

AMP-activated protein kinase and its downstream transcriptional pathways

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Abstract The AMP-activated protein kinase (AMPK) is a key regulator of catabolic versus anabolic processes. Its properties as an energy sensor allow it to couple the energy status of the cell to the metabolic environment. These adaptations not only take place through the acute modulation of key metabolic enzymes via direct phosphorylation, but also through a slower transcriptional adaptative response. The question of how AMPK regulates the expression of a number of gene sets, such as those related to mitochondrial biogenesis, energy production and oxidative protection, is only beginning to be elucidated, and still many questions remain to be answered. In this review we will try to integrate our current knowledge on how AMPK regulates transcription in muscle and liver, which will serve as examples to illustrate the major advances in the field and the key challenges ahead.

Keywords AMPK · Energy metabolism · PGC-1 α · SIRT1

Introduction

One and a half centuries ago, Darwin shocked the world with one of the brightest concepts to ever impact biological sciences, i.e., that the ability of organisms to respond and adapt to environmental challenges has been vital for evolution. To the amazement of the scientific community, this

remarkable feat to adapt to environmental changes is consistently found not only in organisms as a whole, but also at the tissue and cellular levels. Given that most biological processes (cell growth, division, movement, etc.) depend on energy consumption, it is not surprising that one outcome of evolution is that cells and organisms can sense energy levels and adapt their energy production to their energy demands.

In order to sustain proper biological functions, ATP levels, the energy currency in cells, are maintained in the low millimolar range, hinting at the existence of molecular mechanisms that keep an appropriate balance between energy-consuming and -producing processes. AMP-activated protein kinase (AMPK), an enzyme that senses AMP levels and that is conserved along the eukaryote kingdom, could be a key molecular player in this adaptation process. This review will focus on mammalian AMPK, but we refer the reader to some recent reviews in order to gain some insight on AMPK homologs in different eukaryotes [1–4].

Deconstructing AMPK: enzyme bricks and regulation of its activity

AMPK is a heterotrimeric enzyme

AMPK is a heterotrimeric Ser/Thr kinase composed of an α , β and γ subunit [3]. There are two different forms of α (α_1 and α_2) and β (β_1 and β_2) subunits, while three different γ isoforms (γ_1 , γ_2 and γ_3) exist [3]. The α subunits are the catalytic subunits of the functional heterotrimer and contain the Thr¹⁷² residue, whose phosphorylation is required for full enzymatic activity [5]. The α subunit partners with the β and γ subunits through its C-terminal region [6]. The β subunit also interacts with both the α and γ

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subunits, and its mid-region contains an evolutionarily conserved carbohydrate-binding domain, which allows AMPK to interact with glycogen particles [7]. The γ subunits contain one of the critical features of the enzyme, the four tandem repeats known as cystathionine β -synthase (CBS) motifs, which form an interface for interaction with two AMP or ATP molecules in a mutually exclusive way and a third AMP molecule in a non-exchangeable fashion [8]. While the binding of ATP keeps the activity of the enzyme low, the exchange of ATP for AMP is enough to promote a mild, less than fivefold, activation of the kinase through an allosteric mechanism [5]. More importantly, AMP binding renders AMPK a poorer substrate for the α subunit Thr¹⁷² phosphatase, which results in increased Thr¹⁷² phosphorylation [9]. The combination of the allosteric and phosphorylation effects promoted by AMP leads to a >1,000-fold activation of the enzyme [10]. Due to the reaction catalyzed by adenylate kinase, transforming two ADP molecules into one ATP and one AMP, the AMP/ATP ratio is a very sensitive reflection of metabolic disturbances of the cell [11], and, therefore, transforms AMPK into an exquisite sensor of energy balance.

Regulation of AMPK phosphorylation

As described above, AMPK is maximally active when phosphorylated. Consequently, there has been great interest in identifying the regulators of the phosphorylation state of this enzyme. During the last decade, a number of upstream kinase activities have been identified, and, even though the exact identity of the phosphatase activity remains elusive, it seems to belong to the PP2C family [9, 12]. Among the different kinases proposed to act as AMPKKs, LKB1 and CAMKK are now widely accepted to be key. Others, like transforming growth factor- β -activated kinase 1 (TAK1), can certainly phosphorylate AMPK *in vitro* [13], but the “*in vivo*” evidence of their capacity to activate AMPK is still not conclusive. The reasons and scenarios justifying the need for different AMPK upstream kinases are yet to be fully understood.

LKB1/STRAD/MO25

LKB1 is a Ser/Thr kinase that was originally identified as a tumor suppressor mutated in an inherited form of susceptibility to cancer, the Peutz-Jeghers syndrome [14]. LKB1 requires the formation of a heterotrimeric complex with two additional proteins in order to function, Sterile-20-related adaptor (STRAD) and Mouse protein 25 (MO25) [15]. In their absence, LKB1 is weakly active [15]. A number of post-translational modifications can impact LKB1 and potentially modulate its activity [14, 16]. However, most evidence points towards the hypothesis

that, in normal physiological settings, the LKB1/STRAD/MO25 complex is a constitutively active kinase [17] and that the regulation of AMPK happens through different accessibility for the phosphatase activity [9]. This particularity might be explained by the fact that the LKB1 complex acts as a master kinase for the 13 members of the entire family of AMPK-related kinases [18], making it necessary to create substrate specificity through additional methods. In this sense, increased AMP only leads to activation of AMPK, and not of the other 12 family members [17]. Studies in the LKB1-deficient mouse have shown that LKB1 is the main AMPK kinase in muscle and liver [19–21]. Muscle-specific LKB-1 KO mice display severely impaired AMPK α_2 phosphorylation after stimulation of AMPK with the pharmacological AMP-mimetic AICAR (aminoimidazole-4-carboxamide-1- β -D-ribofuranoside) or *ex-vivo* contraction, demonstrating that LKB1 is the major AMPK kinase in skeletal muscle [19, 21]. In liver, deletion of LKB1 prevented the effects of metformin on AMPK activation and glucose production [20].

CaMKK

Simultaneous work by David Carling and Grahame Hardie’s groups found a second alternative AMPK kinase in brain and LKB1-deficient cells: the Ca²⁺/calmodulin-dependent kinase kinases (CaMKKs) [22, 23]. Other tissues, like muscle, also express CAMKK α and, not so clearly, CAMKK β , although at lower levels than brain [24, 25]. The activity of CAMKKs depend on increases in intracellular Ca²⁺ levels and act on AMPK independently of changes in AMP [10]. It has been hypothesized that CAMKKs could be the main AMPKK during the initial phase of mild-tetanic muscle contraction [26]. Overexpression of CAMKK α or CAMKK β in muscle is enough to increase AMPK phosphorylation [27], and muscle overload is known to increase AMPK activity in LKB1 knock-out mice, in correlation with an increase in CAMKK expression [25]. However, it must be said that a number of experiments studying the role of CAMKK in muscle have relied on the use of STO-609 as a CAMKK inhibitor, whose specificity is not fully clear [19, 26].

