADILOV 2012. The potential role of soluble adenylylcyclase in beta1-adrenergic stimulation-induced hypertrophy. *Cardiovascular Research,* 93**,** S52–S87.

SCHMIDT, M., EVELLIN, S., WEERNINK, P. A. O., DORP, F. V., REHMANN, H., LOMASNEY, J. W. & JAKOBS, K. H. 2001. A new phospholipase-C–calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. *Nature Cell Biology,* 3**,** 1020.

SCHUSTER, I., MAHMOODZADEH, S., DWORATZEK, E., JAISSER, F., MESSAOUDI, S., MORANO, I. & REGITZ-ZAGROSEK, V. 2016. Cardiomyocyte-specific overexpression of oestrogen receptor β improves survival and cardiac function after myocardial infarction in female and male mice. *Clinical Science,* 130**,** 365.

SCHWOCH, G., TRINCZEK, B. & BODE, C. 1990. Localization of catalytic and regulatory subunits of cyclic AMP-dependent protein kinases in mitochondria from various rat tissues. *Biochemical Journal,* 270**,** 181-188.

SHAW, R. J., KOSMATKA, M., BARDEESY, N., HURLEY, R. L., WITTERS, L. A., DEPINHO, R. A. & CANTLEY, L. C. 2004. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 3329-3335.

SHEN, T., DING, L., RUAN, Y., QIN, W., LIN, Y., XI, C., LU, Y., DOU, L., ZHU, Y., CAO, Y., MAN, Y., BIAN, Y., WANG, S., XIAO, C. & LI, J. 2014. SIRT1 Functions as an Important Regulator of Estrogen-Mediated Cardiomyocyte Protection in Angiotensin II-Induced Heart Hypertrophy. *Oxidative Medicine and Cellular Longevity,* 2014**,** 713894.

SHIRIHAI, O., SONG, M. & DORN, G. W. 2015. How mitochondrial dynamism orchestrates mitophagy. *Circulation research,* 116**,** 1835-1849.

STAPLETON, D., MITCHELHILL, K. I., GAO, G., WIDMER, J., MICHELL, B. J., TEH, T., HOUSE, C. M., FERNANDEZ, C. S., COX, T., WITTERS, L. A. & KEMP, B. E. 1996. Mammalian AMP-activated Protein Kinase Subfamily. *Journal of Biological Chemistry,* 271**,** 611-614.

STEEGBORN, C. 2014. Structure, mechanism, and regulation of soluble adenylyl cyclases — similarities and differences to transmembrane adenylyl cyclases. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1842**,** 2535-2547.

STEEGBORN, C., LITVIN, T. N., HESS, K. C., CAPPERM, A. B., TAUSSIG, R., BUCK, J., LEVIN, L. R. & WU, H. 2005a. A Novel Mechanism for Adenylyl Cyclase Inhibition from the Crystal Structure of Its Complex with Catechol Estrogen(). *The Journal of biological chemistry,* 280**,** 31754-31759.

STEEGBORN, C., LITVIN, T. N., LEVIN, L. R., BUCK, J. & WU, H. 2005b. Bicarbonate activation of adenylyl cyclase via promotion of catalytic active site closure and metal recruitment. *Nature structural & molecular biology,* 12**,** 32-37.

STORK, P. J. S. & SCHMITT, J. M. 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends in Cell Biology,* 12**,** 258-266.

SUN, N., YUN, J., LIU, J., MALIDE, D., LIU, C., ROVIRA, I. I., HOLMSTRÖM, K. M., FERGUSSON, M. M., YOO, Y. H., COMBS, C. A. & FINKEL, T. 2015. Measuring in vivo mitophagy. *Molecular cell,* 60**,** 685-696.

SUZUKI, T., BRIDGES, D., NAKADA, D., SKINIOTIS, G., MORRISON, S. J., LIN, J., SALTIEL, A. R. & INOKI, K. 2013. Inhibition of AMPK catabolic action by GSK3. *Molecular cell,* 50**,** 407-419.

TECHNIKOVA-DOBROVA, Z., SARDANELLI, A. M., SPERANZA, F., SCACCO, S., SIGNORILE, A., LORUSSO, V. & PAPA, S. 2001. Cyclic Adenosine Monophosphate-Dependent Phosphorylation of Mammalian Mitochondrial Proteins: Enzyme and Substrate Characterization and Functional Role. *Biochemistry,* 40**,** 13941-13947.

THORNTON, C. 2017. AMPK: keeping the (power)house in order? *Neuronal Signaling,* 1.

THORNTON, C., SNOWDEN, M. A. & CARLING, D. 1998. Identification of a Novel AMP-activated Protein Kinase β Subunit Isoform That Is Highly Expressed in Skeletal Muscle. *Journal of Biological Chemistry,* 273**,** 12443-12450.

