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AMPK: an energy-sensing pathway with multiple inputs and outputs

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21 **Summary**

22 The AMP-activated protein kinase (AMPK) is a key regulator of energy balance expressed
23 ubiquitously in eukaryotic cells. Here, we review the canonical adenine nucleotide-dependent
24 mechanism that activates AMPK when cellular energy status is compromised, as well as other, non-
25 canonical activation mechanisms. Once activated, AMPK acts to restore energy homeostasis by
26 promoting catabolic pathways, resulting in ATP generation, and inhibiting anabolic pathways that
27 consume ATP. We also review the various hypothesis-driven and unbiased approaches that have
28 been used to identify AMPK substrates, which have revealed substrates involved in both metabolic
29 and non-metabolic processes. We particularly focus on methods for identifying the AMPK target
30 recognition motif, and how it can be used to predict new substrates.
31

32 **AMPK – subunit structure and regulation**

33 The AMP-activated protein kinase (AMPK) is a key sensor of cellular energy status present in
34 essentially all eukaryotic cells, where it occurs as heterotrimers comprising catalytic α subunits and
35 regulatory β and γ subunits [1-3]. Genes encoding at least one of these subunits are found in the
36 genomes of essentially all eukaryotes, while mammals have genes encoding multiple isoforms (α 1,
37 α 2; β 1, β 2; γ 1, γ 2, γ 3). AMPK heterotrimers are normally only significantly active after
38 phosphorylation of a conserved threonine residue within the activation loop of the α subunit kinase
39 domain (Thr172 in rat α 2 [4]; the numbering may differ in other species). Mammalian AMPK is
40 activated through binding of 5'-AMP by three complementary effects (Fig. 1): i. promotion of
41 Thr172 phosphorylation by upstream kinases; ii. inhibition of Thr172 dephosphorylation by protein
42 phosphatases; and iii. allosteric activation. Although allosteric activation is only triggered by
43 binding of AMP, effect #1 (promotion of Thr172 phosphorylation [5]) and #2 (inhibition of Thr172
44 dephosphorylation [6, 7]) can be mimicked by ADP. Since ATP antagonizes these effects, AMPK
45 acts as a sensor of cellular AMP:ATP and ADP:ATP ratios, both of which increase during cellular
46 energy stress (although the changes in AMP:ATP are always larger due to the adenylate kinase
47 reaction [7]). AMPK can sense small changes in AMP even in the presence of concentrations of
48 ATP two to three orders of magnitude higher [7, 8].
49 In this review, we will discuss recent studies of the molecular mechanisms by which AMPK is
50 activated by the canonical inputs AMP and ADP (introduced above), as well as by the non-
51 canonical inputs that are being increasingly recognized. We also discuss recent approaches aimed at
52 establishing the full complement of downstream targets that are phosphorylated in cells when
53 AMPK is activated.

54 **Canonical and non-canonical inputs into the AMPK system**

55 ***Canonical inputs - adenine nucleotide binding to the AMPK- γ subunit***

56 AMPK senses changes in AMP through its direct binding to the γ subunit. AMPK- γ subunits in all
57 species contain four tandem repeats of sequence motifs known as cystathionine β -synthase (CBS)
58 repeats. These also occur in a small number of other proteins in the human genome, although
59 usually as just two tandem repeats. In many cases, each tandem pair of repeats bind a regulatory

adenosine-containing ligand, such as ATP or S-adenosyl methionine, in the cleft between the repeats [9]. In AMPK- γ subunits, the four repeats assemble into a disc-like shape with one repeat in each quadrant, generating four potential nucleotide-binding sites that are numbered according to which repeat binds the ribose ring of each nucleotide. Of these, site 2 appears to be always vacant, while site 4 is thought to contain only a permanently-bound AMP [10]. Although the latter view has been challenged [11], this would leave sites 1 and 3 as the sites where AMP, ADP and ATP bind in competition.

A crystal structure [8] of the human $\alpha 1\beta 2\gamma 1$ complex containing several bound ligands (AMP, the kinase inhibitor staurosporine and the glycogen mimetic β -cyclodextrin) is shown in Fig. 2; it is similar to previous structures of $\alpha 2\beta 1\gamma 1$ [12] and $\alpha 1\beta 1\gamma 1$ [13] complexes. The kinase domain on the α subunit (α -KD), containing the small N-terminal and larger C-terminal lobes of a typical protein kinase, is immediately followed by the autoinhibitory domain (α -AID), which is so-called because KD:AID constructs are 10-fold less active than those containing the KD alone [14-16]. Structures of KD:AID constructs from fission yeast [17], and human $\alpha 1$ [8], reveal the α -AID to be a compact bundle of three α -helices that inhibits the kinase by binding of its helix $\alpha 3$ to the N- and C-lobes of the α -KD, on the opposite surface to the active site (Fig. 3A). In all structures of active kinase domains, four hydrophobic side chains known as the *regulatory spine* are stacked in alignment, indicating the correct disposition of active site residues [18]. In the inactive KD:AID structure shown in Fig. 3A, these residues (shown in white, red, magenta and blue) are not aligned. By contrast, in the structures of active AMPK heterotrimers, the α -AID has rotated away from the α -KD, with its $\alpha 3$ helix now interacting with the second CBS repeat of the γ subunit instead (Fig. 2), and the side chains of the regulatory spine are now aligned (e.g. Fig. 3B).

The α -AID is connected to the C-terminal domain of the α -subunit (α -CTD) by a critical region of extended polypeptide termed the α -linker. In the view of Fig. 2 this linker (shown in space-filling representation in blue, red and magenta) wraps around the front face of the γ subunit. It contains two conserved motifs, termed α -RIM1 and α -RIM2 (*RIM* = *regulatory subunit interacting motif*) [19]. In structures of active heterotrimers, α -RIM1 (in blue in Fig. 2) binds to the surface of the γ subunit containing the vacant site 2, while α -RIM2 (in magenta) interacts with site 3 containing bound AMP. This tight association of the α -linker with the AMP-bound form of the γ subunit is proposed to cause the observed rotation of the α -AID away from the α -KD, thus explaining how

binding of AMP at site 3 causes allosteric activation [12, 19]. This model requires that binding of ATP at site 3 would not allow the same interaction with α -RIM2. Supporting this, the interaction between an α -AID:linker fragment and a construct containing the γ subunit was shown by luminescence energy transfer to be enhanced by AMP binding, but decreased by ATP binding [8].

Recent experiments with a novel AMPK activator suggest that the ability of AMP analogs to protect against Thr172 dephosphorylation (effect #2) is also due to binding at site 3. C13 (see Fig. 4A) is a phosphonate diester that is taken up into cells and converted by cellular esterases to C2, a potent AMP analog [20]. In cell-free assays, C2 promoted effects #2 (protection against Thr172 dephosphorylation) and #3 (allosteric activation) using α 1-containing complexes, but in α 2-containing complexes, it was only a partial allosteric agonist compared to AMP, and failed to protect against Thr172 dephosphorylation. However, the full effects could be transferred to α 2-containing complexes merely by replacing part of the α -linker from α 2 (including α -RIM2) with the equivalent region from α 1 [21]. Since α -RIM2 contacts site 3, but no part of the α -linker contacts sites 1 or 4 (which are on the opposite face of the γ subunit), binding of C2 at site 3 seems to be crucial for effect #2 as well as #3. This leaves open the question of the functions, if any, of AMP binding at sites 1 and 4.

What is the exact mechanism for effect #2 (protection against Thr172 dephosphorylation)? Current structures of the heterotrimer, which are in active conformations with AMP bound in site 3, can be divided into two major regions termed the “catalytic module” (upper left in Fig. 2) and “nucleotide-binding” module (lower right in Fig. 2), with Thr172 located in the cleft between them. The α -linker can be regarded as a flexible connector linking these two modules, and its release from the γ subunit when ATP replaces AMP at site 3 may allow the catalytic and nucleotide-binding modules to move apart, increasing the accessibility of Thr172 to phosphatases, thus inducing dephosphorylation [6]. This movement would also allow the α -AID to move back into its inhibitory position behind the α -KD, thus reversing allosteric activation. In support of this model, measurements of small angle X-ray scattering [22], and luminescence energy transfer [8] both suggest that heterotrimers adopt less compact conformations when ATP, rather than AMP, is bound.

The explanation for effect #1 (promotion of Thr172 phosphorylation by LKB1) remains unknown, although an intriguing recent proposal is that AMP binding causes AMPK to co-localize

120 with LKB1 due to their mutual interactions with the scaffold protein axin, which in turn binds to
121 LAMTOR1 at the surface of the lysosome [23, 24]. However, effect #1 can also be observed on
122 reconstitution of highly purified LKB1 and AMPK [7], suggesting that it does not strictly require
123 these additional components.

