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## AMPK: mechanisms of cellular energy sensing and restoration of metabolic balance

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### Abstract

AMPK is a highly conserved master regulator of metabolism, which restores energy balance during metabolic stress both at the cellular and physiological levels. The identification of numerous AMPK targets has helped explain how AMPK restores energy homeostasis. Recent advancements, however, demonstrate that regulation of AMPK is also affected by novel contexts, such as subcellular localization and phosphorylation by non-canonical upstream kinases. Notably, the therapeutic potential of AMPK is widely recognized and heavily pursued for treatment of metabolic diseases such as diabetes, but also obesity, inflammation and cancer. Moreover, the recently solved crystal structure of AMPK has shed light both into how nucleotides activate AMPK but, importantly, also into the sites bound by small molecule activators, thus providing a path for improved drugs.

### Keywords

AMPK; trimeric complex; energy restoration; metabolic adaptation; therapeutic activation

### Introduction

Maintenance of energy homeostasis and the execution of adaptive responses during periods of low nutrients are critical functions of all cells. The main sensor of cellular energy status in effectively all eukaryotic cells is the AMP-activated protein kinase (AMPK), which is highly conserved across all eukaryotic species. In general, AMPK is activated in response to energy stress by sensing increases in AMP:ATP and ADP:ATP ratios and restores energy balance by inhibiting anabolic processes that consume ATP, while promoting catabolic processes that generate ATP. Moreover, the activity of AMPK is extensively regulated by multiple upstream signals, thus making AMPK a central node exploited by cells to coordinate their metabolism with specific energy demands. Importantly, the primordial role of AMPK as an energy sensor has been co-opted in higher eukaryotes, such as mammals, to also coordinate growth and metabolism both in specialized tissues and at the whole body level. Therefore, this ability of AMPK to reprogram metabolism is heavily pursued as a therapeutic avenue

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for the treatment of several metabolic diseases, especially diabetes, but also for obesity, inflammation, and cancer. Here we review recent advances in our understanding of AMPK structure, its complex upstream regulation and downstream effects, and provide perspective on novel approaches for the therapeutic activation of AMPK.

## The AMPK trimeric complex

AMPK exists as a trimeric complex consisting of a catalytic subunit ( $\alpha$ -subunit) and two regulatory subunits ( $\beta$ - and  $\gamma$ -subunits). In mammals, the  $\alpha$ -subunit is encoded by two isoforms, and the  $\beta$ - and  $\gamma$ -subunits are encoded by two and three isoforms, respectively (Fig 1A). The expression levels of these AMPK isoforms vary across tissues, leading to diverse subunit combinations in different cell types. Although the distinct complexes are for the most part functionally redundant, they can exhibit somewhat different biochemical properties (Ross et al., 2016). While AMPK $\alpha$ 1, AMPK $\beta$ 1, and AMPK $\gamma$ 1 are ubiquitously expressed, the other isoforms show a more restricted expression pattern. AMPK  $\alpha$ 2 is expressed at high levels in skeletal and cardiac muscle, where it is the dominant  $\alpha$ -subunit, but is also expressed in the liver and at lower levels in other tissues. Similarly, AMPK $\beta$ 2 is the dominant  $\beta$ -subunit in skeletal and cardiac muscle, but it is found at lower levels in many other tissues. The expression of AMPK $\gamma$ 2 and AMPK $\gamma$ 3 appears to be restricted to skeletal and cardiac tissue.

The N-terminus of the  $\alpha$ -subunit is comprised of the kinase domain (KD). Phosphorylation of a conserved threonine in the activation loop (by convention referred to as Thr172) of the kinase domain is required for full activation of AMPK. The C-terminus of the  $\alpha$ -subunit binds the  $\beta$ - and  $\gamma$ -subunits and contains important regulatory domains, namely, a so-called autoinhibitory domain (AID), the  $\alpha$ -linker (which interacts with the  $\gamma$ -subunit through two regulatory-subunit interacting motifs, or  $\alpha$ -RIM motifs) and a serine/threonine rich domain referred to as the “ST-loop”. The  $\beta$ -subunit contains two conserved domains: a carbohydrate-binding module (CBM) (also called the glycogen-binding domain), which allows AMPK to sense glycogen, and a C-terminus domain that binds the  $\alpha$ - and  $\gamma$ -subunits. The  $\beta$ -subunit also contains a myristoylation site at its N-terminus, which facilitates targeting of AMPK to cellular membranes. The  $\gamma$ -subunit is comprised of two Bateman domains, each of which contains two cystathionine  $\beta$ -synthase repeats (CBS). The four CBS repeats form the four sites in AMPK that can potentially bind AMP, ADP or ATP in a competitive manner. In mammals, site 1 and site 3 exchange adenine nucleotides competitively, whereas site 2 is missing the key aspartate required for nucleotide binding, which renders it non-functional (Xiao et al., 2011). Site 4 can also bind AMP and ATP, but has higher affinity for AMP (Calabrese et al., 2014; Chen et al., 2012). The ability of the  $\gamma$ -subunit to bind AMP, ADP and ATP confers AMPK with its exquisite ability to sense the energy state of the cell.

## AMPK structure

Our molecular understanding of the regulation and function of AMPK has significantly been advanced by the elucidation of the crystal structures of a variety of AMPK holoenzymes (Calabrese et al., 2014; Chen et al., 2012; 2013; Li et al., 2015a; Xiao et al., 2011; 2013; Xin

et al., 2013). Although the reports differ in certain details, together they provide a detailed view of the architecture of the AMPK complex. The structure of the AMPK trimeric complex consists of three major segments or “modules”: the catalytic module, the carbohydrate-binding module (CBM) and the nucleotide-binding module (also called “regulatory fragment”) (Fig 1B). The activation loop of the  $\alpha$ -subunit resides at the interface between the catalytic and nucleotide-binding modules, in close proximity to the C-terminus of the  $\beta$ -subunit and the CBS repeats of the  $\gamma$ -subunit. This structural arrangement ensures that phosphorylation and dephosphorylation of Thr172 is sensitive to conformational rearrangements induced by nucleotide binding. The catalytic domain exhibits a typical eukaryotic serine/threonine kinase domain structure with a small N-lobe and a large C-lobe. The CBM directly contacts the N-lobe of the kinase domain and the interface between these two modules forms a discrete pocket that was identified as the binding site for many direct AMPK-activating compounds. It is speculated that natural metabolites might bind this site to regulate AMPK, however, no such metabolite has been yet identified. The nucleotide-binding module is made up mostly by the  $\gamma$ -subunit, which forms a flattened disk with the CBS repeats symmetrically arranged around the disk, one in each quadrant.