TONDERA, D., GRANDEMANGE, S., JOURDAIN, A., KARBOWSKI, M., MATTENBERGER, Y., HERZIG, S., DA CRUZ, S., CLERC, P., RASCHKE, I., MERKWIRTH, C., EHSES, S., KRAUSE, F., CHAN, D. C., ALEXANDER, C., BAUER, C., YOULE, R., LANGER, T. & MARTINOU, J.-C. 2009. SLP-2 is required for stress-induced mitochondrial hyperfusion. *The EMBO Journal,* 28**,** 1589-1600.

TOYAMA, E. Q., HERZIG, S., COURCHET, J., LEWIS, T. L., LOSÓN, O. C., HELLBERG, K., YOUNG, N. P., CHEN, H., POLLEUX, F., CHAN, D. C. & SHAW, R. J. 2016. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. *Science (New York, N.Y.),* 351**,** 275-281.

TRESGUERRES, M., LEVIN, L. R. & BUCK, J. 2011. Intracellular cAMP signaling by soluble adenylyl cyclase. *Kidney international,* 79**,** 1277-1288.

TWIG, G., ELORZA, A., MOLINA, A. J. A., MOHAMED, H., WIKSTROM, J. D., WALZER, G., STILES, L., HAIGH, S. E., KATZ, S., LAS, G., ALROY, J., WU, M., PY, B. F., YUAN, J., DEENEY, J. T., CORKEY, B. E. & SHIRIHAI, O. S. 2008. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *The EMBO Journal,* 27**,** 433-446.

VALSECCHI, F., RAMOS-ESPIRITU, L. S., BUCK, J., LEVIN, L. R. & MANFREDI, G. 2013. cAMP and Mitochondria. *Physiology,* 28**,** 199-209.

WANG, Z., LIU, D., VARIN, A., NICOLAS, V., COURILLEAU, D., MATEO, P., CAUBERE, C., ROUET, P., GOMEZ, A. M., VANDECASTEELE, G., FISCHMEISTER, R. & BRENNER, C. 2016. A cardiac mitochondrial cAMP signaling pathway regulates calcium accumulation, permeability transition and cell death. *Cell Death & Disease,* 7**,** e2198.

WATSON, R. L., BUCK, J., LEVIN, L. R., WINGER, R. C., WANG, J., ARASE, H. & MULLER, W. A. 2015. Endothelial CD99 signals through soluble adenylyl cyclase and PKA to regulate leukocyte transendothelial migration. *The Journal of Experimental Medicine,* 212**,** 1021-1041.

WOODS, A., DICKERSON, K., HEATH, R., HONG, S.-P., MOMCILOVIC, M., JOHNSTONE, S. R., CARLSON, M. & CARLING, D. 2005. Ca2+/calmodulin-dependent protein kinase kinase-

β acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metabolism,* 2**,** 21-33.

WOODS, A., JOHNSTONE, S. R., DICKERSON, K., LEIPER, F. C., FRYER, L. G. D., NEUMANN, D., SCHLATTNER, U., WALLIMANN, T., CARLSON, M. & CARLING, D. 2003. LKB1 Is the Upstream Kinase in the AMP-Activated Protein Kinase Cascade. *Current Biology,* 13**,** 2004-2008.

WRIGHT, G., HIGGIN, J. J., RAINES, R. T., STEENBERGEN, C. & MURPHY, E. 2003. Activation of the Prolyl Hydroxylase Oxygen-sensor Results in Induction of GLUT1, Heme Oxygenase-1, and Nitric-oxide Synthase Proteins and Confers Protection from Metabolic Inhibition to Cardiomyocytes. *Journal of Biological Chemistry,* 278**,** 20235-20239.

WU, K. Y., ZIPPIN, J. H., HURON, D. R., KAMENETSKY, M., HENGST, U., BUCK, J., LEVIN, L. R. & JAFFREY, S. R. 2006. Soluble adenylyl cyclase is required for netrin-1 signaling in nerve growth cones. 9**,** 1257.

WU, N., ZHENG, B., SHAYWITZ, A., DAGON, Y., TOWER, C., BELLINGER, G., SHEN, C.-H., WEN, J., ASARA, J., MCGRAW, T. E., KAHN, B. B. & CANTLEY, L. C. 2013. AMPK-dependent degradation of TXNIP upon energy stress leads to enhanced glucose uptake via GLUT1. *Molecular cell,* 49**,** 1167-1175.

WU, W., TIAN, W., HU, Z., CHEN, G., HUANG, L., LI, W., ZHANG, X., XUE, P., ZHOU, C., LIU, L., ZHU, Y., ZHANG, X., LI, L., ZHANG, L., SUI, S., ZHAO, B. & FENG, D. 2014. ULK1 translocates to mitochondria and phosphorylates FUNDC1 to regulate mitophagy. *EMBO Reports,* 15**,** 566-575.