124 ***Non-canonical inputs - activation by ligands that bind between the α and β subunits***

125 Many screens of candidate molecules, as well as unbiased screens, have been conducted in the hunt
126 for AMPK activators that might have therapeutic potential. A small selection of known activators is
127 shown in Fig. 4. One class (Fig. 4A), including C13 [20] and 5-aminoimidazole-4-carboxamide
128 ribonucleoside (AICAR) [25], are pro-drugs converted by cellular enzymes into AMP analogs that
129 bind to the γ subunit. A second class (Fig. 4B) is exemplified by A-769662 [26], which does not
130 bind at the AMP-binding sites even though, like AMP, it causes both allosteric activation and
131 protection against Thr172 dephosphorylation [15, 27]. Binding of A-769662 involves the β -subunit
132 carbohydrate-binding module (β -CBM), which is related to non-catalytic domains found in
133 enzymes that metabolize glycogen or starch, and has been shown to cause binding of AMPK to
134 glycogen particles within cells [28, 29]. In the structure shown in Fig. 2, the synthetic
135 oligosaccharide β -cyclodextrin was bound at the presumed glycogen-binding site. In other recent
136 structural analyses [12, 13], A-769662 or another activator, 991 (also known as ex229 [30]), was
137 bound at the opposite surface of the β -CBM, in the cleft between it and the α -KD N-lobe; this cleft
138 is labeled in Fig. 2 although unoccupied. A-769662 and 991 are synthetic molecules, but the natural
139 plant product and AMPK activator, salicylate, was suggested to bind at the same site [31]; this was
140 recently confirmed by a crystal structure containing bound 5-iodosalicylate [13]. Salicylate (in the
141 form of willow bark extracts) has been used as a medicine since ancient times, with acetyl salicylic
142 acid (aspirin) being a synthetic derivative that is rapidly broken down to salicylate *in vivo*.
143 However, this leaves open the question as to whether there is a naturally occurring metabolite in
144 mammals that regulates AMPK by binding this site. Although such a metabolite has not yet been
145 found, the site has been referred to as the allosteric drug and metabolite (ADaM) binding pocket
146 [32].

147 **Non-canonical inputs – activation by the Ca^{2+} /CaMKK β pathway**

148 Hormones that increase intracellular Ca^{2+} activate AMPK via phosphorylation of Thr172 by the
149 calmodulin-dependent protein kinase CaMKK β (Fig. 1) [33-35]. This represents an alternate, Ca^{2+} -
150 dependent pathway for AMPK activation that is independent of changes in adenine nucleotides and
151 is therefore considered to be non-canonical. However, because AMP binding inhibits Thr172
152 dephosphorylation, the two pathways can act synergistically [36].

153 **Indirect mechanisms for AMPK activation**

154 Most other activators (Fig. 4C) activate AMPK indirectly by inhibiting ATP synthesis, thus
155 increasing intracellular AMP:ATP and ADP:ATP ratios [37]. These include the glycolytic inhibitor
156 2-deoxyglucose, the anti-diabetic drugs metformin and phenformin, and many natural plant
157 products that are either used in traditional medicines or are being tested for health-promoting
158 activities (e.g. berberine [38], arctigenin [39], and resveratrol [40]). Another interesting activation
159 mechanism is displayed by the tetrahydrofolate analogs pemetrexed and methotrexate, used for
160 treatment of cancer or inflammatory disorders such as rheumatoid arthritis. These analogs inhibit
161 tetrahydrofolate-utilizing enzymes, including the transformylase that catalyzes the first step in the
162 metabolism of ZMP, the phosphorylated form of AICAR, to purine nucleotides (although inhibition
163 by methotrexate is most likely secondary to inhibition of dihydrofolate reductase [41]). They
164 therefore cause accumulation of cellular ZMP, which binds to the γ subunit and activates AMPK
165 (Fig. 4D) [41, 42].

166 **Outputs: identification of downstream targets**

167 Once AMPK is activated, it acts to restore energy homeostasis by activating catabolic pathways that
168 generate ATP and inhibiting anabolic pathways that consume ATP. However, since the great
169 majority of cellular processes consume energy and most are coupled to ATP hydrolysis, there is no
170 reason why processes switched off by AMPK should be restricted to metabolic roles. There has
171 therefore been a need to understand how AMPK recognizes its target phosphorylation sites, and to
172 develop methods to screen for, and even predict, novel downstream targets. This will form the
173 central theme of the remainder of this review, and some of the methods used are illustrated in Fig.
174 5.

175 **Initial hypothesis-driven approaches to determine the AMPK recognition motif**

176 Sequencing of the first few sites phosphorylated by AMPK led to the identification of conserved
177 residues close to the phosphoacceptor site (P), including basic residues at P-3 and/or P-4 and
178 hydrophobic residues at P-5 and P+4, which were proposed to be important in recognition. This was
179 confirmed by making replacements within the *SAMS* peptide [43], which was based on the sequence
180 around Ser79 on rat acetyl-CoA carboxylase-1 (ACC1) and was the first peptide substrate for
181 AMPK [44]. This study also showed that AMPK would phosphorylate threonine, although serine
182 was preferred (this preference for serine is a feature of serine/threonine kinases in general). A
183 follow-up study utilized a specially designed peptide sequence (*AMARAASAAALA*) where all
184 residues other than the serine and the critical positions at P-5, P-3, and P+4 were alanine. This study
185 showed that the mammalian, budding yeast, and higher plant orthologs of AMPK had very similar
186 specificities, and that the *position* as well as the chemical nature of the P-5, P-3 and P+4 residues
187 was crucial, although the basic residue could be at P-4 or P-3 [45]. Another approach used a
188 bacterially expressed peptide containing 34 residues around the Ser79 site on rat ACC1 as the
189 substrate. A model for the binding of this sequence to the kinase domain was generated by
190 homology to structures of closely related kinases. By making complementary mutations of kinase
191 and substrate, and analyzing their effects on phosphorylation kinetics (Fig. 5A), this model was
192 validated [46]. An additional positive determinant identified in this study was a basic residue at P-6,
193 although not all known AMPK substrates contain this. This study also suggested that the sequence
194 N-terminal to P-5 in ACC1, in which conserved hydrophobic residues occur every 3-4 residues (at
195 P-5, P-9, P-13 and P-16), form an amphipathic α -helix that fits into a hydrophobic groove within
196 the C lobe of the α -KD. Interestingly, a partial structure of another AMPK target, HMG-CoA
197 reductase [47], revealed that residues from Gly860 to Arg871 (corresponding to P-12 to P-1 with
198 respect to the phosphorylation site, Ser872) do form such an α -helix. However, this amphipathic
199 helix cannot be essential for target recognition, because it is not present in the peptide substrates, or
200 in the liver or muscle isoforms of glycogen synthase, two well-validated targets for AMPK where
201 the P-6 residues actually form the N-termini of the mature proteins. These α -helices should
202 therefore be regarded as docking sites that enhance the affinity of the kinase-substrate interaction,
203 rather than as essential determinants for substrate recognition.

204 *Unbiased approaches to determine the recognition motif*

205 The studies described in the previous section were built on hypotheses about substrate recognition
206 derived from sequences of a relatively small number of known targets. More recently, unbiased
207 screens to identify the preferred AMPK recognition motif have been developed. The first was a
208 peptide library approach using spatially arrayed sets of peptide mixtures [48], a method originally
209 developed for studies of other kinases [49]. Each mixture contained a fixed amino acid at a given
210 position relative to a central fixed serine/threonine phosphoacceptor, with mixtures at all other
211 positions (Fig. 5B). From the relative amount of phosphate incorporated into each mixture, a
212 measure of selectivity for and against individual amino acids at each position was obtained.
213 Gratifyingly, the results yielded a recognition motif remarkably similar to the earlier approaches,
214 with hydrophobic side chains being preferred at P-5 (especially M or L) and P+4 (I, L, M or F), and
215 basic side chains (R>K) at the P-4 or P-3 position; a preference for polar side chains at P+3 was
216 also noted [48]. One drawback of this approach is that it is not feasible to study the effects of amino
217 acids more than about five residues away from the phosphoacceptor, because the peptide mixtures
218 become too degenerate.

219 A second method, represented in Fig. 5C, involved identification of large numbers of direct
220 targets in intact cells and allowed analysis beyond the P-5 and P+4 positions [50]. This approach
221 utilized a method where the ATP binding pocket of the kinase was enlarged by mutation to accept a
222 bulky (N⁶-phenethyl) derivative of ATP containing γ -thiophosphate. The bulky nucleotide was
223 introduced into intact cells through digitonin permeabilization and was used by the mutant kinase to
224 thiophosphorylate direct targets [51, 52]. The thiophosphorylated substrates were then
225 immunoprecipitated using a thiophosphate-specific antibody, resulting in identification of 28 new
226 targets [50]. This initial approach did not identify the actual phosphorylation sites, but the known
227 AMPK recognition motif was used to predict the phosphorylation sites on four (PPP1R12C,
228 BAIAP2, CDC27 and PAK2), which were subsequently independently validated [50].