AMPK actions

As mentioned before, AMPK acts as an energy sensor by sensing the AMP/ATP ratio. AMPK activation is generally linked to the stimulation of metabolic responses in order to prevent metabolic and energetic crisis in situations where ATP synthesis is compromised (hypoxia, ischemia, low nutrient availability) or ATP consumption is accelerated. Consequent to this principle, AMPK activation stimulates catabolic processes to generate ATP and

inhibits ATP-consuming anabolic processes that are not required for the immediate survival of the cell. Even though this review aims to focus on the transcriptional events regulated by AMPK, it is necessary to understand the acute effects of AMPK activation in order to understand the global physiological actions of AMPK and the implications of its pharmacological activation. Therefore, we will briefly mention the most notable acute effects of AMPK and refer the reader to some recent reviews for more details [3, 28, 29].

One of the immediate whole-body consequences of AMPK activation is an increase in glucose uptake by skeletal muscle through the induction of GLUT4 translocation to the plasma membrane [30]. In fact, AMPK has for a long time been hypothesized as a crucial mediator of the effects of muscle contraction on glucose transport [19, 28, 31]. Muscle contraction activates AMPK as a consequence of ATP depletion [19], and, probably, also through the activation of CAMKK in response to the fluctuations in cytosolic Ca^{2+} during contraction [26]. The downstream events bridging AMPK activation to GLUT4 translocation are still nebulous. A number of studies have focused their attention on the attractive link provided by TBC1D1 and TBC1D4, two highly related proteins of the same family, that are predominant in glycolytic and oxidative muscle, respectively [32]. TBC1D1 and D4 are Rab GTPase-activating proteins (GAPs), which are believed to slow down or prevent GLUT4 exocytosis by keeping GLUT4-vesicle associated Rab proteins in their GDP-bound form [33]. AMPK phosphorylates TBC1D1 and D4, and this dissociates them from GLUT4 vesicles, allowing GLUT4 translocation [33]. While this conforms an interesting mechanism of action, a number of concerns [28] indicate that there are still many questions open regarding the molecular mechanisms by which AMPK regulates glucose uptake.

Acute activation of AMPK is also associated with decreases in glycogen synthesis rates. This can be achieved through the direct phosphorylation of glycogen synthase on Ser⁷, which inhibits its activity [34]. The decreased glycogen synthesis rates upon acute AMPK activation are generally coupled to an increase in the glycolytic flux, thanks to the activation of 6-phosphofructo-2-kinase (PFK-2) through direct phosphorylation on Ser⁴⁶⁶ [35]. PFK-2 catalyzes the synthesis of fructose 2,6-bisphosphate, a potent stimulator of glycolysis. Therefore, activation of AMPK rapidly mobilizes glucose into ATP-generating processes.

AMPK also stimulates fatty acid oxidation as a way to increase energy levels. To achieve this goal, AMPK directly phosphorylates acetyl-coA carboxylase (ACC) 1 and 2 isoforms on Ser⁷⁹ and Ser²¹² [36], respectively. ACC is the enzyme that catalyzes the reaction forming malonyl

coA from acetyl coA and that constitutes the initial step in lipid synthesis [36]. In addition, malonyl coA is an allosteric inhibitor of CPT1b [37], the protein responsible for fatty acid intake into the mitochondria for β -oxidation. The phosphorylation of ACC by AMPK renders ACC inactive [36], which translates into a decrease in lipid synthesis rates and the relieve of CPT-1b inhibition, leading to increased fatty acid flux into the mitochondria for β -oxidation. This induction of β -oxidation contributes, together with the increased glycolytic rate, to stimulate ATP synthesis in order to meet the energy requirements of the cell.

Also protein metabolism is affected by AMPK activation. Through phosphorylation of TSC2 [38] and raptor [39], AMPK blocks the mTOR pathway, a major controller of protein synthesis and biomass generation. This not only translates into the attenuation of protein biosynthetic processes [40], but also into the induction of protein degradation through autophagy and the ubiquitin-proteasome system [40]. While AMPK activation is generally linked to both degrading processes, this action seems to be largely indirect via mTOR inhibition and, probably, relying on transcriptional events [28].

The importance of different AMPK trimers

The existence of different isoforms for each AMPK subunit highlights the possibility that 12 different combinations of AMPK trimers can exist. To date, however, we know that all combinations are not found in different tissues and, furthermore, that every trimer combination displays a distinct spectrum of biochemical properties.

At the tissue level, AMPK trimer composition is extremely varied. For example, the α_1 is the predominant isoform in white adipose tissue, blood cells, smooth muscle, endothelial cells and nerve. In contrast, α_2 is the predominant one in tissues such as muscle or heart. Other tissues, like liver, contain both catalytic subunits at similar levels [41]. This tissue-specific pattern is especially clear for the γ_3 subunit of AMPK, whose expression is almost restricted to glycolytic skeletal muscle, where it is the predominant γ isoform [42]. A second degree of specificity, yet to be understood, is how a similar subunit repertoire in different tissues does not necessarily lead to equal trimer composition. A clear example of this can be found in the fact that the β_1 subunit is the predominant subunit associated to α_2 in oxidative muscle, while both β_1 and β_2 equally bind α_2 in glycolytic muscle [43]. An additional layer of complexity is composed by the observation that different trimer compositions can also influence the intracellular localization. Several AMPK subunits (i.e., α_2 , β_2 , γ_1 , γ_3) have been found to partly reside in the nuclear compartment [44–46] (see below for discussion), suggesting that they might be involved in the regulation of gene expression.

Most of the studies on AMPK trimer composition have been performed in mouse skeletal muscle, where it has been postulated that distinct trimers might have different biochemical properties. For example, while AMPK activity has long been known to increase in response to muscle contraction [47, 48], recent studies indicate that ex-vivo contraction at different intensities and time periods can promote trimer-specific activation (see [28] for review).

The use of transgenic mice has contributed to the understanding of isoform-specific contributions to general AMPK functions and global metabolism. For example, the AMPK α_2 knock-out mice, but not the α_1 , are insulin resistant, glucose intolerant and resistant to the hypoglycemic action of AICAR [49, 50]. This is a clear indication that the lack of one subunit cannot be compensated by the other by specificity in the localization, the activation mechanism or the functional output.

As of now, it is clear that we are only at the tip of the iceberg on our knowledge of the significance of the different AMPK trimers. However, the fact that the AMPK trimer composition is regulated in a tissue/compartment-specific fashion, that different AMPK trimers can be selectively activated and that different isoforms can affect specific processes clearly indicates that AMPK trimer composition is non-random and aimed to the regulation of specific functions and/or respond to different kinds/intensities of stresses.