WU, Z., PUIGSERVER, P., ANDERSSON, U., ZHANG, C., ADELMANT, G., MOOTHA, V., TROY, A., CINTI, S., LOWELL, B., SCARPULLA, R. C. & SPIEGELMAN, B. M. 1999. Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1. *Cell,* 98**,** 115-124.

XIAO, B., DENG, X., ZHOU, W. & TAN, E.-K. 2016. Flow Cytometry-Based Assessment of Mitophagy Using MitoTracker. *Frontiers in Cellular Neuroscience,* 10**,** 76.

XIAO, B., HEATH, R., SAIU, P., LEIPER, F. C., LEONE, P., JING, C., WALKER, P. A., HAIRE, L., ECCLESTON, J. F., DAVIS, C. T., MARTIN, S. R., CARLING, D. & GAMBLIN, S. J. 2007. Structural basis for AMP binding to mammalian AMP-activated protein kinase. *Nature,* 449**,** 496-500.

XIAO, B., SANDERS, M. J., CARMENA, D., BRIGHT, N. J., HAIRE, L. F., UNDERWOOD, E., PATEL, B. R., HEATH, R. B., WALKER, P. A., HALLEN, S., GIORDANETTO, F., MARTIN, S. R., CARLING, D. & GAMBLIN, S. J. 2013. Structural basis of AMPK regulation by small molecule activators. *Nature Communications,* 4**,** 3017.

XIE, M., ZHANG, D., DYCK, J. R. B., LI, Y., ZHANG, H., MORISHIMA, M., MANN, D. L., TAFFET, G. E., BALDINI, A., KHOURY, D. S. & SCHNEIDER, M. D. 2006. A pivotal role for endogenous

TGF-β-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *Proceedings of the National Academy of Sciences,* 103**,** 17378-17383.

XING, Y., MUSI, N., FUJII, N., ZOU, L., LUPTAK, I., HIRSHMAN, M. F., GOODYEAR, L. J. & TIAN, R. 2003. Glucose Metabolism and Energy Homeostasis in Mouse Hearts Overexpressing Dominant Negative α2 Subunit of AMP-activated Protein Kinase. *Journal of Biological Chemistry,* 278**,** 28372-28377.

YAMAUCHI, T., KAMON, J., MINOKOSHI, Y., ITO, Y., WAKI, H., UCHIDA, S., YAMASHITA, S., NODA, M., KITA, S., UEKI, K., ETO, K., AKANUMA, Y., FROGUEL, P., FOUFELLE, F., FERRE, P., CARLING, D., KIMURA, S., NAGAI, R., KAHN, B. B. & KADOWAKI, T. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. 8**,** 1288.

YAN, Y., TSUKAMOTO, O., NAKANO, A., KATO, H., KIOKA, H., ITO, N., HIGO, S., YAMAZAKI, S., SHINTANI, Y., MATSUOKA, K., LIAO, Y., ASANUMA, H., ASAKURA, M., TAKAFUJI, K., MINAMINO, T., ASANO, Y., KITAKAZE, M. & TAKASHIMA, S. 2015. Augmented AMPK activity inhibits cell migration by phosphorylating the novel substrate Pdlim5. *Nature Communications,* 6**,** 6137.

YANG, C.-S., KIM, J.-J., LEE, H.-M., JIN, H. S., LEE, S.-H., PARK, J.-H., KIM, S. J., KIM, J.-M., HAN, Y.-M., LEE, M.-S., KWEON, G. R., SHONG, M. & JO, E.-K. 2014. The AMPK-PPARGC1A pathway is required for antimicrobial host defense through activation of autophagy. *Autophagy,* 10**,** 785-802.

ZAHA, V. G., QI, D., SU, K. N., PALMERI, M., LEE, H.-Y., HU, X., WU, X., SHULMAN, G. I., RABINOVITCH, P. S., RUSSELL, R. & YOUNG, L. H. 2016. AMPK Is Critical for Mitochondrial Function during Reperfusion after Myocardial Ischemia. *Journal of molecular and cellular cardiology,* 91**,** 104-113.

ZHAO, X., LEÓN, I. R., BAK, S., MOGENSEN, M., WRZESINSKI, K., HØJLUND, K. & JENSEN, O. N. 2011. Phosphoproteome Analysis of Functional Mitochondria Isolated from Resting Human Muscle Reveals Extensive Phosphorylation of Inner Membrane Protein Complexes and Enzymes. *Molecular & Cellular Proteomics : MCP,* 10**,** M110.000299.