Mechanistically, these crystallographic studies reveal the molecular details of how adenine nucleotides and small molecule activators activate AMPK. In the case of nucleotides, the crystal structures show that when AMP is bound to site 3, the  $\gamma$ -subunit forms stable interactions with a few amino acids within the  $\alpha$ -linker’s  $\alpha$ -RIM1 and  $\alpha$ -RIM2, which interact with the unoccupied site 2 and the AMP molecule bound at site 3, respectively (Chen et al., 2013; Xiao et al., 2013; Xin et al., 2013). The binding of the  $\alpha$ -RIM motifs to the  $\gamma$ -subunit restricts the flexibility of the  $\alpha$ -linker, resulting in tighter association of the catalytic and nucleotide-binding modules, which physically protects Thr172 from dephosphorylation. Interestingly, the same effect is proposed to occur when ADP binds site 3, raising the possibility that in some contexts ADP might be the relevant AMPK activating signal (Xiao et al., 2011). Moreover, the binding of the  $\alpha$ -RIM motifs to the  $\gamma$ -subunit shifts the AID in the  $\alpha$ -subunit away from the kinase domain when AMP is bound, thus releasing the AID’s negative effects on the kinase domain (Calabrese et al., 2014; Chen et al., 2013; Li et al., 2015a). This rearrangement of the AID domain may represent the molecular basis for the allosteric activation effect of AMP. According to this model, the AID can shift between kinase domain-bound (inactive AMPK) and nucleotide-module-bound states (active AMPK) depending on the nucleotide binding status. In summary, the published crystal structures concur that binding of AMP, especially at site 3, induces a conformational change that is transmitted to the kinase domain by changes in the interaction of the  $\alpha$ -RIM motifs and the AID with the nucleotide-binding module. These structural changes, which are opposed by ATP, result in the allosteric activation of AMPK and a compaction of the interface between the catalytic module and the nucleotide-binding module, which protects Thr172 from dephosphorylation. However, it is not clear whether these structural rearrangements also promote Thr172 phosphorylation.

On the other hand, activating compounds, such as A769662, activate AMPK by a different mechanism. Binding of these compounds, together with phosphorylation of serine 108 in the  $\beta$ -subunit, stabilizes the CBM and strengthens the interaction of the CBM with the kinase domain (Calabrese et al., 2014; Li et al., 2015a; Xiao et al., 2013). Specifically, binding of

activating compounds induces the formation of an  $\alpha$ -helix in the  $\beta$ -subunit, termed the C-interacting helix, which interacts with the so-called C-helix of the kinase domain (a conserved helix across multiple kinases which is important for ATP binding). This conformational change results in a shift towards a closed, active conformation of the kinase domain, protection from Thr172 dephosphorylation, and increased substrate affinity. Interestingly, glycogen inhibits the CBM-KD interaction and this may be the mechanism by which glycogen inhibits AMPK (McBride et al., 2009).

## Nucleotide-dependent and nucleotide-independent regulation of AMPK

The regulation of AMPK as a sensor of changes in intracellular levels of AMP, ADP and ATP, such as in the case of energy stress, is significantly understood and is well established as the canonical regulation of AMPK (Figure 2). Accordingly, AMPK becomes fully activated through a three-pronged mechanism. First, binding of AMP or ADP to the  $\gamma$ -subunit promotes Thr172 phosphorylation in the activation loop in the kinase domain by upstream kinases. The main upstream kinase responsible for Thr172 phosphorylation in response to energy stress is the serine/threonine kinase LKB1 (liver-kinase-B1) (Hawley et al., 2003; Shaw et al., 2004; Woods et al., 2003). Phosphorylation of Thr172 in the  $\alpha$ -subunit is the principal event required for full activation of AMPK. This phosphorylation event can increase AMPK activity up to 100-fold *in vitro*, although fold activation in intact cells is usually more modest (Gowans et al., 2013; Oakhill et al., 2011; Suter et al., 2006). Second, binding of AMP or ADP to the  $\gamma$ -subunit induces a conformational change that protects against Thr172 dephosphorylation by protein phosphatases (Gowans et al., 2013; Xiao et al., 2011). Notably, the phosphatases that normally dephosphorylate AMPK under physiological conditions remain largely unknown, though recent reports implicate roles for different phosphatases (Garcia-Haro et al., 2010; Joseph et al., 2015). Lastly, binding of AMP, but not ADP, results in up to 10 fold allosteric activation of AMPK (Gowans et al., 2013). Of note, ATP inhibits all three mechanisms.

In addition to changes in adenine nucleotide levels, it has become increasingly clear that there are other important, non-canonical modes of AMPK regulation (Figure 2). The best characterized nucleotide-independent regulation of AMPK is phosphorylation of Thr172 by CAMKK2 (calcium/calmodulin-dependent kinase kinase 2, also known as CAMKK $\beta$ ), the other major upstream kinase of AMPK (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). CAMKK2 is activated by increases in intracellular  $\text{Ca}^{2+}$  levels. Indeed,  $\text{Ca}^{2+}$ -mediated CAMKK2 activation of AMPK is a frequent mechanism by which metabolically relevant hormones induce transient activation of AMPK. Thus, although CAMKK2 does not itself sense cellular energy status, it is nonetheless critical for the regulation of many aspects of whole body metabolism by AMPK.

Besides phosphorylation of Thr172, phosphorylation of the ST-loop has emerged as an important site for the regulation and inhibition of AMPK by other kinases (Hardie, 2014). Examples of this include phosphorylation of serine 485 (Ser485) in AMPK $\alpha$ 1 and the equivalent serine 491 (Ser491) in AMPK $\alpha$ 2 by PKA (cyclic-AMP-dependent protein kinase), which was proposed to be important for counteracting AMPK activity during gluconeogenic periods (Hurley et al., 2006), and by AKT (insulin-activated protein kinase),

which was proposed to be a mechanism by which insulin inhibits AMPK (Horman et al., 2008). Similarly, S6K (p70S6 kinase) has been reported to inhibit AMPK activity by phosphorylating Ser491 in AMPK $\alpha$ 2, and this phosphorylation was proposed as the mechanism by which leptin inhibits AMPK in the hypothalamus (Dagon et al., 2012). Other kinases reported to inhibit AMPK by phosphorylating various residues in the ST-loop are GSK3 (glycogen synthesis kinase 3) (Suzuki et al., 2013), PKD1 (protein kinase D) (Coughlan et al., 2016) and PKC (protein kinase C) (Heathcote et al., 2016), though it remains to be determined which of these kinases are relevant in different tissues in vivo. Although the mechanism of AMPK inhibition is not entirely clear, it seems that phosphorylation of the ST-loop reduces net phosphorylation of Thr172, by either physically interfering with its phosphorylation or by promoting its dephosphorylation (Hawley et al., 2014). Collectively, these phosphorylation events on the ST-loop may represent an important mechanism to keep AMPK activity low during periods when anabolic metabolism is required. Indeed, phosphorylation of AMPK by oncogenically deregulated AKT, a common feature in tumor cells, could be a mechanism used by tumors to downregulate AMPK activity, whose activity would otherwise oppose their proliferative metabolism (Hawley et al., 2014). This requirement for some tumors to reduce AMPK activity is further demonstrated by the identification of yet another mechanism to downregulate AMPK, namely, through its ubiquitination and degradation. Thus, for instance, MAGE (melanoma antigen genes)-A3 and MAGE-A6 proteins, which are testis-specific genes that are abnormally expressed in a variety of tumors, potently promote tumor growth by, in part, mediating the ubiquitin-dependent degradation of AMPK $\alpha$ 1 by the TRIM28 ubiquitin ligase (Pineda et al., 2015). In addition, UBE20 (ubiquitin-conjugating enzyme E20) was similarly reported to promote tumor growth by targeting AMPK $\alpha$ 2 for ubiquitination and degradation (Vila et al., 2017). Moreover, another ubiquitin ligase, called WWP1, was reported to degrade AMPK $\alpha$ 2 under high-glucose conditions in muscle cells (Lee et al., 2013), suggesting that ubiquitin-dependent degradation of AMPK may be a more broad mechanism for the regulation of AMPK than is currently recognized.