Transcriptional actions of AMPK

Nuclear localization of AMPK

The consequences of AMPK activation expand far beyond acute responses. This is due to the ability of AMPK to directly and indirectly regulate transcriptional programs through phosphorylation events. AMPK modulates the transcription of a number of genes that increase ATP production through glycolysis and the use of lipid as a mitochondrial energy source. Studies in yeast described how snf1, the AMPK α subunit yeast homolog, is present in the nucleus and regulates transcription even through the direct phosphorylation of histones [51]. Pioneering studies by Grahame Hardie's laboratory showed how mammalian AMPK complexes containing the α_2 subunit were, at least partly, distributed in the nuclear compartment [44]. This work was further extended by the demonstration that complexes containing the α_2 subunit translocate to the nucleus in response to muscle contraction [45] or leptin treatment [52]. This specificity by which AMPK α_2 translocates to the nucleus is still largely unknown, but seems to depend on the presence of a nuclear localization signal that is not found in the α_1 subunit [52]. A recent report,

however, also suggests that α_1 -trimers might translocate to the nucleus, too [53]. Elucidating how AMPK shuttles in and out of the nucleus warrants future investigation.

By merging the observations that activation of AMPK promotes its nuclear translocation and that AMPK leads to specific changes in gene-expression patterns, it is easy to postulate that AMPK might be targeting nuclear proteins involved in transcriptional regulation. In the chapters below, we will discuss AMPK-regulated gene expression in different tissues, the key transcriptional regulators involved in this process, and how AMPK modulates their activity.

AMPK transcriptional regulation in muscle

Skeletal muscle is the predominant site of post-prandial glucose uptake and the major affected tissue in insulin-resistant subjects [54]. Upon nutrient scarcity, as occurs during fasting or calorie restriction, the muscle decreases glucose consumption and switches to fatty acid utilization as main energy source [55]. Similarly, with endurance training, skeletal muscle suffers a number of changes, such as fiber-type switch from type IIx to IIa and an increase in mitochondrial biogenesis [56–58], aimed to optimize and enhance energy production. As we will see below, AMPK might act as a key mediator of these adaptations.

Chronic treatment of rodents with AMPK-activating compounds, such as AICAR, β -guanadinopropionic acid (a phosphocreatine depleting agent) or resveratrol, all increase mitochondrial biogenesis in skeletal muscle [59–62]. The actions of these agents on mitochondrial content and gene expression is robustly impaired in models with defective AMPK activity [61, 63–65], implying that AMPK is a master regulator of the transcriptional mechanisms controlling mitochondrial biogenesis. This notion was further confirmed by a number of different gain-of function and loss-of function transgenic approaches. For example, mice overexpressing a kinase dead (KD) AMPK α_2 subunit in muscle displayed less voluntary running activity and reduced endurance performance than wild-type littermates [31], indicating impaired mitochondrial function. Similarly, muscle-specific expression of an inactive form of AMPK α_2 , in which Asp¹⁵⁷ is mutated to Ala, promoted a marked decrease in mitochondrial gene expression and rendered the mice exercise intolerant [66, 67]. These defects in mitochondrial gene expression were also prominent in resting muscles from global AMPK α_2 knock-out mice [63, 68]. Conversely, different genetic manipulations aimed to promote AMPK activation clearly illustrate the positive effects of AMPK activation on mitochondrial activity. Genetic AMPK activation in mice is achieved through different mutations in the γ subunits. Muscle-specific overexpression of a mutated form of AMPK, in which Arg⁷⁰ from the γ_1 subunit is mutated to

Gln, promoted a three-fold higher basal AMPK activity [69], which translated into an increase in mitochondrial markers' gene expression [67]. A different gain of function model, in which a mutated form of the γ_3 subunit (Arg225Gln) is overexpressed, also displayed a prominent increase in mitochondrial gene expression and muscle oxidative profile [70]. Altogether, both pharmacological and transgenic manipulations clearly indicate that AMPK acts as a master transcriptional regulator of mitochondrial genes.

The effects of AMPK activation on mitochondrial genes can be achieved through the regulation of a number of transcriptional factors and cofactors (Fig. 1). For example, AMPK is a master controller of PGC-1 α [60, 65, 71, 72], a transcriptional coactivator that orchestrates a constellation of transcription factors, such as ERR α , NRF1, NRF2 or PPARs, to induce mitochondrial gene expression [73–75]. The link between AMPK and PGC-1 α is further reinforced by the phenotypic similarities of mice with muscle-specific deletions of PGC-1 α or AMPK, both of which have a general reduction in mitochondrial gene expression and exercise intolerance [31, 66–68, 76]. Conversely, a number of pharmacological or transgenic strategies that increase AMPK or PGC-1 α activity in muscle have all consistently potentiated the endurance capabilities of mice and led to a higher oxidative profile of muscle fibers [60, 62, 70, 77, 78]. Firm proof for this link was provided by the fact that AICAR was unable to increase mitochondrial gene expression in muscles of mice lacking PGC-1 α [71]. As such, PGC-1 α seems the key downstream mediator of the effects of AMPK on mitochondrial biogenesis. Several mechanisms explain how AMPK impacts PGC-1 α . AMPK can directly phosphorylate PGC-1 α at Thr¹⁷⁷ and Ser⁵³⁸ in *in vitro* assays [71]. PGC-1 α phosphorylation might not directly affect its intrinsic coactivation activity, but, rather, release it from its repressor protein p160myb [79] and/or allow deacetylation and subsequent activation by SIRT1 [65, 72]. Additionally, AMPK activation increases PGC-1 α expression in muscle [60, 80], an effect that is likely to be achieved through PGC-1 α autoregulation on its own promoter [72, 81–83]. Trimers containing the γ_3 subunit are responsible for the majority of the effect of AMPK on PGC-1 α deacetylation and activation upon exercise or fasting [65]. This is an interesting finding with long-reaching consequences, as the γ_3 subunit is enriched in fast glycolytic muscle, while it is almost absent in oxidative muscle [42]. This helps explain why PGC-1 α is not deacetylated in the oxidative soleus muscle or in the heart upon AMPK activation, but only in glycolytic skeletal muscle [62, 72]. Similarly, trimers containing the γ_3 subunit are the ones more sensitive to exercise-induced energy stress in mouse muscle [28], making them the more apt to fine-tune exercise intensity/duration to transcriptional outputs.