229 Since existing AMPK recognition motifs were used in this approach to identify the likely sites,
230 sites that did not adhere to these motifs would have been missed. Another drawback was that it
231 might identify as false positives proteins that co-precipitated with the thiophosphorylated substrates.
232 A recent refinement of this method allows concurrent identification of both the substrate and its
233 thiophosphorylation site(s): the thiophosphorylated proteins are digested with trypsin and the

modified peptides isolated via a peptide capture approach, allowing the sites to be directly identified by tandem mass spectrometry [53]. When applied to AMPK (Fig. 5C) [54], the 32 phosphorylation sites (27 of them novel) most frequently identified across replicate screens showed striking resemblance to the established AMPK phosphorylation motif [43, 45, 46, 48]. Hydrophobic residues (L, M, I) were observed at P-5, basic residues (R>K), usually at P-3 but also occasionally at P-4 or P-2, neutral polar residues (S, N, T, Q) at P-2, polar and/or charged residues (S, D, N) at P+3, and hydrophobic residues (usually L) at P+4 [54]. As suggested by an earlier approach [46], a subset of sites contained basic residues at P-6, although they were not present at all sites. Some of the novel sites [54], as well as many established AMPK target sites (see supplementary Table 1 for an updated list of 64 well-validated AMPK target sites, and Fig. 6), contain proline at P+2, which is not selected in screens utilizing cell-free assays [48]. This is most likely due to the functional overlap between AMPK phosphorylation motifs and 14-3-3 binding sites, with the latter usually having a phosphoserine with R at P-3, S at P-2, and P at P+2 [55]. No strong distal motif outside of P-5 to P+4 emerged from analysis of the phosphorylation sites identified in this study [54] or of 64 well-validated AMPK sites (supplementary Table 1), suggesting that the primary recognition motif is contained within this window. It should also be noted that the target sequences listed in supplementary Table 1 that were used to generate Fig. 6 excluded autophosphorylation sites, such as Ser108 on AMPK- β 1 [56], Ser491 on AMPK- α 2 [57], and several others [58]. While they can have critical functions, autophosphorylation sites do not always conform to the recognition motif found on other substrates, perhaps because their close proximity to the kinase domain means that this is not necessary.

Prediction of novel targets using the AMPK recognition motif

AMPK now has a recognition motif (Fig. 6) that is among the best defined of any protein kinase, making it potentially useful for predicting novel targets. It is important to note, however, that there are likely to be many sites that conform to this motif that are not targets for AMPK, either because they are not accessible to the kinase due to some aspect of structure or protein:protein interaction, or because of their subcellular location. Since important phosphorylation sites are likely to be conserved, several groups have searched protein databases to find motifs matching earlier versions of the recognition motif, and then filtered their results by evolutionary conservation. This led to the

identification of phosphorylation sites with important regulatory functions on RAPTOR [48], ULK1 [59], Class IIA histone deacetylases [60], and AMOTL1, the latter involved in Hippo signaling [61]. Since AMPK phosphorylation often triggers 14-3-3 binding, the identification of some of these was aided by screening for candidates that bound 14-3-3 proteins in an AMPK-dependent manner. Recently, a similar analysis was conducted, searching for motifs in the proteome containing basic residues at P-6 and P-4 and hydrophobic residues at P-5 and P+4, and several sites predicted were validated as AMPK targets in cell-free assays [62]. In a parallel approach, an antibody recognizing the core recognition motif (LxRxxpS/pT) was used to immunoprecipitate proteins following AMPK activation in hepatocytes, which were then identified by mass spectrometry. The majority of the proteins identified did contain that motif, and several of these were validated as AMPK targets [63].

These successes suggest that it should be possible to explore the full network of AMPK substrates, and several resources for this are available. The motif prediction platform Scansite [64] (<http://scansite.mit.edu>) provides a position weight matrix (PWM)-based algorithm for querying a single protein, or a predefined protein database, for optimal AMPK motifs. Building on this, two of us [54] developed an AMPK motif prediction platform (available at https://github.com/BrunetLabAMPK/AMPK_motif_analyzer) by constructing a PWM from a list of substrates, which contains the frequencies of each amino acid at each position in the motif from P-5 to P+4, and controls for a background of general phosphorylation motifs. The platform allows users to upload tab-delimited files, and the output retains all data associated with each scored site. Thus, large quantitative phosphoproteomic datasets can be queried for AMPK-like sites. The PWM can also be used independently in other web-accessible platforms, such as FIMO [65] or Scansite to query entire proteomes or other databases. In addition, the consensus motif may be used to identify the likely AMPK phosphorylation sites on the many proteins that have been identified as in substrate screens [50, 66] or as AMPK interacting proteins, as some of the latter may also be direct AMPK targets [67, 68].

Finally, it might be worth noting that AMPK does seem to tolerate a little more flexibility in its recognition motif than many other protein kinases. Thus, it will accept histidine rather than arginine or lysine as the basic residue at P-3 or P-4, which many other “Basophilic” kinases do not. Moreover, it appears to accept a little slippage in the exact position of the hydrophobic residue at P-

293 5. Thus, in NOS1, NOS3, PAK2 and PFKFB2 the hydrophobic residue is at P-4 rather than P-5,
294 although in all these cases it is followed by an arginine at P-3 (Supplementary Table 1).

295 **Concluding remarks**

296 Some important unresolved issues regarding the inputs and outputs impinging on AMPK are
297 outlined in the “Outstanding questions” Box. AMPK is now known to be activated through a
298 variety of upstream inputs, including the canonical mechanism involving sensing of adenine
299 nucleotide ratios, and non-canonical mechanisms such as those used by compounds that bind at the
300 ADaM site, and the Ca^{2+} -dependent pathway involving CaMKK β .

301 The number of outputs, i.e. identified downstream targets, is also expanding rapidly. The
302 availability of phosphorylation site prediction tools should greatly assist in elucidation of the
303 complete network of downstream targets. However, the current site prediction tools do have some
304 limitations. AMPK- α 1 and - α 2 lie in the same branch of the kinome as up to twelve AMPK-related
305 kinases, and some of the latter, notably the MARKs [69], have similar recognition motifs. Thus,
306 using the AMPK recognition motif to predict sites may also yield targets for AMPK-related kinases.
307 Covalent modifications within the proximity of the target site (such as oxidation of methionine
308 when this is the hydrophobic residue at P-5 [70]) might also affect recognition. In addition,
309 sequences and/or higher order structures outside of the P-5 to P+4 window, such as the amphipathic
310 helix from P-15 to P-5 upstream of Ser79 on ACC1, can enhance the recognition of specific sites.
311 Because these are not essential, the increased power they could bring to site prediction is currently
312 lost. One future direction might be to investigate whether multiple subsets of distal recognition
313 motifs exist, which might contain elements that increase predictive power. Further exploration of
314 the network in different contexts, for example when activated by different inputs, will be a key step
315 in better understanding the physiological and pathological roles of this central energy-sensing
316 kinase.

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323 cite due to constraints on the length of the article.
324

325 **FIGURE LEGENDS**

326 **Figure 1: Canonical mechanism of activation of AMPK by adenine nucleotides, and the**
327 **Ca²⁺-dependent mechanism mediated by CaMKK β .** AMP binding activates AMPK
328 by three effects, i.e. promotion of Thr172 phosphorylation by LKB1 (effect #1),
329 inhibition of Thr172 dephosphorylation by protein phosphatases (PP) (effect #2), and
330 allosteric activation (effect #3). All three effects are opposed by binding of ATP, while
331 binding of ADP mimics effect #2 and #1, but not #3. CaMKK β phosphorylates the
332 same site as LKB1 (Thr172) in response to increases in cellular Ca²⁺.

333 **Figure 2: Crystal structure of the human $\alpha 1\beta 2\gamma 1$ heterotrimer in complex with β -**
334 **cyclodextrin, staurosporine, and AMP, with Thr172 phosphorylated.** Atomic co-
335 ordinates are from the PDB file 4RER [8]. The model was rendered in PyMOL v1.7.4.2
336 with the majority of the polypeptide in “cartoon” view and the α -linker in “sphere”
337 view. The domains referred to in the text are color coded and labeled. The kinase
338 inhibitor staurosporine in the active site, and the side chain of phospho-Thr172, are in
339 “sphere” view, and β -cyclodextrin in the glycogen-binding site of the β -CBM in “stick”
340 view, all with C atoms in green, O red, and N blue (H omitted). The curved dotted line
341 in the center shows the approximate boundary between the “catalytic module”
342 (containing the α -KD and β -CBM) and the “nucleotide-binding module” (containing
343 the γ subunit and the C-terminal domains of α and β); the α -AID and α -linker form one
344 of the flexible connectors linking these two modules. Note how the α -RIM2 section of
345 the α -linker (in magenta) contacts site 3 of the γ subunit with its bound AMP.

Figure 3: Structures of the kinase domain (α -KD) and auto-inhibitory domains (α -AID) of the α subunit in (A) inactive and (B) active conformations. Atomic co-ordinates are from the PDB files 4RED (A) and 4RER (B) [8], with only the α -KD, α -AID and the start of the α -linker being displayed in (B). The color-coding of domains is as in Fig. 2. Most of the structures are rendered in “cartoon” view (PyMOL v1.7.4.2), but the side chains of the regulatory spine [18] (Leu81, white; Leu70, red; Phe160, magenta; His139, blue), and phosphorylated Thr172 in (B), are in “sphere” view. Note how the residues of the regulatory spine are stacked in alignment in (B) but not in (A). In (A), the α -AID shown is that attached to the other molecule of α -KD: α -AID within the crystal dimer, but in solution the α -AID from the same molecule is thought to adopt this position [8].