Another context that is increasingly appreciated as important for the regulation of AMPK activity is its localization to cellular membranes. Indeed, myristoylation of the  $\beta$ -subunit can promote localization of AMPK to membranes (Liang et al., 2015; Oakhill et al., 2010). Interestingly, LKB1 is farnesylated and can also localize to membranes, which raised the question of whether co-localization of AMPK and LKB1 into two-dimensional surfaces of cellular membranes could promote activation of AMPK by LKB1. Strong support for this hypothesis has come from the analysis of knock-in mice that express an LKB1 mutant that cannot be farnesylated (LKB1<sup>C433S</sup>) and does not, therefore, localize to membranes (Houde et al., 2014). Although LKB1<sup>C433S</sup> was fully capable of activating AMPK in vitro, tissues and cells from LKB1<sup>C433S</sup> mice exhibited a significant reduction of both basal and induced AMPK activation (Houde et al., 2014), suggesting that the reduced AMPK activation was due to defective membrane localization of LKB1<sup>C433S</sup> in cells. Notably, which cellular membranes were important for LKB1-AMPK activation was left unanswered.

Interestingly, recent studies suggest that the lysosome might be one relevant membrane surface where LKB1 phosphorylates AMPK (Zhang et al., 2014; 2013). In brief, these studies describe a complex mechanism in which, under conditions of low nutrients, AMPK

and LKB1 form a complex with the scaffolding protein AXIN (Zhang et al., 2013) and localize to the lysosomes via binding to the lysosomal protein LAMTOR1 (Zhang et al., 2014), a component of the mTORC1-activating RAGULATOR complex. Moreover, LAMTOR1 knockout cells and tissues had defective AMPK activation, suggesting that recruitment of AMPK to the lysosome might be necessary for proper activation (Zhang et al., 2014). Although this was not tested, it is tempting to speculate that the lipid modifications in AMPK and LKB1 might be necessary for their lysosomal localization. Intriguing as this might be, a systematic analysis of the intracellular location of AMPK activity found it to be enriched not only in lysosomal membranes, but also in Golgi, endoplasmic reticulum, mitochondrial and plasma membranes, suggesting AMPK membrane localization is broadly exploited in cells (Miyamoto et al., 2015). Consequently, even though most AMPK is cytosolic, a critical question for now is whether discrete subcellular pools of AMPK (such as membrane-bound AMPK) respond to specific stress stimuli, and perhaps in turn may target subsets of downstream targets.

Finally, there is increasing evidence suggesting that AMPK may also be a redox-sensing protein. Reactive oxygen species (ROS) are naturally produced by many metabolic reactions, most notably by the production of ATP in mitochondria, and management of their levels is important for cellular homeostasis. Although ROS can indirectly activate AMPK through increases in AMP (Hawley et al., 2010), a few studies have demonstrated that ROS can modulate AMPK activity by direct posttranslational modification (Shao et al., 2014; Zmijewski et al., 2010). The nature and the effects of such modifications, however, seem to be context dependent. In HEK293 and lung cells, H<sub>2</sub>O<sub>2</sub> was shown to induce oxidation and S-glutathionylation of cysteines 299 and 304 in the  $\alpha$ -subunit, resulting in activation of AMPK (Zmijewski et al., 2010). In cardiomyocytes, on the other hand, increased H<sub>2</sub>O<sub>2</sub> levels and ischemia induced oxidation of the highly conserved cysteines 130 and 174 in the  $\alpha$ -subunit, which resulted in inhibition of AMPK through aggregation of AMPK molecules and blockage of phosphorylation by upstream kinases (Shao et al., 2014). Importantly, this study also demonstrated that Trx1 (thioredoxin1), a major reducing enzyme, prevented AMPK oxidation and was necessary for maintenance of AMPK activity. Collectively, even though a comprehensive picture is yet to emerge, the evidence points to AMPK as a nexus between cellular metabolism and cellular redox status.

## Metabolic consequences of AMPK activation

Our understanding of the mechanisms by which AMPK regulates metabolism has been greatly expanded over the last few years by the identification of numerous AMPK substrates, aided substantially by the decoding of the AMPK substrate motif (Gwinn et al., 2008; Hardie et al., 2016). Moreover, novel proteomic approaches have considerably expanded the list and the scope of putative AMPK substrates (Banko et al., 2011; Hoffman et al., 2015; Schaffer et al., 2015), although rigorous analysis of the functional roles of these novel substrates will require further investigation. As explained above, AMPK restores ATP levels during metabolic stress by acutely inhibiting ATP-consuming biosynthetic pathways while simultaneously activating pathways that regenerate ATP through the breakdown of macromolecules (Figure 2). In addition, AMPK phosphorylates several transcription factors (or co-factors) that are themselves master regulators of biosynthetic pathways and

metabolism (Mouchiroud et al., 2014). In this way, AMPK can acutely restore energy balance but also reprogram cell metabolism transcriptionally in response to prolonged energetic decreases. Some of the best established AMPK substrates are depicted in Figure 3. We discuss some of the metabolic effects of AMPK in the following section.

### Glucose and lipid metabolism

Glucose and lipids are major sources for the supply and storage of energy in cells. AMPK increases ATP levels by promoting their breakdown and inhibiting their synthesis and storage. AMPK promotes glucose uptake by phosphorylating TBC1D1 (TBC domain family, member 1) and TXNIP (thioredoxin-interacting protein), which control the translocation and cell-surface levels of glucose transporters GLUT4 and GLUT1, respectively (Hardie, 2013; Wu et al., 2013), though through distinct mechanisms. AMPK also acutely regulates glycolysis in some tissue types by phosphorylating PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3), while inhibiting storage of glucose in some tissues by inhibiting multiple isoforms of GYS (glycogen synthase) (Hardie, 2013). AMPK controls overall cellular lipid metabolism through direct phosphorylation of ACC1 (acetyl-CoA carboxylase 1) and ACC2, suppressing fatty acid synthesis and simultaneously promoting fatty acid oxidation by relieving the suppression of CPT1 (carnitine palmitoyltransferase 1) by malonyl-CoA locally produced at the mitochondria outer membrane by ACC2, which has a mitochondrial targeting sequence in its amino terminus. AMPK also phosphorylates and inhibits HMGCR (3-hydroxy-3-methyl-glutaryl-coA reductase), which, collectively with its effects on ACC1 and ACC2, leads to a preprogramming of lipid and sterol synthesis in the cell. AMPK also promotes lipid absorption and release by phosphorylating lipases such as HSL (hormone-sensitive lipase) and ATGL (adipocyte-triglyceride lipase) (Ahmadian et al., 2011; Kim et al., 2016).