However, PGC-1 α is a coactivator, and its transcriptional effects depend on the transcription factors it coactivates. Therefore, it is also likely that AMPK can somehow target PGC-1 α towards the transcription factors of interest. This is important, as discussed below for liver metabolism, and helps to understand how AMPK activation does not activate all possible PGC-1 α -regulated gene programs. A key transcriptional factor coactivated by PGC-1 α in muscle to promote oxidative metabolism is MEF2 [78], which in turn also regulates PGC-1 α expression through directly binding the PGC-1 α promoter [84]. Interestingly, MEF2 activity is also crucially regulated by AMPK [85, 86], as demonstrated by studies on the GLUT4 promoter [86]. Activation of AMPK leads to the translocation of MEF2 to the nucleus and its binding to its target promoters *in vivo* in a time frame concordant with the increased expression of GLUT4 and PGC-1 α in exercised or AICAR-treated mice [84, 86, 87]. The mechanism by which AMPK impacts on MEF2 is likely to be indirect, as AMPK does not phosphorylate MEF2 [86] and no interaction has been reported to date. One suggested hypothesis was that MEF2 translocation could be aided by its interacting partner GEF (GLUT4 Enhancer Factor) [86, 88]. Interestingly, AMPK phosphorylates GEF and promotes its import into the nucleus and DNA binding [86], strengthening the possibility that both transcription factors are regulated in coordination by AMPK as a unit.

The CREB family of transcription factors has also been implicated in muscle metabolism through the regulation of hexokinase II or PGC-1 α , among others [84, 89]. Recent data indicate that AMPK can phosphorylate the CREB family of transcription factors, including CREB1, ATF1 and CREM [90]. AMPK phosphorylates CREB at the same residue as PKA, Ser¹³³, and enhances CREB-dependent transcription [90]. As discussed in the next chapter this coordination between AMPK and CREB might be conditioned by a number of circumstances and display some tissue/time specificity, as AMPK is also known to block the action of some CREB coactivators [91]. While phosphorylation of CREB is not essential for the binding of CREB to CRE sites, it promotes the recruitment of essential coactivators like CBP/p300 [92]. Interestingly, AMPK has also been shown to directly phosphorylate CBP/p300 at Ser⁸⁹ [93]. This phosphorylation presumably alters the structure of the N-terminal region of the protein, impeding its interaction with nuclear receptors, such as PPARs, but not with other families of transcription factors, such as CREB [93]. While this constitutes a beautiful model to explain a “channelled” activation of gene expression, it potentially contradicts the notion that AMPK exerts a number of its biological effects on lipid oxidative genes through the activation of PPAR α [52, 94]. Indeed, PPAR α and PPAR β/δ constitute attractive mediators for the

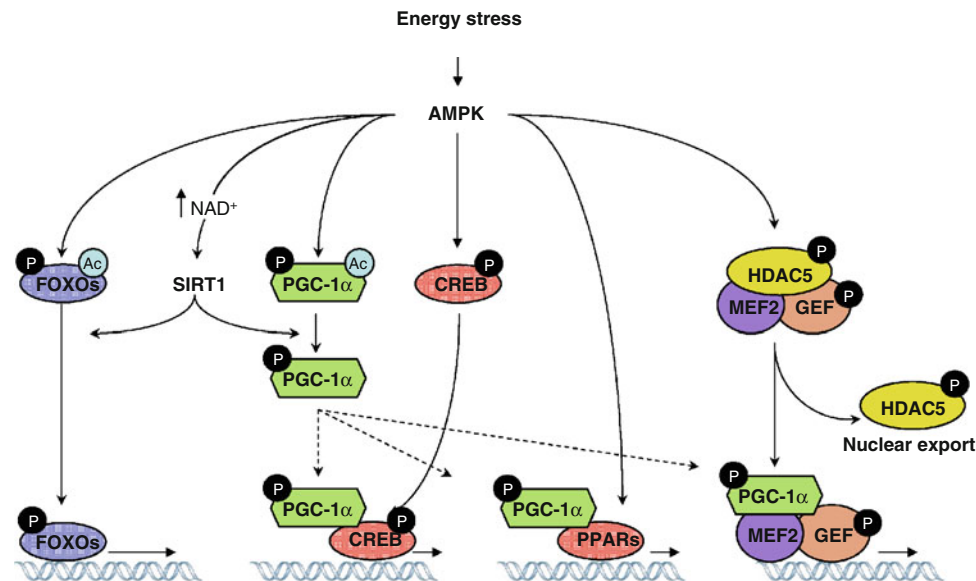


Fig. 1 AMPK regulates muscle transcriptional events through distinct mechanisms. Activation of AMPK upon energy stress increases mitochondrial and oxidative metabolism gene expression through direct and indirect events. SIRT1 is an example of a transcriptional regulator whose activity is increased by AMPK through an indirect mechanism (i.e., by promoting an increase in NAD⁺). Direct phosphorylation of AMPK occurs, for example, on the coactivator PGC-1 α and the FOXO family of transcription factors, whose subsequent deacetylation by SIRT1 increases their activity. The

activation of PGC-1 α leads to the coactivation of a myriad of transcription factors, such as PPAR α , PPAR β/δ and CREB, which is also phosphorylated and activated by AMPK. Phosphorylation of GEF promotes co-translocation with MEF2 to the nucleus. Furthermore, phosphorylation of HDAC5 by AMPK relieves the inhibition on the MEF2/GEF complex and allows transcriptional activation. These examples illustrate the mechanisms involved when AMPK directly and indirectly regulates transcriptional events

transcriptional actions of AMPK, as the metabolic profile achieved by AMPK activation shares many common features with that obtained through PPAR α and PPAR β/δ activation, i.e., stimulation of mitochondrial biogenesis, of endurance performance and of lipid oxidation metabolism [95–99]. Some results already support that PPAR α mediates the transcriptional actions of AMPK on oxidative metabolism [94], and recent data suggest that simultaneous AMPK and PPAR α or PPAR β/δ activation may act synergistically in the induction of such genes [77, 100]. It has also been proposed that the AMPK can interact with PPAR α or PPAR β/δ through the α subunit, leading to a synergistic effect with the ligand-dependent activation of the nuclear receptor [77, 100, 101]. Interesting in this context, despite many efforts, no consistent evidence exists for the requirement of a direct phosphorylation event to link AMPK with PPAR α or PPAR β/δ activity [77, 101]. Another plausible explanation for the synergism between AMPK and PPAR activation could be the fact that the activation of PGC-1 α by AMPK would further increase transcriptional co-activation of the ligand-bound PPAR α or PPAR β/δ . The ability of AMPK to acutely promote lipid oxidation could provide endogenous ligands for PPARs, hence contributing as such to the synergism between the kinase and the PPARs. Unravelling these links between AMPK and PPARs will constitute a promising ground for

investigation in the years to come. Expanding on this field, it will be interesting to test the possible relationship and synergistic effects that AMPK could have with other nuclear receptors that strongly influence mitochondrial biogenesis, such as the estrogen-related receptors (ERRs) [102].