Figure 4: Selection of AMPK activating compounds grouped according to their mechanisms of action. (A) pro-drugs converted into AMP analogs by cellular enzymes; (B) compounds that bind at the ADaM site; (C) compounds that act by inhibiting mitochondrial ATP synthesis and thus increase cellular AMP and ADP; (D) antifolate drugs that activate AMPK by inhibiting AICAR transformylase in the pathway of purine nucleotide biosynthesis (shown), thus increasing cellular ZMP. Figure modified from Fig. 1 in [71].

Figure 5: Approaches used to characterize the AMPK recognition motif. (A) Hypothesis-driven approach using mutations. Constructs containing 34 residues around Ser79 on ACC1, with and without the indicated mutations, were expressed in bacteria and phosphorylated by AMPK in cell-free assays. Changes in kinetic parameters (k_{cat}/K_m) for each mutant relative to the wild type are represented by the lengths of the bars above (increases) or below (decreases) the indicated amino acid [46]. **(B) Positional scanning peptide library.** Phosphorylation by AMPK in cell-free assays of peptide mixtures (10-mers) containing serine and threonine at position 6, a fixed amino acid at one position (e.g. P-5, illustrated), and random mixtures at all others, revealed preferences for specific amino acids at each position [48]; “x”, any amino acid. **(C) Direct thiophosphorylation with gatekeeper mutation.** An AMPK mutant uses a bulky derivative of ATP- γ -S (A*TP γ S) to thiophosphorylate direct targets in permeabilized cells [50]. Many direct AMPK phosphorylation sites were identified through isolation and identification by tandem mass spectrometry of the thiophosphorylated peptides [54]. Hydrophobic amino acids are in green, neutral polar in blue, acidic in orange, and basic in red.

Figure 6: AMPK recognizes a well-defined phosphorylation motif. The logo motif of 64 known AMPK phosphorylation sites from P-7 to P+7 is presented, and points of interest in the AMPK recognition motif are noted. Color scheme as in Figure 5. The logo motif was generated by WebLogo [72], using a previously described list of 50 well-validated phosphorylation sites [54] updated with 14 additional sites from the literature. This full list of 64 well-validated AMPK targets is provided as supplementary Table 1. Human sequences were used to generate this motif.

389 **Glossary:**

390 **α -AID:** auto-inhibitory domain, the domain that follows the kinase domain in AMPK- α subunits,
391 and which inhibits the kinase domain in the absence of AMP

392 **α -KD:** kinase domains on the α subunits of AMPK, which are related to the catalytic domains in
393 other members of the “eukaryotic” protein kinase (ePK) family

394 **α -linker:** region of the AMPK- α subunit that connects the α -AID to the C-terminal domain,
395 important in the mechanism of regulation by AMP

396 **α -RIM1/ α -RIM2:** conserved sequences within the α -linker, which interact with the AMPK- γ
397 subunit when AMP is bound at site 3

398 **ADaM site:** the “Allosteric Drug and Metabolite” binding pocket, located between the α subunit
399 kinase domain and the β subunit carbohydrate-binding module, where drugs such as A-769662
400 bind, and where naturally occurring metabolites are speculated to bind

401 **CBS repeat:** a sequence motif, first found in the enzyme cystathionine β -synthase, that always
402 occurs as tandem repeats; a single twin repeat often binds a ligand containing adenosine, such as
403 AMP, ATP or S-adenosylmethionine

404 **kinase recognition motif:** amino acid sequence surrounding a phosphorylation site, which
405 promotes a given protein kinase to target that residue
406

407 **References**

- 408 1 Hardie, D.G. (2014) AMPK--sensing energy while talking to other signaling pathways. *Cell*
409 *Metab.* 20, 939-952
- 410 2 Oakhill, J.S., *et al.* (2012) AMPK functions as an adenylate charge-regulated protein kinase.
411 *Trends Endocrinol. Metab.* 23, 125-132
- 412 3 Carling, D., *et al.* (2012) AMP-activated protein kinase: new regulation, new roles?
413 *Biochem. J.* 445, 11-27
- 414 4 Hawley, S.A., *et al.* (1996) Characterization of the AMP-activated protein kinase kinase
415 from rat liver, and identification of threonine-172 as the major site at which it
416 phosphorylates and activates AMP-activated protein kinase. *J. Biol. Chem.* 271, 27879-
417 27887
- 418 5 Oakhill, J.S., *et al.* (2011) AMPK is a direct adenylate charge-regulated protein kinase.
419 *Science* 332, 1433-1435
- 420 6 Xiao, B., *et al.* (2011) Structure of mammalian AMPK and its regulation by ADP. *Nature*
421 472, 230-233
- 422 7 Gowans, G.J., *et al.* (2013) AMP is a true physiological regulator of AMP-activated protein
423 kinase by both allosteric activation and enhancing net phosphorylation. *Cell Metab.* 18,
424 556-566
- 425 8 Li, X., *et al.* (2015) Structural basis of AMPK regulation by adenine nucleotides and
426 glycogen. *Cell Res.* 25, 50-66
- 427 9 Scott, J.W., *et al.* (2004) CBS domains form energy-sensing modules whose binding of
428 adenosine ligands is disrupted by disease mutations. *J. Clin. Invest.* 113, 274-284
- 429 10 Xiao, B., *et al.* (2007) Structural basis for AMP binding to mammalian AMP-activated
430 protein kinase. *Nature* 449, 496-500
- 431 11 Chen, L., *et al.* (2012) AMP-activated protein kinase undergoes nucleotide-dependent
432 conformational changes. *Nat. Struct. Mol. Biol.* 19, 716-718
- 433 12 Xiao, B., *et al.* (2013) Structural basis of AMPK regulation by small molecule activators.
434 *Nature Commun.* 4, 3017

- 435 13 Calabrese, M.F., *et al.* (2014) Structural basis for AMPK activation: natural and synthetic
436 ligands regulate kinase activity from opposite poles by different molecular mechanisms.
437 Structure 22, 1161-1172
- 438 14 Pang, T., *et al.* (2007) Conserved alpha-helix acts as autoinhibitory sequence in AMP-
439 activated protein kinase alpha subunits. J. Biol. Chem. 282, 495-506
- 440 15 Goransson, O., *et al.* (2007) Mechanism of action of A-769662, a valuable tool for
441 activation of AMP-activated protein kinase. J. Biol. Chem. 282, 32549-32560
- 442 16 Crute, B.E., *et al.* (1998) Functional domains of the alpha1 catalytic subunit of the AMP-
443 activated protein kinase. J. Biol. Chem. 273, 35347-35354
- 444 17 Chen, L., *et al.* (2009) Structural insight into the autoinhibition mechanism of AMP-
445 activated protein kinase. Nature 459, 1146-1149
- 446 18 Taylor, S.S. and Kornev, A.P. (2011) Protein kinases: evolution of dynamic regulatory
447 proteins. Trends Biochem. Sci. 36, 65-77
- 448 19 Xin, F.J., *et al.* (2013) Coordinated regulation of AMPK activity by multiple elements in the
449 alpha-subunit. Cell Res. 23, 1237-1240
- 450 20 Gomez-Galeno, J.E., *et al.* (2010) A potent and selective AMPK activator that inhibits de
451 novo lipogenesis. ACS Med. Chem. Lett. 1, 478-482
- 452 21 Hunter, R.W., *et al.* (2014) Mechanism of action of Compound-13: an alpha1-selective
453 small molecule activator of AMPK. Chem. Biol. 21, 866-879
- 454 22 Riek, U., *et al.* (2008) Structural properties of AMP-activated protein kinase. Dimerization,
455 molecular shape, and changes upon ligand binding. J. Biol. Chem. 283, 18331-18343
- 456 23 Zhang, C.S., *et al.* (2014) The lysosomal v-ATPase-Ragulator complex is a common
457 activator for AMPK and mTORC1, acting as a switch between catabolism and anabolism.
458 Cell Metab. 20, 526-540
- 459 24 Zhang, Y.L., *et al.* (2013) AMP as a low-energy charge signal autonomously initiates
460 assembly of AXIN-AMPK-LKB1 complex for AMPK activation. Cell Metab. 18, 546-555
- 461 25 Corton, J.M., *et al.* (1995) 5-Aminoimidazole-4-carboxamide ribonucleoside: a specific
462 method for activating AMP-activated protein kinase in intact cells? Eur. J. Biochem. 229,
463 558-565