AMPK inhibits the transcriptional induction of gluconeogenesis, the process of *de novo* synthesis of glucose, via phosphorylation and nuclear exclusion of CRTC2 (cyclic-AMP-regulated transcriptional co-activator 2) and class II HDACs (histone deacetylases), which are necessary co-factors for the transcription of gluconeogenic genes (Koo et al., 2005; Mihaylova et al., 2011). In addition, AMPK phosphorylates and inhibits transcription factors that activate glycolytic and lipogenic transcriptional programs, most notably SREBP1 (sterol regulatory element binding protein 1), a master transcriptional regulator of lipid synthesis (Li et al., 2011), but also HNF4 $\alpha$  (hepatocyte nuclear factor-4 $\alpha$ ) and ChREBP (carbohydrate-responsive element binding protein) (Hong et al., 2003; Kawaguchi et al., 2002). Thus, acute AMPK activation favors glucose uptake to promote ATP restoration, while sustained AMPK activation reprograms cells to limit glucose and lipid synthesis and favor oxidation of fatty acids as an energy source.

### mTOR and protein metabolism

The ability of AMPK to inhibit protein synthesis is mediated in large part by direct inhibition of the mTORC1 complex (mechanistic target of rapamycin complex 1). mTOR is a central integrator of nutrient and growth factor signals that activates many biosynthetic pathways, especially protein translation, and stimulates cellular growth. At many levels, AMPK and mTORC1 function antithetically in the regulation of cellular metabolism.

AMPK inhibits mTORC1 activity by a two-pronged mechanism, through phosphorylation and activation of TSC2 (tuberous sclerosis complex 2) (Inoki et al., 2003), a negative regulator of mTORC1, and phosphorylation and inhibition of Raptor (regulatory-associated protein of mTOR), a subunit of the mTORC1 complex (Gwinn et al., 2008). Besides inhibition of mTOR, AMPK has been reported to limit protein synthesis by blocking ribosomal RNA synthesis through phosphorylation and inhibition of TIF-IA (transcription initiation factor IA), a transcription factor for RNA-polymerase I (Hoppe et al., 2009). AMPK also inhibits protein elongation, through phosphorylation and activation of eEF2K (eukaryotic elongation factor 2 kinase), an inhibitor of elongation (Leprivier et al., 2013). Importantly, mTORC1 is also a dominant regulator of eEF2K (Faller et al., 2015), providing an example of the many downstream targets of AMPK that are also directly phosphorylated by mTORC1 or S6K1 to antagonistically regulate their function from AMPK phosphorylation. In this fashion, AMPK and mTOR control anabolism and catabolism by traveling around the cell flipping on and off a limited set of master metabolic switches.

### Autophagy and mitophagy

Autophagy is a cellular process in which proteins, organelles and other macromolecules are delivered to the lysosomes for degradation. It is a process used by cells both for normal turnover and for the generation of nutrients in response to energy shortages. AMPK potently promotes autophagy through several mechanisms. AMPK phosphorylates and activates ULK1 (unc-51-like autophagy-activating kinase 1), which triggers the initiation of the autophagic cascade (Egan et al., 2011; Kim et al., 2011; Mack et al., 2012). Importantly, mTOR strongly suppresses autophagy, in part by directly phosphorylating and inhibiting ULK1 (Kim et al., 2011). Accordingly, AMPK promotes autophagy not only by direct activation of ULK1 but also by negatively regulating mTORC1 and blocking its inhibitory effect on ULK1. Thus, ULK1 is yet another node at which AMPK and mTOR regulate a specific metabolic process in opposing fashion. AMPK also stimulates autophagy initiation by differential regulation of VPS34 (vacuolar protein sorting 34) containing complexes (Kim et al., 2013), which are important for the initiation and formation of autophagosomes. AMPK was reported to directly phosphorylate and inhibit VPS34 in non-autophagic complexes that do not contain autophagy adaptor proteins, while enhancing VPS34 activity in pro-autophagic complexes that contain Beclin-1 by directly phosphorylating Beclin-1 (Kim et al., 2013). In this way, AMPK presumably suppresses nonessential vesicle trafficking in favor of membrane trafficking into the autophagy pathway during nutrient starvation. Given that both AMPK and ULK1 have been reported to directly phosphorylate distinct sites in both Beclin-1 and Vps34, much remains to be clarified about the temporal and spatial control of autophagy initiation in response to different stresses. In addition, AMPK and ULK1 have also both been reported to phosphorylate and control the localization of Atg9, a transmembrane protein involved in early autophagosome formation (Mack et al., 2012; Weerasekara et al., 2014; Zhou et al., 2017).

AMPK has also recently been shown to promote autophagy through transcriptional mechanisms, via regulation of Tfeb (transcription factor EB), a master transcriptional regulator of lysosomal genes and autophagy. Although no direct link between AMPK and Tfeb has been reported, AMPK can activate Tfeb through inhibition of mTORC1, thus



blocking the ability of mTOR to phosphorylate and translocate Tfeb out of the nucleus (Young et al., 2016). Furthermore, through phosphorylation and activation of the transcription factor FOXO3a (Forkhead box O3) (Greer et al., 2007), AMPK has been reported to increase the levels of CARM1 (co-activator-associated arginine methyltransferase 1), an important cofactor for Tfeb transcription (Shin et al., 2016).