The FOXO family of transcription factors is another seducing target for AMPK. The actions of FOXO have been linked to lifespan extension [103], and in muscle they are commonly associated with protection against oxidative stress, enhancement of lipid metabolism and induction of autophagy [104]. The relation of AMPK with FOXOs was brought to light when FOXOs were reported as possible mediators of the effects of AMPK on autophagy [105]. Furthermore, AMPK can directly phosphorylate different members of the FOXO family of transcription factors [106]. Among them, FOXO3 is phosphorylated by AMPK in up to six residues [106]. Mutation of these residues impaired the ability of AMPK to promote key transcriptional responses during glucose deprivation, including the transcriptional activation of oxidative protection genes [106]. FOXO phosphorylation by AMPK does not influence FOXO sub-cellular localization, but rather its activity [106]. However, it must be noted that, as with PGC-1 α , FOXO activity is also critically controlled through acetylation/deacetylation, which is altered by SIRT1 [107–109]. It is tempting to

speculate that AMPK phosphorylation of FOXO could also serve as a signal for the deacetylation by SIRT1, which, in turn, seems to provide FOXO with specificity towards the regulation of oxidative stress genes [107], suggesting that the modifications of FOXO by AMPK and SIRT1 might be interconnected.

The transcriptional actions of AMPK in muscle not only take place through the activation of transcriptional factors, but also through the modulation of corepressors and histone deacetylase activities. For example, SIRT1 has already been mentioned as an enzyme whose activity is highly linked to AMPK [72, 110]. SIRT1 is an evolutionarily conserved NAD⁺-dependent deacetylase, whose action impacts on a number of transcriptional regulators [111]. Activation of SIRT1 has generally been linked to the induction of lipid oxidation and mitochondrial metabolism in muscle [112]. The similar phenotypic outputs from AMPK and SIRT1 activation suggest that there might be a functional link between both activities. Direct interaction or phosphorylation events, however, do not seem to take place between these enzymes [72, 106]. Rather, AMPK seems to influence SIRT1 activity through an AMPK-induced modulation of NAD⁺ metabolites [72, 110], which are critical determinants of SIRT1 activity [113, 114]. For example, pharmacological or physiological activation of AMPK is followed by a robust increase in NAD⁺ within hours, which derives from the metabolic rearrangements promoted by an increase in fatty acid oxidation rates [72]. This metabolic and fast increase in NAD⁺ levels induced by AMPK is sustained by the induction of Namp1 expression, a gene that resynthesizes NAD⁺ from its metabolic breakdown product, nicotinamide [110]. This constitutes a two-way impact of AMPK on SIRT1 activity as it generates the SIRT1 activator NAD⁺, while reducing the levels of nicotinamide, a physiological inhibitor of SIRT1 activity [114]. The intimate link between SIRT1 and AMPK is further reinforced by studies using resveratrol, a polyphenol compound that has long been used as a SIRT1 agonist. Resveratrol increases lifespan in a number of lower eukaryotes [115]. In higher eukaryotes, resveratrol increases muscle mitochondrial content and enhances endurance performance [62]. This increased ability to oxidize lipids confers the mice with protection against metabolic disease upon high-fat feeding [62, 116]. While it is true that an important number of biological actions of resveratrol depend on SIRT1 [115], the initial belief that resveratrol could act as a direct SIRT1 agonist [117] is long gone now, as *in vivo* evidence suggests that resveratrol rather acts primordially through AMPK, and any effect on SIRT1 activity is a downstream consequence of AMPK activation [64, 65]. These observations stress the relevance of AMPK/SIRT1 as a conserved signaling axis that is activated upon energy stress. Resveratrol effects on AMPK probably

derive from the overlooked fact that resveratrol can act as a mild mitochondrial “poison” by inhibiting complex III and V of the mitochondrial respiratory chain [118, 119]. Therefore, resveratrol’s actions, like those of metformin [20, 120], likely derive from a mild impairment in ATP synthesis.

Another enzyme, HDAC5, is the predominant type II histone deacetylase in adult skeletal muscle. In general, HDAC5 acts as a transcriptional repressor by directly deacetylating histone lysine residues within the nucleosome, forming a compact structure that limits the accessibility of transcriptional regulators to DNA [121]. The specificity of genes repressed by HDAC5 is provided by the ability of this deacetylase to bind only certain transcription factors, such as MEF2 [122, 123]. This way, HDAC5 controls a myriad of processes in skeletal muscle, from glucose and oxidative metabolism [124, 125] to myocyte differentiation [126]. GLUT4 expression is controlled by interactions among AMPK, HDAC5 and MEF2. This involves an interesting cascade of events in which translocation of certain AMPK trimers to the nucleus upon activation allows the direct phosphorylation of HDAC5 in two residues, Ser²⁵⁹ and Ser⁴⁹⁸ [124]. This AMPK-dependent phosphorylation of HDAC5 triggers its dissociation from MEF2 and provides binding sites for 14-3-3 proteins, which export HDAC5 out from the nucleus [124]. The release of HDAC5 will increase histone acetylation and enable the recruitment of MEF2 coactivators, such as PGC-1 α [127], and the basic transcriptional machinery to the GLUT4 promoter. The mutation of these HDAC5 phosphorylatable residues is enough to prevent AMPK-dependent induction of the GLUT4 gene [124], clearly illustrating the relevance of this mechanism of action.

Transcriptional regulation by AMPK in liver

The liver is key to maintain the whole body’s nutrient homeostasis, as it adapts its ability to store and release carbohydrates to the metabolic needs of the organism. Deficiencies in this regulatory mechanism are manifested in type 2 diabetic patients, where elevated hepatic glucose production leads to hyperglycemia. Consequent to the fact that energy stress triggers its activity, AMPK activation in liver shuts down glucose, cholesterol and triglyceride biosynthetic pathways in liver while promoting fatty acid oxidation [41]. Most manipulations of AMPK activity in liver confirm this paradigm. Deletion of the α_2 subunit of AMPK in the liver promotes hyperglycemia and glucose intolerance because of increased hepatic glucose production [128]. Similarly, defective AMPK activity compromises fatty acid metabolism as a consequence of decreased mitochondrial gene expression [129], leading to increased plasma free fatty acids and decreased production of ketone

bodies. Conversely, overexpression of an active form of AMPK α in liver is enough to improve glucose profiles in diabetic mice [130]. Similarly, overexpression of the α_2 subunit in the liver decreases plasma triglycerides and increases production of ketone bodies, reflecting an increase in lipid oxidation versus synthesis [130].

Some of the above-mentioned actions of AMPK happen through direct phosphorylation of key enzymes. This is the case with, for example, the regulation of cholesterol biosynthesis, which is rapidly decreased by AMPK through direct phosphorylation and inhibition of the rate-limiting enzyme hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase [131]. Another example is ACC, whose phosphorylation by AMPK prevents lipid synthesis and favors fatty acid import into the mitochondria for oxidation [36]. However, processes like gluconeogenesis and lipid biosynthesis are also highly regulated by transcriptional changes. Most of them are crucially affected by AMPK, as described below.