- 464 26 Cool, B., *et al.* (2006) Identification and characterization of a small molecule AMPK
465 activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell*
466 *Metab.* 3, 403-416
- 467 27 Sanders, M.J., *et al.* (2007) Defining the mechanism of activation of AMP-activated protein
468 kinase by the small molecule A-769662, a member of the thienopyridone family. *J. Biol.*
469 *Chem.* 282, 32539-32548
- 470 28 Hudson, E.R., *et al.* (2003) A novel domain in AMP-activated protein kinase causes
471 glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. *Current*
472 *Biol.* 13, 861-866
- 473 29 Polekhina, G., *et al.* (2003) AMPK β -Subunit targets metabolic stress-sensing to glycogen.
474 *Current Biol.* 13, 867-871
- 475 30 Lai, Y.C., *et al.* (2014) A small-molecule benzimidazole derivative that potently activates
476 AMPK to increase glucose transport in skeletal muscle: comparison with effects of
477 contraction and other AMPK activators. *Biochem. J.* 460, 363-375
- 478 31 Hawley, S.A., *et al.* (2012) The ancient drug salicylate directly activates AMP-activated
479 protein kinase. *Science* 336, 918-922
- 480 32 Langendorf, C.G. and Kemp, B.E. (2015) Choreography of AMPK activation. *Cell Res.* 25, 5-
481 6
- 482 33 Hawley, S.A., *et al.* (2005) Calmodulin-dependent protein kinase kinase- β is an
483 alternative upstream kinase for AMP-activated protein kinase. *Cell Metab.* 2, 9-19
- 484 34 Woods, A., *et al.* (2005) Ca^{2+} /calmodulin-dependent protein kinase kinase- β acts
485 upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab.* 2, 21-33
- 486 35 Hurley, R.L., *et al.* (2005) The Ca^{2+} /calmodulin-dependent protein kinase kinases are
487 AMP-activated protein kinase kinases. *J. Biol. Chem.* 280, 29060-29066
- 488 36 Fogarty, S., *et al.* (2010) Calmodulin-dependent protein kinase kinase- β activates
489 AMPK without forming a stable complex - synergistic effects of Ca^{2+} and AMP. *Biochem. J.*
490 426, 109-118
- 491 37 Hawley, S.A., *et al.* (2010) Use of cells expressing gamma subunit variants to identify
492 diverse mechanisms of AMPK activation. *Cell Metab.* 11, 554-565

- 493 38 Lee, Y.S., *et al.* (2006) Berberine, a natural plant product, activates AMP-activated protein
494 kinase with beneficial metabolic effects in diabetic and insulin-resistant states. *Diabetes*
495 55, 2256-2264
- 496 39 Huang, S.L., *et al.* (2012) Arctigenin, a natural compound, activates AMP-activated protein
497 kinase via inhibition of mitochondria complex I and ameliorates metabolic disorders in
498 ob/ob mice. *Diabetologia* 55, 1469-1481
- 499 40 Baur, J.A., *et al.* (2006) Resveratrol improves health and survival of mice on a high-calorie
500 diet. *Nature* 444, 337-342
- 501 41 Racanelli, A.C., *et al.* (2009) Therapeutics by cytotoxic metabolite accumulation:
502 pemetrexed causes ZMP accumulation, AMPK activation, and mammalian target of
503 rapamycin inhibition. *Cancer Res.* 69, 5467-5474
- 504 42 Pirkmajer, S., *et al.* (2015) Methotrexate promotes glucose uptake and lipid oxidation in
505 skeletal muscle via AMPK activation. *Diabetes* 64, 360-369
- 506 43 Weekes, J., *et al.* (1993) Specificity determinants for the AMP-activated protein kinase and
507 its plant homologue analysed using synthetic peptides. *FEBS Lett.* 334, 335-339
- 508 44 Davies, S.P., *et al.* (1989) Tissue distribution of the AMP-activated protein kinase, and lack
509 of activation by cyclic AMP-dependent protein kinase, studied using a specific and
510 sensitive peptide assay. *Eur. J. Biochem.* 186, 123-128
- 511 45 Dale, S., *et al.* (1995) Similar substrate recognition motifs for mammalian AMP-activated
512 protein kinase, higher plant HMG-CoA reductase kinase-A, yeast SNF1, and mammalian
513 calmodulin-dependent protein kinase I. *FEBS Lett.* 361, 191-195
- 514 46 Scott, J.W., *et al.* (2002) Protein kinase substrate recognition studied using the
515 recombinant catalytic domain of AMP-activated protein kinase and a model substrate. *J.*
516 *Mol. Biol.* 317, 309-323
- 517 47 Istvan, E.S., *et al.* (2000) Crystal structure of the catalytic portion of human HMG-CoA
518 reductase: insights into regulation of activity and catalysis. *EMBO J.* 19, 819-830
- 519 48 Gwinn, D.M., *et al.* (2008) AMPK phosphorylation of raptor mediates a metabolic
520 checkpoint. *Mol. Cell* 30, 214-226
- 521 49 Songyang, Z., *et al.* (1994) Use of an oriented peptide library to determine the optimal
522 substrates of protein kinases. *Current Biol.* 4, 973-982

- 523 50 Banko, M.R., *et al.* (2011) Chemical genetic screen for AMPK α 2 substrates uncovers a
524 network of proteins involved in mitosis. *Mol. Cell* 44, 878-892
- 525 51 Habelhah, H., *et al.* (2001) Identification of new JNK substrate using ATP pocket mutant
526 JNK and a corresponding ATP analogue. *J. Biol. Chem.* 276, 18090-18095
- 527 52 Shah, K. and Shokat, K.M. (2002) A chemical genetic screen for direct v-Src substrates
528 reveals ordered assembly of a retrograde signaling pathway. *Chem. Biol.* 9, 35-47
- 529 53 Blethrow, J.D., *et al.* (2008) Covalent capture of kinase-specific phosphopeptides reveals
530 Cdk1-cyclin B substrates. *Proc. Natl. Acad. Sci. USA* 105, 1442-1447
- 531 54 Schaffer, B.E., *et al.* (2015) Identification of AMPK phosphorylation sites reveals a
532 network of proteins involved in cell invasion and facilitates large-scale substrate
533 prediction. *Cell Metabolism*, in press
- 534 55 Yaffe, M.B., *et al.* (1997) The structural basis for 14-3-3:phosphopeptide binding
535 specificity. *Cell* 91, 961-971
- 536 56 Mitchelhill, K.I., *et al.* (1997) Posttranslational modifications of the 5'-AMP-activated
537 protein kinase β 1 subunit. *J. Biol. Chem.* 272, 24475-24479
- 538 57 Hawley, S.A., *et al.* (2014) Phosphorylation by Akt within the ST loop of AMPK- α 1 down-
539 regulates its activation in tumour cells. *Biochem. J.* 459, 275-287
- 540 58 Tuerk, R.D., *et al.* (2009) Tracking and quantification of ^{32}P -labeled phosphopeptides in
541 liquid chromatography matrix-assisted laser desorption/ionization mass spectrometry.
542 *Anal. Biochem.* 390, 141-148
- 543 59 Egan, D.F., *et al.* (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein
544 kinase connects energy sensing to mitophagy. *Science* 331, 456-461
- 545 60 Mihaylova, M.M., *et al.* (2011) Class IIa histone deacetylases are hormone-activated
546 regulators of FOXO and mammalian glucose homeostasis. *Cell* 145, 607-621
- 547 61 DeRan, M., *et al.* (2014) Energy stress regulates Hippo-YAP signaling involving AMPK-
548 mediated regulation of Angiotensin-like 1 protein. *Cell Rep.* 9, 495-503
- 549 62 Marin, T.L., *et al.* (2015) Identification of AMP-activated protein kinase targets by a
550 consensus sequence search of the proteome. *BMC Syst. Biol.* 9, 13

- 551 63 Ducommun, S., *et al.* (2015) Motif affinity and mass spectrometry proteomic approach for
552 the discovery of cellular AMPK targets: Identification of mitochondrial fission factor as a
553 new AMPK substrate. *Cell. Signal.* 27, 978-988
- 554 64 Obenauer, J.C., *et al.* (2003) Scansite 2.0: Proteome-wide prediction of cell signaling
555 interactions using short sequence motifs. *Nucl. Acids Res.* 31, 3635-3641
- 556 65 Grant, C.E., *et al.* (2011) FIMO: scanning for occurrences of a given motif. *Bioinformatics*
557 27, 1017-1018
- 558 66 Tuerk, R.D., *et al.* (2007) New candidate targets of AMP-activated protein kinase in
559 murine brain revealed by a novel multidimensional substrate-screen for protein kinases.
560 *J. Proteome Res.* 6, 3266-3277
- 561 67 Behrends, C., *et al.* (2010) Network organization of the human autophagy system. *Nature*
562 466, 68-76
- 563 68 Huttlin, E.L., *et al.* (2015) The BioPlex Network: a systematic exploration of the human
564 interactome. *Cell* 162, 425-440
- 565 69 Goodwin, J.M., *et al.* (2014) An AMPK-independent signaling pathway downstream of the
566 LKB1 tumor suppressor controls Snail1 and metastatic potential. *Mol. Cell* 55, 436-450
- 567 70 Michell, B.J., *et al.* (1996) Isoform-specific purification and substrate specificity of the 5'-
568 AMP-activated protein kinase. *J. Biol. Chem.* 271, 28445-28450
- 569 71 Hardie, D.G. (2015) Regulation of AMP-activated protein kinase by natural and synthetic
570 activators. *Acta Pharm. Sin. B*, in press
- 571 72 Crooks, G.E., *et al.* (2004) WebLogo: a sequence logo generator. *Genome Res.* 14, 1188-
572 1190
- 573

A) α -KD:AID (inactive conformation)

B) α -KD:AID (active conformation)

A) Pro-drugs converted within cells to AMP analogs

B) Compounds that bind between α -KD and β -CBM

C) Compounds that inhibit mitochondrial ATP synthesis and increase cellular AMP

D) Mechanism by which antifolates (e.g. pemetrexed, methotrexate) cause ZMP accumulation:

A. Hypothesis-driven mutations

B. Positional scanning peptide library

adapted from Gwinn et al, 2008

C. Direct thiophosphorylation of substrates

adapted from Banko et al, 2011 and Schaffer et al, 2015

Figure 6

A

Supplementary Material

AMPK: an energy-sensing pathway with multiple inputs and outputs

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Table S1: List of 64 validated sites phosphorylated by AMPK.