In addition to general autophagy, several lines of evidence indicate that AMPK promotes mitophagy, the process of degradation of defective mitochondria. Indeed, activation of ULK1 by AMPK was shown to be required for proper removal of damaged mitochondria via mitophagy, though the details of how ULK1 regulates mitophagy are not fully resolved (Egan et al., 2011). A necessary step preceding removal of damaged mitochondria is the fragmentation of mitochondria in response to mitochondrial insults, in order to separate and target damaged mitochondrial fragments to turnover via the mitophagy pathway. This highly conserved process is known as mitochondrial fission. Recently, a novel mechanism was elucidated by which AMPK promotes mitochondrial fission (Toyama et al., 2016). In this study, AMPK was demonstrated to induce mitochondrial fission during energy stress through direct phosphorylation of MFF (mitochondrial fission factor), which then serves as a receptor for DRP1 (dynamin-related protein 1), the enzyme that catalyzes mitochondrial fission (Toyama et al., 2016). Once at the mitochondria, DRP1 splits damaged mitochondria into smaller fragments that are presumably more efficiently cleared by autophagosomes. Furthermore, AMPK activates PGC1 $\alpha$  (peroxisome proliferator-activated receptor gamma, coactivator 1 $\alpha$ ), a master regulator of mitochondrial biogenesis, reportedly via direct phosphorylation of PGC1 $\alpha$  (Jäger et al., 2007) but also by promoting NAD<sup>+</sup>-dependent activation of PGC1 $\alpha$  by Sirt1 (sirtuin 1) (Cantó et al., 2009). Interestingly, Tfeb, similar to its family member Tfe3, was recently reported to drive mitochondrial biogenesis as well (Mansueto et al., 2017; Wada et al., 2016), which offers the possibility that activation of Tfeb, or Tfe3, might be yet another mechanism by which AMPK can promote the regeneration of mitochondria. In all, AMPK coordinates mitochondrial fission and mitophagy in the acute response to mitochondrial insults, and after sustained energy stress, AMPK promotes transcriptional induction of mitochondrial biogenesis. In this fashion, AMPK serves as a central mediator of mitochondrial quality, ensuring metabolic efficiency in cells and tissues.

### **AMPK activity in other biological contexts**

A great deal of evidence points to a role for AMPK as an inhibitor of cell growth and proliferation. Of considerable interest, however, are recent studies that further document this ability of AMPK to inhibit cell proliferation through phosphorylation of novel substrates in important mitogenic pathways. Specifically, AMPK was reported to inhibit Hedgehog signaling through phosphorylation and destabilization of the transcription factor GLI1 (Glioma-associated oncogene 1) (Li et al., 2015b). Similarly, AMPK was shown to inhibit the Hippo-YAP pathway during energy stress via phosphorylation and stabilization of AMOTL1 (angiomin-like 1) (DeRan et al., 2014), a negative regulator of YAP (Yes-associated protein), and by phosphorylation and inhibition of YAP itself (Mo et al., 2015; Wang et al., 2015). Furthermore, AMPK was reported to phosphorylate and inhibit MDMX (mouse double minute X), a negative regulator of the tumor suppressor p53, resulting in p53

activation and subsequent cell cycle arrest (He et al., 2014). All of these pathways have well-established roles in cell growth and proliferation and are frequently deregulated in tumor cells. Their regulation by AMPK may indicate novel mechanisms by which AMPK serves as a “metabolic checkpoint” in cells.

Novel biological contexts in which AMPK function might be relevant are constantly being reported. One such notable context is management of reactive oxygen species. AMPK is not only a redox sensitive protein, as described above, but is also involved in the response to oxidative stress. Indeed, AMPK was reported to phosphorylate Nrf2 (nuclear factor erythroid 2-related factor 2), a master transcriptional regulator of antioxidant gene programs, which resulted in nuclear accumulation of Nrf2 and subsequent expression of antioxidant genes (Joo et al., 2016). Moreover, AMPK can indirectly mitigate ROS by maintaining high levels NADPH and GSH (which are cellular antioxidants) through inhibition of fatty acid synthesis, a process that consumes NADPH, and by promotion of fatty acid oxidation, a process that generates NADPH (Jeon et al., 2012). Intriguingly, this mechanism of indirect regulation of key metabolites by AMPK was recently described for yet another novel biological context. In a recent study, AMPK was shown to promote the epigenetic remodeling of the *Prdm16* promoter by maintaining high levels of  $\alpha$ -ketoglutarate in brown adipocytes (Yang et al., 2016). Briefly, high  $\alpha$ -ketoglutarate levels (maintained by AMPK $\alpha$ 1) were shown to be required for demethylation of the *Prdm16* promoter by TET (ten-eleven translocation hydroxylases) enzymes, which then led to expression of PRDM16 (PR domain containing 16 protein), the key transcription factor for brown adipogenesis.

Finally, AMPK has been implicated in the regulation of circadian rhythms. The core circadian clock is set by the activity of transcription factors CLOCK and BMAL1 and their cyclical repression by cryptochrome (CRY1, CRY2) and period proteins (PER1, PER2, PER3). AMPK directly regulates the circadian core clock by phosphorylating CRY1 and CRY2, which targets them for degradation (Lamia et al., 2009). AMPK also indirectly promotes degradation of PER proteins (Um et al., 2007). Thus, the ability of AMPK to regulate the circadian clock may be important for the entrainment of circadian rhythms in tissues. Indeed, AMPK-dependent degradation of clock core components may have evolved as a light-independent clock-setting signal that allows coupling of circadian rhythms to energy availability (Jordan and Lamia, 2013). Given the newly appreciated role of CRY1 and CRY2 to act as repressors of specific nuclear receptors (NRs), AMPK dependent degradation of CRY proteins may also serve to promote specific NR-dependent gene expression programs (Lamia et al., 2011).

## Therapeutic activation of AMPK

Given the therapeutically beneficial effects associated with AMPK activation, it is not surprising that many AMPK activating compounds have been identified (Hardie, 2013). How much of the effect of these compounds can be ascribed to AMPK depends not only on drug specificity but also on the particular mechanism of AMPK activation. The mechanisms by which these compounds activate AMPK can be broadly divided into three classes: 1) agents that increase intracellular AMP and ADP, thus indirectly activating AMPK; 2) compounds that mimic AMP and are thus able to bind AMPK at the  $\gamma$ -subunit; 3)

compounds that activate AMPK by selectively binding the interface between the CBM and the kinase domain (Fig 4).

The majority of drugs that activate AMPK by increasing cellular AMP/ADP levels do so by inhibiting mitochondrial respiration and its production of ATP. Biguanides, which include metformin and its more potent analog phenformin, are modest inhibitors of Complex I in the respiratory chain and thus activate AMPK via this mechanism. Metformin is the most widely prescribed drug for type II diabetes and a large proportion of its blood glucose lowering properties are thought to be mediated by AMPK activation (Cao et al., 2014; Duca et al., 2015; Fullerton et al., 2013; He and Wondisford, 2015; Shaw et al., 2005), although AMPK-independent effectors are likely also involved in metformin action (Madiraju et al., 2015; Miller et al., 2013). Mitochondrial poisons (such as oligomycin, CCCP, rotenone, etc.) and a large number of plant-derived drugs (such as resveratrol, berberine, galegine, etc.) have also been shown to activate AMPK by inhibiting different components of the respiratory chain (Hardie, 2013; Hawley et al., 2010). Glycolysis inhibitors, such as 2-deoxyglucose (a non-hydrolysable form of glucose), also elevate AMP levels to activate AMPK, by suppressing glycolysis in cells that are reliant on it for ATP-generation. In a more novel approach, inhibition of AMP deaminase (AMPD) has been tested as yet another way to increase intracellular AMP. AMPD converts AMP into IMP, so its inhibition also increases AMP pools, which in turn activate AMPK (Plaideau et al., 2014). Indeed, one report suggested that metformin may control AMP levels via effects on AMPD (Ouyang et al., 2011).