Gluconeogenesis, the *de novo* synthesis of glucose, takes place in liver through the fast induction of genes encoding rate-limiting enzymes of this process, such as phospho-enol pyruvate carboxykinase (PEPCK) or glucose-6-phosphatase (G6P). Gluconeogenesis is triggered by an increase in intracellular cAMP, as a consequence of low insulin and increasing glucagon blood levels. Through a cascade of events, increased cAMP levels will activate the transcription factor CREB, which binds to and activates the promoters of the above-mentioned genes [132]. Furthermore, binding of the CREB coactivator CRTC2 to CREB allows the recruitment of the transcriptional machinery [133]. AMPK regulates CRTC2 in a similar fashion to that described above for HDAC5 [91]. AMPK can directly phosphorylate CRTC2 on Ser¹⁷¹ [91]. Interestingly, the ability to phosphorylate this residue is shared by other members of the AMPK-related kinases subfamily, such as SIK2 [133]. This phosphorylation event promotes the binding of 14-3-3 to CRTC2 and induces its export to the cytosol [133]. The immediate consequence of this is that CREB loses the interaction with its coactivator and, consequently, CREB-dependent gluconeogenic gene expression is reduced. It is important to note that activation of AMPK led to increased CRTC2 cytoplasmic localization even in the presence of cAMP agonists [91], indicating that cellular energy stress overrides the systemic needs for glucose synthesis. Importantly, this characteristic is unique to AMPK, as phosphorylation of CRTC2 by SIK2 is prevented by cAMP agonists [133]. This model also raises a number of questions. For example, there are situations in which agents that increase cAMP, such as forskolin, isoproterenol or glucagon, lead to AMPK activation [134, 135] which, in liver, would be antagonistic with the induction of gluconeogenic genes. Recent results indicate

that PKA can phosphorylate and negatively regulate certain AMPK trimers containing the α_1 subunit [136], which could keep AMPK activity low during gluconeogenic periods. Another complexity relies in the fact that, at least in muscle, AMPK can phosphorylate and activate CREB [90]. If this happened in liver, then there should be additional mechanisms targeting CREB to non-gluconeogenic gene sets.

An additional critical transcription factor regulating glucose metabolism in liver is HNF4 α , which controls the expression of GLUT2, pyruvate kinase (L-PK) and aldolase B, among others [137]. Initial findings showed how pharmacological activation of AMPK by AICAR led to a downregulation of HNF4 α target genes [138]. This phenomenon was linked to a robust reduction in HNF4 α protein levels, apparently caused by a decrease in HNF4 α protein stability [139]. Furthermore, HNF4 α was identified as a direct target for AMPK. Specifically, AMPK phosphorylated Ser³⁰³ (Ser³¹³ in humans) [139], a residue located in the ligand-binding domain that directly participates in homodimerization, the functional form of these transcription factors. Consequently, it was reported that mutation of Ser³⁰³ to Asp, mimicking constant phosphorylation, impeded HNF4 α homodimerization and DNA binding [139]. Of note, the implications of these findings might not be limited to the liver, as HNF4 α is a critical regulator of glucose metabolism through actions in the pancreas, kidney and intestine [137].

AMPK might also participate in the modulation of a third transcription factor involved in the sensing and regulation of liver glucose metabolism. The carbohydrate response element binding protein (ChREBP) is a liver-specific transcription factor that promotes the expression of glycolytic and fatty acid synthesis genes in situations of high glucose availability [140]. Like HNF4 α , ChREBP induces L-PK expression by binding to its promoter [141]. It has been reported that AMPK phosphorylates ChREBP on Ser⁵⁶⁸, thereby compromising its DNA binding and transcriptional activities [142]. By inhibiting ChREBP, AMPK promotes the use of fatty acids as the main energy source. These findings, however, have been challenged by a report showing that ChREBP nuclear translocation is normal in AMPK-deficient animal models [143]. It must be remembered, however, that as with CREB and CRTC2, AMPK may not be the only kinase acting on ChREBP, and compensatory mechanisms could explain the unaltered phenotype in AMPK-deficient models.

Some conflicting points arise from the extrapolation of AMPK's effects on certain transcriptional regulators in muscle, such as FOXO, SIRT1 and PGC-1 α . AMPK activation in liver promotes an increase in the ratio between β -oxidation and lipogenesis, in part through the induction of mitochondrial content and function ([116], Cantó C and

Auwerx J, unpublished observations). Conversely, ablation of AMPK in liver reduces mitochondrial content and activity [129, 144], probably as a consequence of decreased PGC-1 α expression and activity [129]. However, in contrast to the role of AMPK, most reports to date indicate that PGC-1 α induces gluconeogenesis [145, 146]. Activation of PGC-1 α through SIRT1-mediated deacetylation seems to be a key step in the induction of the gluconeogenic program [147, 148]. Intriguingly, AMPK-induced PGC-1 α expression and deacetylation can also be observed in liver, indicating that AMPK increases SIRT1 and PGC-1 α activity (Cantó C., Auwerx J., unpublished observation). This being so, why does AMPK activation not promote gluconeogenesis? A very likely explanation lies in the fact that PGC-1 α is a coactivator, and consequently, its action depends on the transcription factors it binds to. As AMPK inactivates CRTC2 and HNF4 actions, it is possible that PGC-1 α cannot properly bind CRTC2/CREB and HNF4 transcriptional complexes, therefore redirecting its coactivating activities to other transcription factors linked to mitochondrial biogenesis. While such an explanation might be valid in the case of PGC-1 α , it is more difficult to apply to the case of the FOXO family of transcription factors, which are activated by AMPK and mediate a significant part of AMPK's effects in a number of tissues [106, 149, 150]. Most results to date make it unlikely that this also should be the case in liver, as the FOXO transcription factors are critical positive gluconeogenic regulators [104]. Furthermore, deacetylation by SIRT1 seems to promote nuclear trapping of FOXOs and transcription of gluconeogenic genes [109], which is diametrically opposite to what would be expected for AMPK activation. Strikingly, a great deal of evidence show that resveratrol, which activates AMPK in liver and cultured hepatocytes [116, 151], leads to FOXO deacetylation [109]. Therefore, the paradigm that SIRT1 is pro-gluconeogenic through its actions on FOXO and PGC-1 α need to be revised in light of the number of conflicting observations, for example:

- (1) While SIRT1 downregulation in liver through adenoviral delivery of SIRT1 shRNAs leads to fasting hypoglycemia and decreased expression of gluconeogenic genes [148], liver-specific SIRT1 knock-out mice show normal blood parameters upon fasting and nicely adapt to calorie restriction [152].
- (2) SIRT1 activation in liver does not seem to happen in the initial phase of gluconeogenesis, which is controlled by CRTC2, but rather occurs during a later phase, leading to the deacetylation and degradation of CRTC2, which attenuates gluconeogenic rate [153].
- (3) Mice mildly overexpressing SIRT1 are largely normal when fed a standard chow [154–156], with a tendency towards lower fasting blood glucose levels [155].