Short name	Long name	UNIPROT	Site ^a	Motif (± 10 residues) ^b	Criteria ^c				Refs
					1 ^d	2 ^e	3 ^f	4 ^g	
ACACA	Acetyl-CoA carboxylase 1	Q13085	S80	GLALH I RSSMSGLHLVKQGRD	X	X		X	[3-6]
ACACB	Acetyl-CoA carboxylase 2	O00763	S222	TRVPT M RPSMSGLHLVKRGRE	X	X			[7-10]
AMOTL1	Angiomotin-like protein 1	Q8IY63	S793	TDSSSL R PARSVPS I AAATGT	X	X			[11]
BAIAP2	Brain angiogenesis inhibitor 1-associated protein 2	Q9UQB8	S366	TENK T L P RRSSMAAGLERNGR	X		X	X	[1]
BRAF	Serine/threonine-protein kinase B-raf	P15056	S729	RSLP K I H RSASEPS L NRAGFQ	X	X			[12]
CDC27	Cell division cycle protein 27 homolog	P30260	S379	SPPNAL P RRSSRLFTSDSSTT	X		X		[1]
CDC42EP1	Cdc42 effector protein 1	Q00587	S192	PSEPGL R RS D SLLS F RLDLDL			X	X	[2]
CDKN1B	Cyclin-dependent kinase inhibitor 1B	P46527	T198	PKKPGL R RR Q T	X	X			[13]
CLIP-170	CAP-Gly domain-containing linker protein 1	P30622	S312	TTSAS L K R SPSASS L SSMSSV	X	X			[14]
CRTC2	CREB-regulated transcription coactivator 2	Q53ET0	S171	RLPSAL N RTSSDSALHTSVMN	X	X			[15-18]
CRY1	clock component cryptochrome 1	Q16526	S71	DLNAN L R H LNSRL F VRGQPA	X	X			[19]

EEF2K	eukaryotic elongation factor 2 kinase	O00418	S398	VTFD S L PSSP S SATPHSQKLD	X	X			[20]
EP300	Histone acetyltransferase p300	Q09472	S89	KQLSE L L R S GSS P N L N M G V G G	X	X			[21, 22]
FOXO3a	Forkhead box protein O3	O43524	S413	PTGG L M Q R S S SFPY T T K G S G L	X	X			[23]
FOXO3a	Forkhead box protein O3	O43524	S588	QSM Q T L S D S L S G S S L Y S T S A N	X	X			[23]
GABABR2	GABA B receptor R2 subunit	O75899	S784	STSV T S V N Q A S T S R L E G L Q S E	X	X			[24]
GBF1	Golgi-specific brefeldin A-resistance GEF-1	Q92538	T1337	GRPG K I H R S A S D A D V V N S G W L	X	X			[25]
GFPT1	Glutamine-fructose-6-phosphate aminotransferase-1	Q06210	S261	KGSC N L S R V D S T T C L F P V E E K	X	X			[26, 27]
GLI1	Zinc finger protein GLI1	P08151	S102	S L D L Q T V I R T S P S S L V A F I N S	X	X			[28]
GLI1	Zinc finger protein GLI1	P08151	S408	R G D G P L P R A P S I S T V E P K R E R	X	X			[28]
GLI1	Zinc finger protein GLI1	P08151	T1074	S H D Q R G S S G H S P P S G P P N M A	X	X			[28]
GYS1	Glycogen Synthase 1 (muscle isoform)	P13807	S8	M P L N R T L S M S S L P G L E D W	X	X			[29, 30]
GYS2	Glycogen Synthase 2 (liver isoform)	P54840	S8	M L R G R S L S V T S L G G L P Q W	X	X			[31]
H2B	Histone H2B	Q16778	S37	G K K R K R S R K E S Y S I V Y K V L K	X	X			[32]
HDAC5	Histone Deacetylase 5	Q9UQL6	S259	R D D F P L R K T A S E P N L K V R S R L	X	X			[33]
HDAC5	Histone Deacetylase 5	Q9UQL6	S498	P R H R P L S R T Q S S P L P Q S P Q A L	X	X			[33]
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase	P04035	S872	L V K S H M I H N R S K I N L Q D L Q G A	X				[34-36]
IRS1	insulin receptor substrate 1	P35568	S794	A R H Q H L R L S T S S G R L L Y A A T A	X	X			[37, 38]
KCNB1	Potassium voltage-gated channel subfamily B member 1	Q14721	S444	E A L E R A K R N G S I V S M N M K D A F	X	X			[39]
KCNB1	Potassium voltage-gated channel subfamily B member 1	Q14721	S541	D M Y N K M A K T Q S Q P I L N T K E S A	X	X			[39]
KLC2	Kinesin light chain 2	Q9H0B6	S545	D G S G S L R R S G S F G K L R D A L R R	X			X	[40, 41]
LIPE	Hormone-sensitive lipase	Q05469	S855	P I A E P M R R S V S E A A L A Q P Q G P	X				[42-44]
MAPT	Microtubule-associated protein tau	P10636	S579	D L K N V K S K I G S T E N L K H Q P G G	X	X			[45, 46]
MDM4	Mdm4	O15151	S342	S D C S K L T H S L S T S D I T A I P E K	X	X			[47]
NET1	Neuroepithelial cell-transforming gene 1 protein	Q7Z628	S100	K R V R P L A R V T S L A N L I S P V R N		X	X	X	[2]
NOS1	Neuronal nitric oxide synthase (nNOS)	P29475	S1417	Y E V T N R L R S E S I A F I E S K K D		X			[8, 48]
NOS3	Endothelial nitric oxide synthase (eNOS)	P29474	S1177	Q E V T S R I R T Q S F S L Q E R Q L R G	X				[49]
PAK2	Serine/threonine-protein kinase PAK 2	Q13177	S20	K P P A P P V R M S T I F S T G G K D P	X	X	X		[1]
PEA15	Astrocytic phosphoprotein PEA-15	Q15121	S116	K K Y K D I I R Q P S E E E I I K L A P P	X	X			[50]
PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2	O60825	S466	N Q T P V R M R R N S F T P L S S S N T I	X	X			[51]
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3	Q16875	S461	K G P N P L M R R N S V T P L A S P E P T	X	X			[52, 53]
PGC1A	PPAR-gamma coactivator 1-alpha	Q9UBK2	T178	N H A N H N H R I R T N P A I V K T E N S	X	X			[54]
PGC1A	PPAR-gamma coactivator 1-alpha	Q9UBK2	S539	Q S Y S L F N V S P S C S S F N S P C R D	X	X			[54]
PIKFYVE	1-phosphatidylinositol 3-phosphate 5-kinase	Q9Y2I7	S307	G K S P A R N R S A S I T N L S L D R S G	X	X			[55]

PLD1	Phospholipase D1	Q13393	S505	TDVGS V K R V T S G P S L G S L P P A	X	X			[56]
PPP1R12C	Protein phosphatase 1 regulatory subunit 12C	Q9BZL4	S452	APGAG L Q R S A SS S W L E G T S T Q	X	X	X		[1]
PRKCQ	protein kinase C-theta	Q04759	T538	ENMLGDA K T N T F CGTPDYIAP	X	X			[57]
RAG1	V(D)J recombination-activating protein 1	P15918	S531	EWQPP L K N V S ST D V G I D GL	X	X			[58]
RPTOR	Regulatory-associated protein of mTOR	Q8N122	S792	ETID K M R R A S S Y S L N S L I G V	X	X			[59]
RPTOR	Regulatory-associated protein of mTOR	Q8N122	S722	PCTP R L R SV S Y G N I RAVATA				X	[59]
RRN3	RNA polymerase I-specific transcription initiation factor	Q9NYV6	S635	SSFDT H F R S P SS S V G S P P V L Y	X	X			[60]
SNX17	Sorting nexin-17	Q15036	S437	ESMV K L S S K L S AV S L R G I G S P		X	X	X	[2]
SREBF1	Sterol regulatory element-binding protein 1	P36956	S396	SLRTA V H K S K S L K D L V S A C G S	X	X			[61]
TBC1D1	TBC1 domain family member 1	Q86TI0	S237	PVRRP M R K S F S Q P L R S L A F R	X	X		X	[62]
TBC1D4	TBC1 domain family member 4	O60343	S704	SSLPS L H T S F S A P S F T A P S F L	X	X			[63]
TNNI3	Troponin I, cardiac muscle	P19429	S150	KRPT L R R V R I S AD A M M Q A L L G	X	X			[64, 65]
TP53	p53	P04637	S15	QSDPS V E P P L S Q E T F S D L W K L	X	X			[66, 67]
TP73	Tumor protein p73	O15350	S426	KVHG G M N K L P S V N Q L V G Q P P P	X				[68]
TSC2	tuberous sclerosis complex 2	P49815	S1387	QPSQ P L S K S SS P E L Q T L Q D I	X	X			[69]
TXNIP	Thioredoxin-interacting protein	Q9H3M7	S308	SR S G L SS R T S SM A S R T S SE M S	X	X			[70]
ULK1	Serine/threonine-protein kinase ULK1	O75385	S467	PRSSA I R R S G ST S P L G F AR A S	X	X			[71]
ULK1	Serine/threonine-protein kinase ULK1	O75385	S556	RT S G L G C R L H S A P N L S D L H V V	X	X			[71]
VASP	Vasodilator-stimulated phosphoprotein	P50552	T278	N A M L A R R R K A T Q V G E K T P K D E	X	X			[72]
YAP1	Transcriptional coactivator YAP1	P46937	S94	V P M R L R K L P D S F F K P P E P K S H	X	X			[73]

^aPhosphorylation site (human sequence numbering)

^bPhosphorylation site motif ± 10 residues upstream and downstream of the phosphoamino acid

Red = phosphorylated serine/threonine; brown = bulky hydrophobic residues ((L, I, M, F, V)) at P-5 and P+4; and blue = basic residues at P-6, P-4, P-3 or P-2. In some cases, N-terminal hydrophobic and basic residues are shifted one residue from these standard locations (see main text); these are also color coded. Where no residues are shown, the site is near the N- or C-terminus of the protein.