A second class of compounds activates AMPK by functioning as AMP mimetics. These agents are administered as pro-drugs, which are taken up by cells and then metabolized to the actual AMP analog. The best-known member of this class of compounds is 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR). AICAR is taken up by adenosine transporters in cells and then converted to ZMP, which mimics all of the effects of AMP on AMPK. A new AMPK-activating AMP analog was recently reported, 5-(5-hydroxyl-isoxazol-3-yl)-furan-2-phosphonic acid compound 2 (C2) (Gómez-Galeno et al., 2010), which is administered as the pro-drug C13. C2 is more potent than AMP or ZMP, but unlike AMP or ZMP, it only activates AMPK complexes containing AMPK $\alpha$ 1 (Hunter et al., 2014). Also, although ZMP is expected to affect many AMP-dependent processes independently of AMPK, C2 is reported to have high specificity for AMPK (Hunter et al., 2014). Interestingly, multiple recent reports have exploited modulation of purine biosynthesis as a mechanism to alter ZMP or AMP levels to activate AMPK, including the widely used chemotherapeutics pemetrexed and methotrexate (Asby et al., 2015; Pirkmajer et al., 2015; Racanelli et al., 2009).

The third class of compounds consists of novel small molecules that directly bind and activate AMPK. The first member of this class was the thienopyridone A769662 (Cool et al., 2006). Multiple additional related compounds have since been reported, such as 991 (also known as ex229), MT-63-78, and GSK621 (Li et al., 2015a; Sujobert et al., 2015; Xiao et al., 2013). Like AMP, A769662 was shown to allosterically activate AMPK and to protect from Thr172 dephosphorylation (Göransson et al., 2007; Sanders et al., 2007). However, contrary to AMP, A769662 was demonstrated to specifically require the CBM domain in AMPK $\beta$ 1 and phosphorylation of AMPK $\beta$ 1 at serine 108 (Sanders et al., 2007). Phosphorylation of

AMPK $\beta$ 1 at serine 108 appears to be mostly due to autophosphorylation, although phosphorylation by other kinases cannot be excluded (Sanders et al., 2007; Scott et al., 2014). The crystal structure of the actual single binding site for A769662 and 991 has now been resolved (Xiao et al., 2013). As described above, the CBM domain of the  $\beta$ -subunit directly contacts the kinase domain of the  $\alpha$ -subunit, forming a pocket between the two domains that is bound by 991 and A769662, where these small molecules form hydrophobic interactions with residues from both domains. A769662 and AMP potently synergize to activate AMPK, remarkably, even in the absence of Thr172 phosphorylation (Scott et al., 2014). Of note, salicylate, the breakdown product of aspirin and salsalate, was also found to directly activate AMPK (Hawley et al., 2012). Surprisingly, the CBM-kinase domain pocket is very likely to be the same binding site for salicylate since, like A769662, activation of AMPK by salicylate requires the CBM domain of AMPK $\beta$ 1 and its phosphorylation at Ser108 (Hawley et al., 2012). Consistent with its ability to activate AMPK, mice treated with salicylate were shown to have higher rates of fatty acid oxidation and reduced levels of circulating fatty acids in an AMPK-dependent manner (Hawley et al., 2012). Indeed, some of the beneficial effects of salicylate in humans might be mediated by its ability to activate AMPK, especially its metabolic, anti-tumorigenic and anti-inflammatory effects (Ford et al., 2015; Fullerton et al., 2015).

## Concluding remarks

AMPK is a highly conserved master regulator of metabolism, both at the cellular and organismal levels, whose function is extremely relevant not only for normal physiology, but also for the understanding of many metabolic diseases. The examination of novel mechanisms of AMPK regulation and the identification of additional AMPK substrates continue to enhance our understanding of not only AMPK biology, but also of how cells manage their energy demands generally. Importantly, many AMPK activating compounds, in addition to the clinically approved metformin and salsalate, have enormous therapeutic potential, in particular for the treatment of metabolic disorders such as diabetes. However, these drugs are being experimentally tested for a variety of other applications, including the treatment of obesity, muscle dysfunction, and cancer, and there will undoubtedly be much to learn from these trials.