SIRT1 overexpression, however, effectively protected against hyperglycemia in a number models of metabolic disease because of reduced hepatic glucose output [154, 156] and lower FOXO and PGC-1 α acetylation levels [154], indicating that SIRT1 activity can actually be linked to a decrease in gluconeogenic rates.

- (4) In all murine models of metabolic disease and diabetes tested to date, resveratrol or similar compounds consistently protect against hyperglycemia, triglyceride accumulation and excessive cholesterol production [62, 116, 157, 158], very much in line with the results obtained in mice overexpressing SIRT1 [154, 156]. AMPK is robustly activated in the livers of mice fed with resveratrol [116], and the phenotypic outputs are perfectly in line with those expected for AMPK activation. Since these mice displayed higher SIRT1 and PGC-1 α activity [116], physiological activation of SIRT1 or PGC-1 α in liver is not per se linked to gluconeogenesis. Similar observations were made with the SIRT1 agonist described by Sirtris, SRT1720 [159, 160], even though the direct and specific effects of this compound on SIRT1 activation are controversial [161].
- (5) The observations that resveratrol deacetylates FOXO1 [109] and protects against hyperglycemia [62, 116] indicate that FOXO activation of the gluconeogenic program might be avoided or be very moderate in situations of AMPK activation, while the induction of other FOXO target genes is prioritized. This might be explained by the fact that FOXO actions sometimes require interplay with other transcription factors, such as HNF4 α [162, 163], to modulate glucose metabolism genes. Therefore, AMPK might also channel FOXO activity to specific gene sets through post-translational modifications, such as phosphorylation [106] and deacetylation [107], and by preventing its interplay with certain transcription factors.
- (6) Recent evidence indicates that SIRT1 enhances AMPK action in the liver by deacetylating LKB1, altering its cellular localization and its association with STRAD, ultimately stimulating its activation of AMPK [16]. This suggests that SIRT1 and AMPK might reciprocally activate each other in liver and HepG2 cells [16, 164, 165], creating a positive feedback loop. Such observations imply that AMPK and SIRT1 activities would also go hand in hand in liver, which contradicts the notion of SIRT1 as pro-gluconeogenic factor.

Given these observations, it is clear that we are only at the beginning of our understanding about how the

transcriptional effectors of AMPK are regulated, but it seems clear that different mechanisms of action might be coexisting (Fig. 2). The lack of a linear extrapolation of the way in which SIRT1, PGC-1 and FOXOs act downstream of AMPK complicates the picture. Furthermore, we are still far from grasping how AMPK quickly downregulates some key players in liver lipid metabolism, such as SREBP1c [120, 130]. Given the proven efficacy of AMPK-activating drugs, such as metformin, in type 2 diabetes, the clarification of these enigmas should be a priority for the field.

Additional transcriptional regulators controlled by AMPK

Most of the attention on AMPK has been focused on transcriptional regulation in metabolic tissues, as those described above, or in the immediate phosphorylation of metabolic enzymes and signaling pathways. Still, AMPK

may regulate additional transcriptional events, which are worthy of attention.

Cell cycle and differentiation regulators

A riveting field for future study is the regulation of p53 by AMPK, which potentially will shed light on the link among metabolism and cell cycle and division. Evidence is accumulating that AMPK could control the cell cycle by promoting G1 arrest and reduce the number of S phase cells [166, 167]. Studies showing that AMPK can directly phosphorylate p53 on Ser¹⁵ (Ser¹⁸ in mice) were key to understanding the effects of AMPK on proliferation [166]. In normal circumstances, p53 is rapidly ubiquitinated and degraded. A number of post-translational modifications, such as phosphorylation and acetylation, can stabilize the protein and promote cell cycle arrest and anti-tumorigenic effects [168]. In line with this, phosphorylation of p53 by

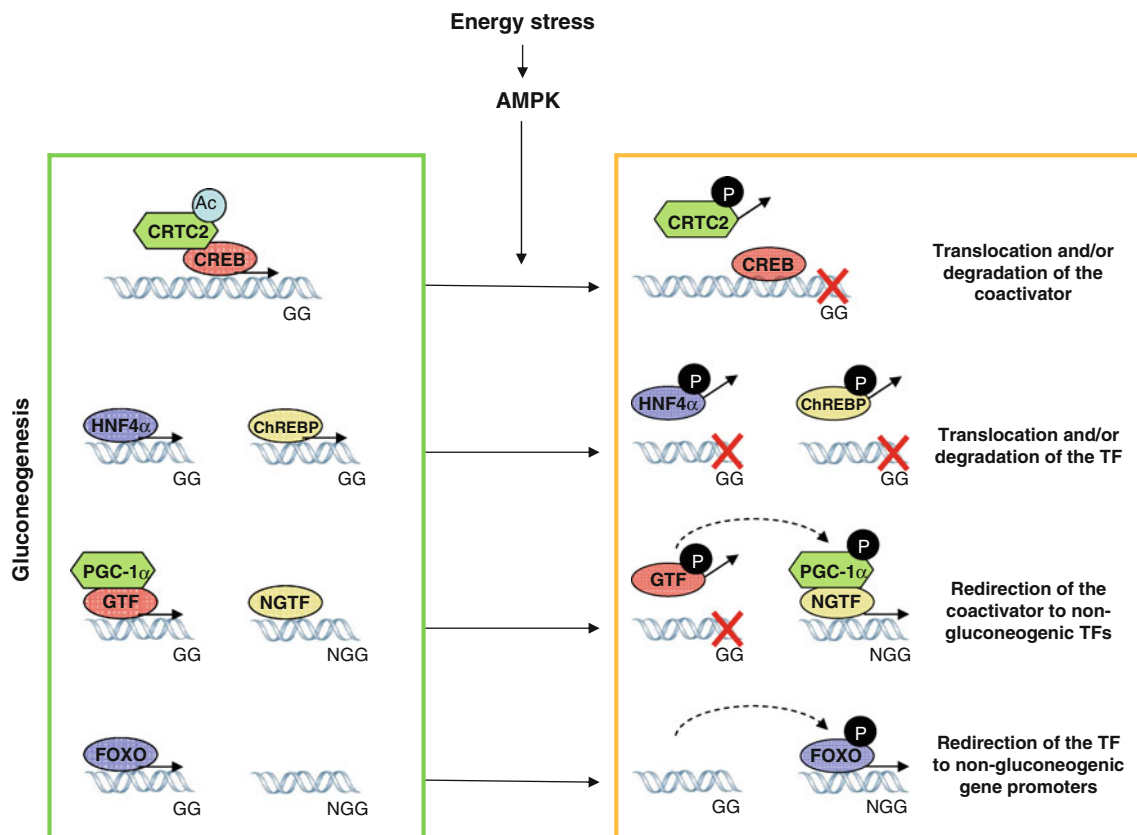


Fig. 2 AMPK anti-gluconeogenic effects are achieved through a combination of different transcriptional mechanisms. A constellation of transcriptional regulators modulates gluconeogenesis, such as CRTC2, FOXO, ChREBP, HNF4 α and PGC-1 α . AMPK impacts on them all through different strategies. For example, AMPK phosphorylates CRTC2 and promotes its nuclear exclusion, disassembling the coactivator from CREB on gluconeogenic genes (GG). AMPK can