^cCriteria that were used to validate AMPK substrates. In general, a site can be considered a well-validated AMPK substrate when both a direct phosphorylation in cell-free assays and AMPK-dependent phosphorylation in intact cells have been demonstrated in a site-specific manner (criteria 1

and 2 together), or the site was identified and validated using the ATP-analog-specific approach in intact, permeabilized cells. In this list, we have generally not taken into account evidence obtained using functional changes in the target protein. Given the size of the AMPK literature, we did not include all sites that have been exclusively validated in cell-free assays, and we may have missed some key substrates or data relevant to the validation of some listed substrates.

^dcell-free assays demonstrating that AMPK directly phosphorylates the site in question (e.g. via mass spectrometry, ³²P incorporation or phosphospecific antibodies, preferably with comparisons of phosphorylation with and without mutation at the site)

^eDemonstration that AMPK phosphorylates the site in intact cells through either; (i) increases in phosphorylation in response to treatment with specific AMPK activators (e.g. A769662, AICAR, 991); (ii) decreases in phosphorylation following knockout or knock-down (e.g. by expression of dominant negative AMPK mutant or interfering RNA); or (iii) the use of less specific AMPK activators (e.g. 2-deoxyglucose, metformin, muscle contraction) in combination with AMPK knockdown or inhibition (e.g. using compound C, although the authors discourage the use of that compound, which is a very non-selective kinase inhibitor).

^fDemonstration that ATP analog-specific (AS) AMPK thiophosphorylates the specific site in intact permeabilized cells [1].

^gIdentification of the site with high confidence in Schaffer et al [2].

References

- 1 Banko, M.R., *et al.* (2011) Chemical genetic screen for AMPK α 2 substrates uncovers a network of proteins involved in mitosis. *Mol. Cell* 44, 878-892
- 2 Schaffer, B.E., *et al.* (2015) Identification of AMPK phosphorylation sites reveals a network of proteins involved in cell invasion and facilitates large-scale substrate prediction. *Cell Metabolism*, in press

- 3 Davies, S.P., *et al.* (1990) Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. *Eur. J. Biochem.* 187, 183-190
- 4 Munday, M.R., *et al.* (1988) Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase. *Eur. J. Biochem.* 175, 331-338
- 5 Laderoute, K.R., *et al.* (2006) 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. *Mol. Cell. Biol.* 26, 5336-5347
- 6 Ha, J., *et al.* (1994) Critical phosphorylation sites for acetyl-CoA carboxylase activity. *J. Biol. Chem.* 269, 22162-22168
- 7 Winder, W.W., *et al.* (1997) Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. *J. Appl. Physiol.* 82, 219-225
- 8 Chen, Z.P., *et al.* (2000) AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. *Am. J. Physiol.* 279, E1202-E1206.
- 9 Dzamko, N., *et al.* (2008) AMPK-independent pathways regulate skeletal muscle fatty acid oxidation. *J. Physiol.* 586, 5819-5831
- 10 Steinberg, G.R., *et al.* (2010) Whole body deletion of AMP-activated protein kinase β_2 reduces muscle AMPK activity and exercise capacity. *J. Biol. Chem.* 285, 37198-37209
- 11 DeRan, M., *et al.* (2014) Energy stress regulates Hippo-YAP signaling involving AMPK-mediated regulation of Angiotensin-like 1 protein. *Cell Rep.* 9, 495-503
- 12 Shen, C.H., *et al.* (2013) Phosphorylation of BRAF by AMPK impairs BRAF-KSR1 association and cell proliferation. *Mol. Cell* 52, 161-172
- 13 Liang, J., *et al.* (2007) The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat. Cell Biol.* 9, 218-224

- 14 Nakano, A., *et al.* (2010) AMPK controls the speed of microtubule polymerization and directional cell migration through CLIP-170 phosphorylation. *Nat. Cell Biol.* 12, 583-590
- 15 Koo, S.H., *et al.* (2005) The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature* 437, 1109-1114
- 16 Foretz, M., *et al.* (2010) Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J. Clin. Invest.* 120, 2355-2369
- 17 Patel, K., *et al.* (2014) The LKB1-salt-inducible kinase pathway functions as a key gluconeogenic suppressor in the liver. *Nat. Commun.* 5, 4535
- 18 Lerner, R.G., *et al.* (2009) A role for the CREB co-activator CRTC2 in the hypothalamic mechanisms linking glucose sensing with gene regulation. *EMBO Rep.*
- 19 Lamia, K.A., *et al.* (2009) AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science* 326, 437-440
- 20 Browne, G.J., *et al.* (2004) Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. *J. Biol. Chem.* 279, 12220-12231
- 21 Yang, W., *et al.* (2001) Regulation of transcription by AMP-activated protein kinase. Phosphorylation of p300 blocks its interaction with nuclear receptors. *J. Biol. Chem.* 276, 38341-38344
- 22 Zhang, Y., *et al.* (2011) AMP-activated protein kinase suppresses endothelial cell inflammation through phosphorylation of transcriptional coactivator p300. *Arterioscler. Thromb. Vasc. Biol.* 31, 2897-2908
- 23 Greer, E.L., *et al.* (2007) The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. *J. Biol. Chem.* 282, 30107-30119
- 24 Kuramoto, N., *et al.* (2007) Phospho-dependent functional modulation of GABA(B) receptors by the metabolic sensor AMP-dependent protein kinase. *Neuron* 53, 233-247

- 25 Miyamoto, T., *et al.* (2008) AMP-activated protein kinase phosphorylates Golgi-specific brefeldin A resistance factor 1 at Thr1337 to induce disassembly of Golgi apparatus. *J. Biol. Chem.* 283, 4430-4438
- 26 Li, Y., *et al.* (2007) Identification of a novel serine phosphorylation site in human glutamine:fructose-6-phosphate amidotransferase isoform 1. *Biochemistry* 46, 13163-13169
- 27 Eguchi, S., *et al.* (2009) AMP-activated protein kinase phosphorylates glutamine : fructose-6-phosphate amidotransferase 1 at Ser243 to modulate its enzymatic activity. *Genes Cells* 14, 179-189
- 28 Li, Y.H., *et al.* (2015) AMP-activated protein kinase directly phosphorylates and destabilizes Hedgehog pathway transcription factor GLI1 in medulloblastoma. *Cell Rep.*
- 29 Carling, D. and Hardie, D.G. (1989) The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim. Biophys. Acta* 1012, 81-86
- 30 Jorgensen, S.B., *et al.* (2004) The α 2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* 53, 3074-3081
- 31 Bultot, L., *et al.* (2012) AMP-activated protein kinase phosphorylates and inactivates liver glycogen synthase. *Biochem. J.* 443, 193-203
- 32 Bungard, D., *et al.* (2010) Signaling kinase AMPK activates stress-promoted transcription via histone H2B phosphorylation. *Science* 329, 1201-1205
- 33 McGee, S.L., *et al.* (2008) AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. *Diabetes* 57, 860-867
- 34 Clarke, P.R. and Hardie, D.G. (1990) Regulation of HMG-CoA reductase: identification of the site phosphorylated by the AMP-activated protein kinase in vitro and in intact rat liver. *EMBO J.* 9, 2439-2446