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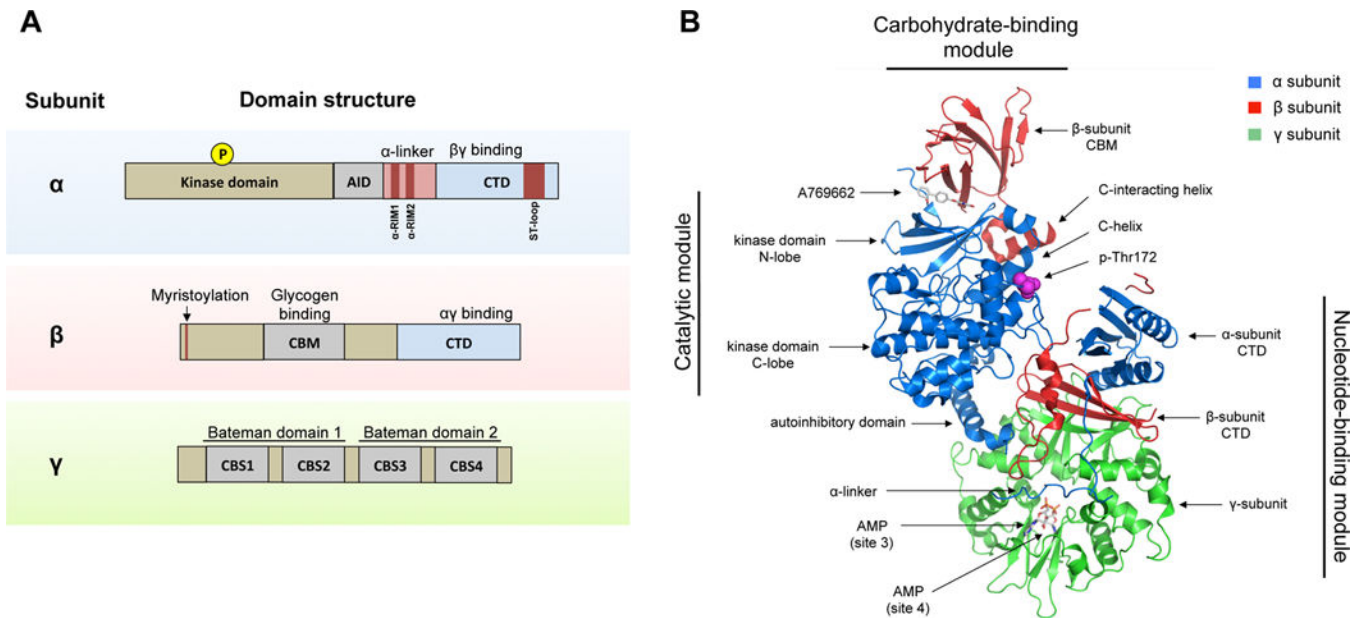
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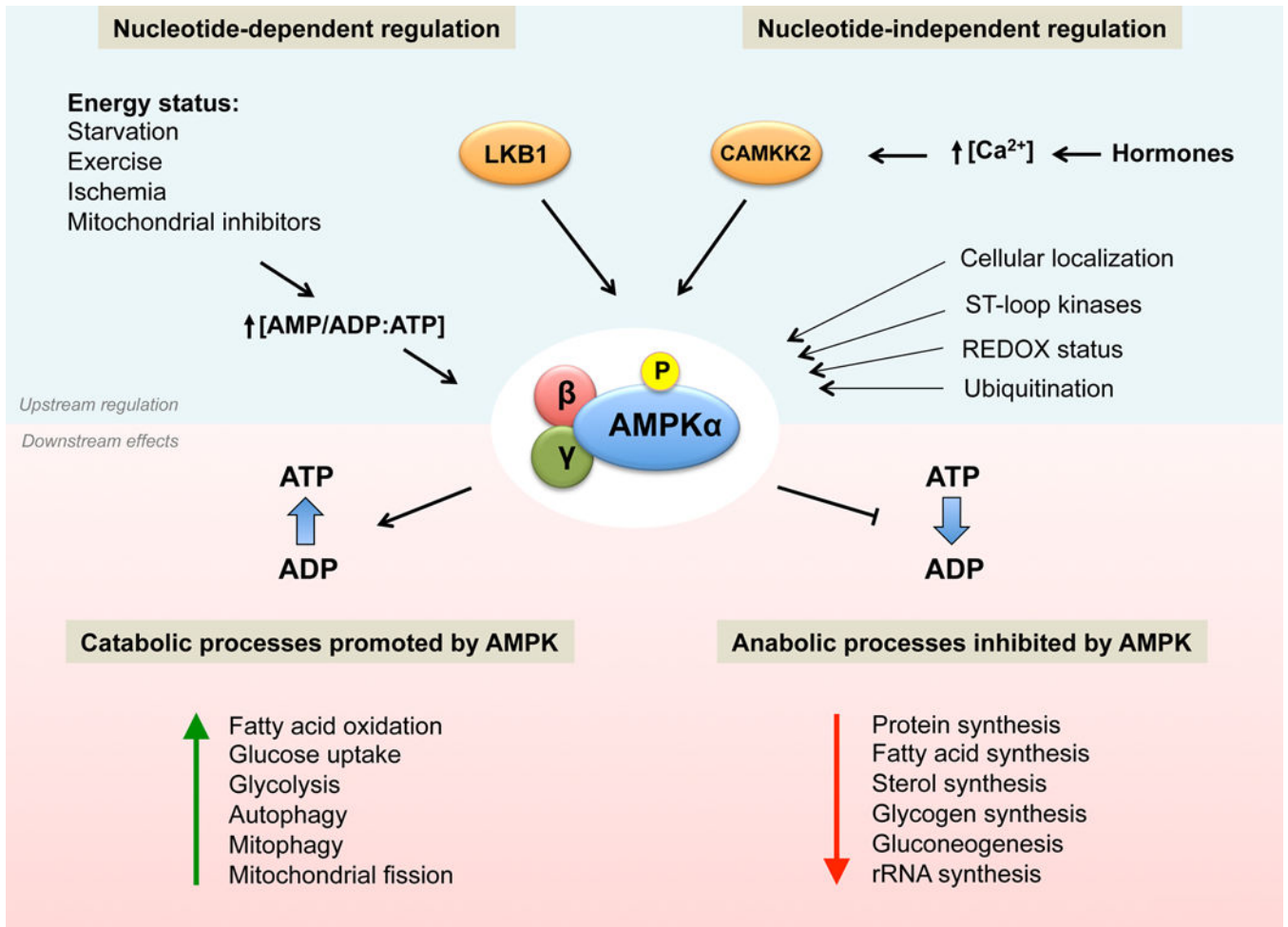
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**Figure 1. Domains and structure of the AMPK complex**

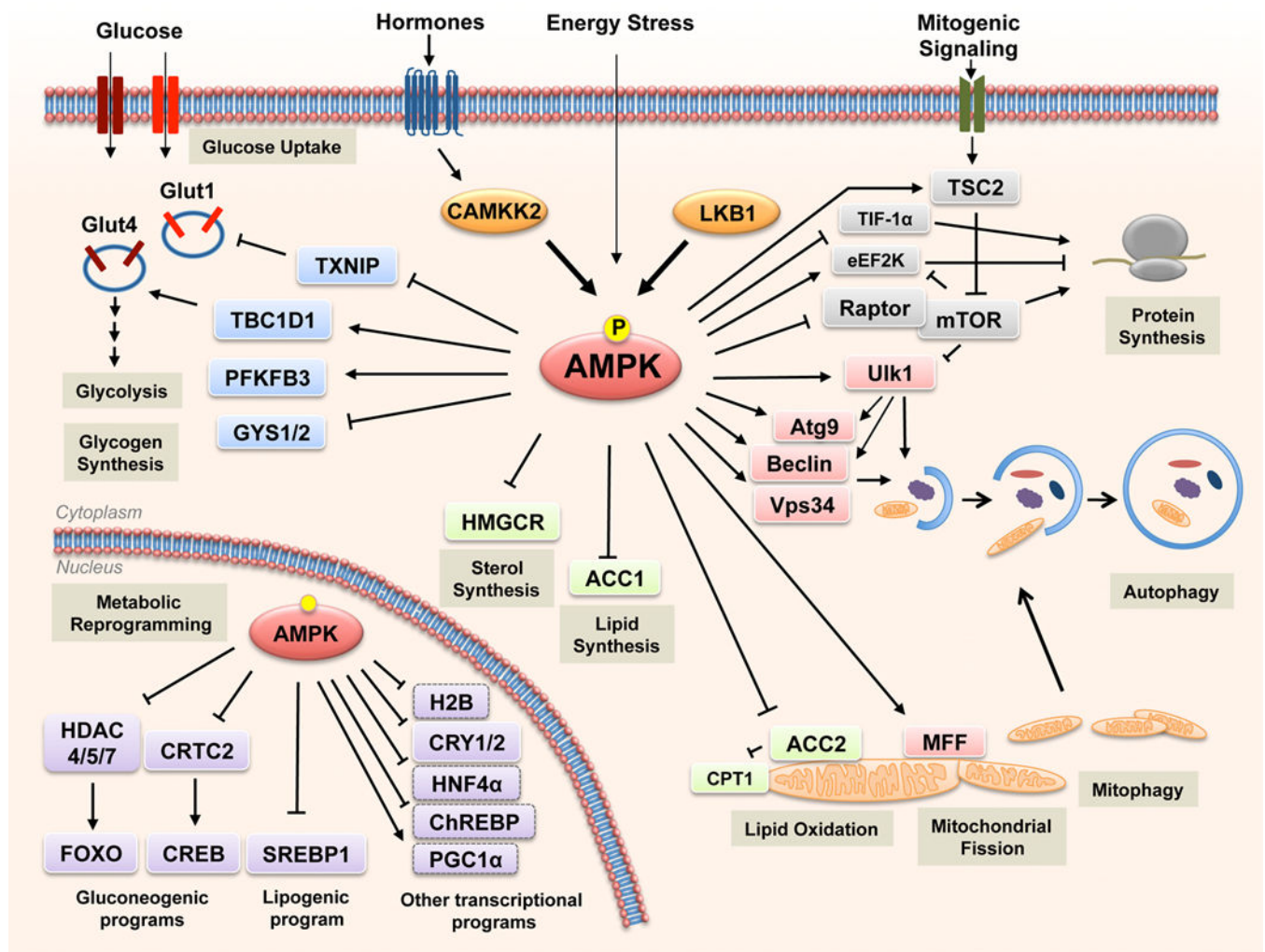
A) AMPK exists as a trimeric complex consisting of a catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ). The main protein domains are shown. Abbreviations: AID (autoinhibitory domain), CTD (C-terminus domain), NTD (N-terminus domain), CBM (carbohydrate-binding module), CBS (cystathionine  $\beta$ -synthase repeats), RIM (regulatory-subunit-interacting motif), ST-loop (serine/threonine enriched loop).