ZHOU, G., MYERS, R., LI, Y., CHEN, Y., SHEN, X., FENYK-MELODY, J., WU, M., VENTRE, J., DOEBBER, T., FUJII, N., MUSI, N., HIRSHMAN, M. F., GOODYEAR, L. J. & MOLLER, D. E. 2001. Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation,* 108**,** 1167-1174.

ZHU, Y., WANG, C., YU, M., CUI, J., LIU, L. & XU, Z. 2013. ULK1 and JNK are involved in mitophagy incurred by LRRK2 G2019S expression. *Protein & Cell,* 4**,** 711-721.

ZIMMERMAN, N. P., ROY, I., HAUSER, A. D., WILSON, J. M., WILLIAMS, C. L. & DWINELL, M. B. 2015. Cyclic AMP regulates the migration and invasion potential of human pancreatic cancer cells. *Molecular carcinogenesis,* 54**,** 203-215.

ZIPPIN, J. H., CHADWICK, P. A., LEVIN, L. R., BUCK, J. & MAGRO, C. M. 2010. Soluble Adenylyl Cyclase Defines a Nuclear cAMP Microdomain in Keratinocyte Hyperproliferative Skin Diseases. *The Journal of investigative dermatology,* 130**,** 1279-1287.

ZIPPIN, J. H., CHEN, Y., NAHIRNEY, P., KAMENETSKY, M., WUTTKE, M. S., FISCHMAN, D. A., LEVIN, L. R. & BUCK, J. 2003. Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. *The FASEB Journal,* 17**,** 82-84.

ZIPPIN, J. H., CHEN, Y., STRAUB, S. G., HESS, K. C., DIAZ, A., LEE, D., TSO, P., HOLZ, G. G., SHARP, G. W. G., LEVIN, L. R. & BUCK, J. 2013. CO(2)/HCO(3)(−)- and Calcium-regulated Soluble Adenylyl Cyclase as a Physiological ATP Sensor. *The Journal of Biological Chemistry,* 288**,** 33283-33291.

ZIPPIN, J. H., FARRELL, J., HURON, D., KAMENETSKY, M., HESS, K. C., FISCHMAN, D. A., LEVIN, L. R. & BUCK, J. 2004. Bicarbonate-responsive “soluble” adenylyl cyclase defines a nuclear cAMP microdomain. *The Journal of Cell Biology,* 164**,** 527-534.

ZONG, H., REN, J. M., YOUNG, L. H., PYPAERT, M., MU, J., BIRNBAUM, M. J. & SHULMAN, G. I. 2002. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 15983-15987.

12 Presentations and publications

¤ RADical reduction of OXidative stress in cardiovascular disease (RADOX) final Conference. *Title:* Soluble adenylyl cyclase controls AMPK activity, mitochondrial function and biogenesis and may play a role in estradiol-dependent protection against oxidative stress in cardiac cells. October 26, 2016. Berlin, Germany. **Oral and poster presentation**.

¤ Keystone Symposia on Mitochondria Communication. *Title:* cAMP pool produced by type 10 adenylyl cyclase controls AMPK activity in cardiac myoblasts. January 14—18, 2017. Taos, NM, USA. **Poster presentation**.

¤ 42nd FEBS conference. *Title:* Soluble adenylyl cyclase controls AMPK activity, mitochondrial function and biogenesis and may play a role in estradiol-dependent protection against oxidative stress in cardiac cells. September 10—14, 2017. Jerusalem, Israel. **Poster presentation**.

¤ EMBO—FEBS lecture series, Mitochondria in life, death and disease. Title: Soluble adenylyl cyclase modulates AMPK activity and mitochondrial function/biogenesis and may mediate estradiol-dependent protection against oxidative stress in cardiac cells. October 9—13, 2017. Brindisi, Italy. **Poster presentation**.

¤ **Jayarajan V**, Appukutan A, Reusch P, Regitz-Zagrosek V, Ladilov Y. Role of soluble adenylyl cyclase in cAMP/AMPK-mediated mitochondrial biogenesis, function and clearance. Manuscript under preparation.

¤ Rinaldi L, Pozdniakova S, **Jayarajan V**, Aslam M, Troidl C, Abdallah Y, Ladilov Y. Role of soluble adenylyl cyclase in reperfusion-induced injury of cardiac cells. Manuscript under preparation.

¤ Pulavendran S, **Jayarajan V**, Rose C. 2010. Differential anti-inflammatory and anti-fibrotic activity of transplanted mesenchymal vs. hematopoietic stem cells in carbon tetrachloride-induced liver injury in mice. *Int Immunopharmacol*. 10(4):513 – 519.

13 Selbstständigkeitserklärung

Hiermit versichere ich, dass ich diese Arbeit selbst verfasst habe sowie keine andere als die angegebenen Quellen und Hilfsmittel in Anspruch genommen habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form keiner anderen Prüfungsbehörde vorgelegt wurde.

Vignesh Jayarajan April, 2018