- 35 Sato, R., *et al.* (1993) Replacement of serine-871 of hamster 3-hydroxy-3-methylglutaryl-CoA reductase prevents phosphorylation by AMP-activated kinase and blocks inhibition of sterol synthesis induced by ATP depletion. *Proc. Natl. Acad. Sci. USA* 90, 9261-9265
- 36 Ching, Y.P., *et al.* (1996) Analysis of the specificity of the AMP-activated protein kinase by site-directed mutagenesis of bacterially expressed 3-hydroxy 3-methylglutaryl-CoA reductase, using a single primer variant of the unique-site-elimination method. *Eur. J. Biochem.* 237, 800-808
- 37 Jakobsen, S.N., *et al.* (2001) 5'-AMP-activated protein kinase phosphorylates IRS-1 on Ser-789 in mouse C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside. *J. Biol. Chem.* 276, 46912-46916
- 38 Zakikhani, M., *et al.* (2010) Metformin and rapamycin have distinct effects on the AKT pathway and proliferation in breast cancer cells. *Breast Cancer Res. Treat.* 123, 271-279
- 39 Ikematsu, N., *et al.* (2011) Phosphorylation of the voltage-gated potassium channel Kv2.1 by AMP-activated protein kinase regulates membrane excitability. *Proc. Natl. Acad. Sci. USA* 108, 18132-18137
- 40 Johnson, C., *et al.* (2011) Visualization and biochemical analyses of the emerging mammalian 14-3-3-phosphoproteome. *Mol. Cell. Proteomics* 10, M110 005751
- 41 Amato, S., *et al.* (2011) AMP-activated protein kinase regulates neuronal polarization by interfering with PI 3-Kinase localization. *Science*
- 42 Garton, A.J., *et al.* (1989) Phosphorylation of bovine hormone-sensitive lipase by the AMP-activated protein kinase. A possible antilipolytic mechanism. *Eur. J. Biochem.* 179, 249-254
- 43 Daval, M., *et al.* (2005) Anti-lipolytic action of AMP-activated protein kinase in rodent adipocytes. *J. Biol. Chem.* 280, 25250-25257
- 44 Roepstorff, C., *et al.* (2004) Regulation of hormone-sensitive lipase activity and Ser563 and Ser565 phosphorylation in human skeletal muscle during exercise. *J. Physiol.* 560, 551-562

- 45 Thornton, C., *et al.* (2011) AMP-activated protein kinase (AMPK) is a tau kinase, activated in response to amyloid beta-peptide exposure. *Biochem. J.* 434, 503-512
- 46 Mairet-Coello, G., *et al.* (2013) The CAMKK2-AMPK kinase pathway mediates the synaptotoxic effects of Abeta oligomers through Tau phosphorylation. *Neuron* 78, 94-108
- 47 He, G., *et al.* (2014) AMP-activated protein kinase induces p53 by phosphorylating MDMX and inhibiting its activity. *Mol. Cell. Biol.* 34, 148-157
- 48 Thomas, M.M., *et al.* (2014) Muscle-specific AMPK beta1beta2-null mice display a myopathy due to loss of capillary density in nonpostural muscles. *FASEB J.* 28, 2098-2107
- 49 Chen, Z.P., *et al.* (1999) AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett.* 443, 285-289
- 50 Hindupur, S.K., *et al.* (2014) Identification of a novel AMPK-PEA15 axis in the anoikis-resistant growth of mammary cells. *Breast Cancer Res. Treat.* 16, 420
- 51 Marsin, A.S., *et al.* (2000) Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Current Biol.* 10, 1247-1255
- 52 Marsin, A.S., *et al.* (2002) The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase. *J. Biol. Chem.* 277, 30778-30783
- 53 Domenech, E., *et al.* (2015) AMPK and PFKFB3 mediate glycolysis and survival in response to mitophagy during mitotic arrest. *Nat. Cell Biol.* 17, 1304-1316
- 54 Jager, S., *et al.* (2007) AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1{alpha}. *Proc. Natl. Acad. Sci. USA* 104, 12017-12022

- 55 Liu, Y., *et al.* (2013) Phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) is an AMPK target participating in contraction-stimulated glucose uptake in skeletal muscle. *Biochem. J.*
- 56 Kim, J.H., *et al.* (2010) Phospholipase D1 mediates AMP-activated protein kinase signaling for glucose uptake. *PLoS One* 5, e9600
- 57 Lee, J.Y., *et al.* (2012) AMP-activated protein kinase mediates T cell activation-induced expression of FasL and COX-2 via protein kinase C theta-dependent pathway in human Jurkat T leukemia cells. *Cellular Signalling* 24, 1195-1207
- 58 Um, J.H., *et al.* (2013) Metabolic sensor AMPK directly phosphorylates RAG1 protein and regulates V(D)J recombination. *Proc. Natl. Acad. Sci. USA* 110, 9873-9878
- 59 Gwinn, D.M., *et al.* (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell* 30, 214-226
- 60 Hoppe, S., *et al.* (2009) AMP-activated protein kinase adapts rRNA synthesis to cellular energy supply. *Proc. Natl. Acad. Sci. USA* 106, 17781-17786
- 61 Li, Y., *et al.* (2011) AMPK phosphorylates and Inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell Metab.* 13, 376-388
- 62 Chen, S., *et al.* (2008) Complementary regulation of TBC1D1 and AS160 by growth factors, insulin and AMPK activators. *Biochem J* 409, 449-459
- 63 Treebak, J.T., *et al.* (2006) AMPK-mediated AS160 phosphorylation in skeletal muscle Is dependent on AMPK catalytic and regulatory subunits. *Diabetes* 55, 2051-2058
- 64 Oliveira, S.M., *et al.* (2012) AMP-activated protein kinase phosphorylates cardiac troponin I and alters contractility of murine ventricular myocytes. *Circ. Res.* 110, 1192-1201
- 65 Nixon, B.R., *et al.* (2012) AMP-activated protein kinase phosphorylates cardiac troponin I at ser-150 to increase myofilament calcium sensitivity and blunt PKA-dependent function. *J. Biol. Chem.* 287, 19136-19147

- 66 Jones, R.G., *et al.* (2005) AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol. Cell* 18, 283-293
- 67 Imamura, K., *et al.* (2001) Cell cycle regulation via p53 phosphorylation by a 5'-AMP activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside, in a human hepatocellular carcinoma cell line. *Biochem. Biophys. Res. Commun.* 287, 562-567
- 68 Adamovich, Y., *et al.* (2014) AMPK couples p73 with p53 in cell fate decision. *Cell Death Differ.*
- 69 Inoki, K., *et al.* (2003) TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115, 577-590
- 70 Wu, N., *et al.* (2013) AMPK-dependent degradation of TXNIP upon energy stress leads to enhanced glucose uptake via GLUT1. *Mol. Cell* 49, 1167-1175
- 71 Egan, D.F., *et al.* (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science* 331, 456-461
- 72 Blume, C., *et al.* (2007) AMP-activated protein kinase impairs endothelial actin cytoskeleton assembly by phosphorylating vasodilator-stimulated phosphoprotein. *J. Biol. Chem.* 282, 4601-4612
- 73 Mo, J.S., *et al.* (2015) Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway. *Nat. Cell Biol.* 17, 500-510

Glossary:

α -AID: auto-inhibitory domain, the domain that follows the kinase domain in AMPK- α subunits, and which inhibits the kinase domain in the absence of AMP

α -KD: kinase domains on the α subunits of AMPK, which are related to the catalytic domains in other members of the “eukaryotic” protein kinase (ePK) family

α -linker: region of the AMPK- α subunit that connects the α -AID to the C-terminal domain, important in the mechanism of regulation by AMP

α -RIM1/ α -RIM2: conserved sequences within the α -linker, which interact with the AMPK- γ subunit when AMP is bound at site 3

CBS repeat: a sequence motif, first found in the enzyme cystathionine β -synthase, that always occurs as two tandem repeats; a single twin repeat often binds a ligand containing adenosine, such as AMP, ATP or S-adenosylmethionine

kinase recognition motif: amino acid sequence surrounding a phosphorylation site, which promotes a given protein kinase to target that residue

TRENDS:

- AMPK is an energy-sensing protein kinase activated by phosphorylation of Thr172 within its catalytic α subunit. It binds AMP and/or ADP, both signals of energy stress, via its regulatory γ subunit. This activates the kinase by promoting Thr172 phosphorylation, inhibiting Thr172 dephosphorylation and allosteric activation.
- AMP binding to the γ subunit causes its interaction with the “ α -linker” region of the α subunit. This pulls the auto-inhibitory domain on the α subunit away from the kinase domain, triggering activation.
- AMPK is also activated by binding of synthetic and naturally occurring drug-like molecules that bind in the allosteric drug and metabolite (ADaM) site, between the α and β subunits
- AMPK has a well-defined recognition motif that has been established both by hypothesis-driven approaches and by various unbiased screens. It now has over 60 well-validated substrates.

Outstanding questions:

1. Given that site 3 on the AMPK- γ subunit seems to be the crucial site where binding of AMP causes activation, what is the function of AMP binding at the other two sites, sites 1 and 4?
2. How does binding of AMP or ADP to the AMPK- γ subunit promote phosphorylation of the AMPK- α subunit at Thr172 by upstream kinases such as LKB1?
3. Are there any naturally occurring metabolites in mammalian cells that bind to the allosteric drug and metabolite (ADaM) binding pocket between the α and β subunits, and thus activate or inhibit AMPK?
4. How are responses to different inputs affected by the numerous post-translational modifications that have been reported to occur on each AMPK subunit?
5. Does AMPK phosphorylate a different subset of substrates, depending on the inputs via which it was activated?
6. Are there subsets of primary or secondary structures, beyond the defined window from amino acids -5 to +4 in the AMPK recognition motif, which can help better predict substrates?
7. How much flexibility is there in the recognition motif surrounding any given AMPK phosphorylation site? For example can more distal features, such as the N-terminal amphipathic α -helices that are observed in acetyl-CoA carboxylase and HMG-CoA reductase, determine how rigidly the motif from P-5 to P+4 must adhere to the canonical recognition motif?