B) The crystal structure of the AMPK  $\alpha_2\beta_1\gamma_1$  trimeric complex is shown. Major structural domains are indicated. The structure shows the activator A769662 bound to a pocket formed by the interface between the kinase domain and the CBM. Also shown are two AMP molecules bound to site 3 and site 4, respectively, and phospho-Thr172. The ST-loop in the  $\alpha$ -subunit and the myristoylation site in the  $\beta$ -subunit are not resolved in this structure. Structure sourced and adapted from PDB file 4CFF, using PyMOL software.



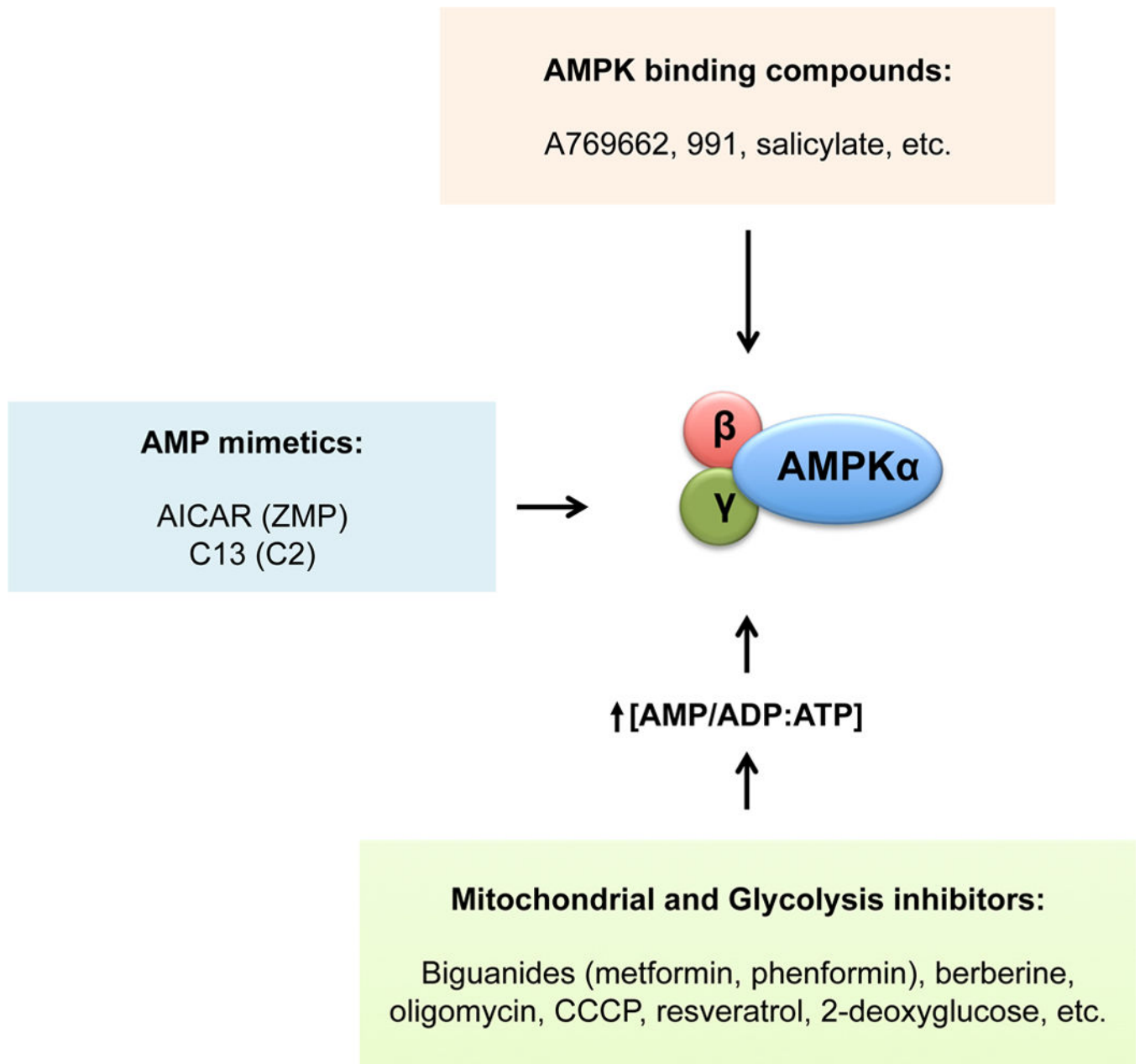
**Figure 2. Upstream regulation of AMPK and metabolic consequences of AMPK activation**

Phosphorylation by upstream kinases is the main AMPK activating event. The regulation of AMPK phosphorylation can be nucleotide-dependent (canonical regulation), resulting from changes in AMP:ATP or ADP:ATP ratios that are induced by a variety of energy stresses. Several important alternative modes of AMPK regulation have been described, which can be classified as nucleotide-independent regulation. In response to energy stress AMPK restores ATP levels by acutely inhibiting ATP-consuming biosynthetic pathways while simultaneously activating catabolic pathways that regenerate ATP through the breakdown of macromolecules. Some of the metabolic processes that are affected by AMPK activation are shown.



**Figure 3. Substrates of AMPK regulate multiple metabolic processes in cells**

AMPK is phosphorylated and activated by LKB1 and CAMKK $\beta$  in response to stimuli that increase AMP/ADP levels (energy stress) or Ca<sup>2+</sup> flux, respectively. Once active, AMPK induces metabolic changes through the phosphorylation of substrates. Some of the best established metabolic processes regulated by AMPK are shown, together with the relevant substrates.



**Figure 4. AMPK activating compounds**

Compounds that activate AMPK can be divided into three categories, depending on the mechanism used to activate AMPK. Examples of some of these compounds are shown.