also directly phosphorylate transcription factors (TFs), as happens with HNF4 α and ChREBP, promoting their nuclear exclusion and/or degradation. In the case of PGC-1 α , phosphorylation by AMPK might direct its coactivating actions towards non-gluconeogenic gene (NGG) regulation. Similarly, phosphorylation of FOXOs by AMPK may drive its action from gluconeogenic genes towards other gene sets, such as oxidative protection

AMPK stabilizes p53 and induces the expression of its target gene p21 [166, 167], an inhibitor of cyclin-dependent kinases, which promotes a cell cycle arrest at the level of G1 and G2 [169]. Therefore, situations of low nutrient availability and/or energy stress could translate into a natural inhibition of cell division in order to ensure cellular sustainability. These findings have serious implications for the possibility of using AMPK-activating compounds as anti-cancer drugs. Additionally, p53 has also been linked to the transcriptional regulation of mitochondrial metabolism [170], providing a new mechanism by which AMPK could impact on mitochondrial gene expression. An intriguing paradox in the link between AMPK and SIRT1 is the fact that SIRT1 is known to deacetylate and inactivate p53 [171, 172], while the role of AMPK seems to be the opposite. Elucidation of this apparent contradiction deserves investigation. To date, most of the studies on the AMPK/SIRT1 link have been done in adult normal tissue, making it possible that this signaling pathway is altered in tumors. Similarly, a recent report indicates that SIRT1 activity can somehow be oriented towards certain targets, as phosphorylation of SIRT1 by JNK leads to specific deacetylation of p53, but not of other substrates [173]. This concept is in line with our observations showing that PGC-1 α needs to be primed by prior AMPK-mediated phosphorylation in order to be deacetylated [72] and makes it possible that in a similar fashion AMPK phosphorylation of p53 could be preventing or not affecting SIRT1 interaction with this substrate.

Another transcriptional regulator controlled by AMPK is the retinoblastoma protein (Rb). Rb regulates the progression, fate and differentiation of a number of cell types by binding and modulating the activity of members of the E2F family of transcription factors [174]. In neuronal precursor and stem cells, AMPK can directly phosphorylate Rb on Ser⁸⁰⁴, which then leads to its dissociation from E2F [175]. This is in line with the fact that low glucose promotes Rb/E2F dissociation [175]. The regulation of the Rb/E2F axis by AMPK has long-reaching consequences. For example, Rb phosphorylation status determines a number of fate choices [176] and interactions with other transcriptional regulators, such as PPAR γ [177]. However, as several kinases can impact on the phosphorylation of the same residue in Rb, it is difficult to extrapolate from these data the relevance of AMPK signaling on the Rb/E2F axis. In fact, a number of scenarios are potentially opposed to the hypothesis that AMPK inhibits Rb and favors E2F transcription, such as those implying that Rb is a tumor suppressor [174] or that E2F can negatively regulate mitochondrial biogenesis [178]. It is also interesting to note that, again, AMPK and SIRT1 find a convergent substrate in Rb [179], even though any possible interplay between AMPK-mediated phosphorylation and SIRT1-dependent deacetylation of Rb is yet to be explored.

Direct regulation of the epigenetic and transcriptional machinery

Other possible substrates of interest that need confirmation are those intimately related to epigenetic phenomena. The finding that AMPK trimer containing the γ_3 subunit could be detected in the nucleoli [46] led to the hypothesis that it could participate in the regulation of rRNA synthesis, which is necessary for the whole ribosomal structure and mRNA translation. As AMPK is known to decrease protein translation by inhibiting the mTOR pathway [180], it would make sense that it could also shut down this process directly through an alternative mechanism. In line with this, AMPK activation decreased RNAPol I activity [46, 181]. This raised the hypothesis of a possible direct regulation through phosphorylation events in the nucleoli, as recently shown by the fact that AMPK phosphorylates the RNA polymerase I (Pol I)-associated transcription factor TIF-IA at Ser⁶³⁵ [181]. Phosphorylation by AMPK impairs the interaction of TIF-IA with SL1, precluding the assembly of functional transcription initiation complexes [181]. Further supporting this hypothesis, mutation of Ser⁶³⁵ prevents down-regulation of Pol I transcription in response to low energy supply [181]. All these results provide evidence that activation of AMPK adapts rRNA synthesis to nutrient availability [181]. Another intriguing link is that between AMPK and histone phosphorylation, which derives from pioneer findings in yeast indicating that the yeast AMPK homolog, snf1, could phosphorylate histone 3 on Ser¹⁰, enabling the subsequent recruitment of the GCN5 acetyltransferase to acetylate Lys¹⁴, unfold DNA strands and initiate transcription [51]. While the possibility of AMPK directly phosphorylating histones on target genes would open doors for innumerable hypothesis, this finding has not yet been confirmed in mammalian cells. Additionally, it would also imply the requirement of a currently unknown additional specificity mechanism in order to select target genes.

Conclusions and future perspectives

The fact that AMPK activation tightly controls the transcriptional regulation of a number of gene sets has been known for years. A number of transcriptional regulators have arisen as immediate AMPK phosphorylation targets, but the implications of such findings at the gene promoter level are far from understood. We are now beginning to elucidate the way phosphorylation by AMPK influences the activity and interaction of transcriptional regulators in different tissues, which will provide clues on how AMPK determines gene set specification. Furthermore, AMPK regulates transcription not only through direct

events (i.e., phosphorylation of transcriptional regulators), but also indirectly (for example, by increasing NAD⁺ and inducing SIRT1 activity). Further possibilities yet to be explored would involve the direct binding of AMPK to target promoters. Another challenging point for future research will be the complete understanding of how AMPK actually shuttles in and out of the nucleus and of how the nuclear functions of AMPK depend on the trimer composition. All these questions will need answers in order to fully understand AMPK action.

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