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AMP-activated protein kinase: a cellular energy sensor with a key role in metabolic disorders and in cancer

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Abstract

It is essential to life that a balance is maintained between processes that produce ATP and those that consume it. An obvious way to do this would be to have systems that monitor the levels of ATP and ADP, although because of the adenylate kinase reaction (2ADP↔ATP+AMP), AMP is actually a more sensitive indicator of energy stress than ADP. Following the discoveries that glycogen phosphorylase and phosphofructokinase were regulated by AMP and ATP, Daniel Atkinson proposed that all enzymes at branch points between biosynthesis and degradation would be regulated by adenine nucleotides. This turned out to be correct, but what Atkinson did not anticipate was that sensing of nucleotides would, in most cases, be performed not by the metabolic enzymes themselves, but by a signalling protein, AMPK (AMP-activated protein kinase). AMPK occurs in essentially all eukaryotes and consists of heterotrimeric complexes comprising catalytic α subunits and regulatory β and γ subunits, of which the latter carries the nucleotide-binding sites. Once activated by a metabolic stress, it phosphorylates numerous targets that alter enzyme activity and gene expression to initiate corrective responses. In lower eukaryotes, it is critically involved in the responses to starvation for a carbon source. Because of its ability to switch cellular metabolism from anabolic to catabolic mode, AMPK has become a key drug target to combat metabolic disorders associated with overnutrition such as Type 2 diabetes, and some existing anti-diabetic drugs (e.g. metformin) and many 'nutraceuticals' work by activating AMPK, usually via inhibition of mitochondrial ATP production. AMPK activators also potentially have anticancer effects, and there is already evidence that metformin provides protection against the initiation of cancer. Whether AMPK activators can be used to treat existing cancer is less clear, because many tumour cells appear to have been selected for mutations that inactivate the AMPK system. However, if we can identify the various mechanisms by which this occurs, we may be able to find ways of overcoming it.

Key words: AMP-activated protein kinase (AMPK), cancer, cell metabolism, energy balance, mitochondrion, Type 2 diabetes.

Abbreviations used: AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; CaMKK, Ca^{2+}/calmodulin-dependent kinase kinase; CBM, carbohydrate-binding module; GLUT, glucose transporter; HCV, hepatitis C virus; HIF-1α, hypoxia-inducible factor 1α; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LKB1, liver kinase B1; PI3K, phosphoinositide 3-kinase; PPARγ, peroxisome-proliferator-activated receptor γ; PGC-1α, PPARγ co-activator 1α; PP2Cα, protein phosphatase 2Cα; TCA, tricarboxylic acid; 5′-TOP, 5′ terminal oligopyrimidine tract; TOR, target of rapamycin; TORC1, TOR complex 1.

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Introduction

Living cells are molecular machines that require a considerable amount of energy to maintain their highly ordered structure, to move, to grow and divide, and to release molecules into their environment. This energy mainly comes, of course, from conversion of ATP into ADP and phosphate, and a useful analogy can be made between these and the chemicals in a rechargeable battery. In heterotrophic organisms, including the entire animal kingdom, reduced carbon compounds are ingested and oxidized by catabolism to carbon dioxide, with some of the energy released being used to convert ADP and phosphate into ATP, equivalent to charging the battery. In phototrophic organisms, including higher plants, this process can also be driven by photosynthesis. Whatever the mechanism, the high ratio of ATP to ADP so produced (analogous to a fully charged battery) is used to drive most of the energy-requiring reactions in the cell, the few exceptions being those that are driven directly by the electrochemical gradient of protons across the mitochondrial or chloroplast membranes.

It is clearly essential that the production of ATP by catabolism (or photosynthesis) is maintained in precise balance with its consumption by all of the various energy-requiring processes, thus keeping the ratio of ATP to ADP and phosphate high and well away from equilibrium. There is no reason *a priori* that these processes should automatically remain in balance, and the fact that the ATP/ADP ratios in cells tend to be rather constant indicates that there are systems that maintain that balance. The obvious way to do this would be to have systems that respond to changes in energy state by sensing ATP and ADP. An increase in the ADP/ATP ratio would activate catabolism (and/or photosynthesis) while inhibiting energy-consuming processes. This is in fact essentially how it is done, but an interesting twist is that most of these systems respond to AMP as well as, or instead of, ADP. This requires a few words of explanation. All eukaryotic cells express adenylate kinases that catalyse the reversible reaction 2ADP↔ATP+AMP. Interestingly, in *Schizosaccharomyces pombe*, where there is a single isoform of adenylate kinase, it is an essential gene [1]. Since the reaction catalysed merely exchanges one phosphate–phosphate (acid anhydride) bond for another, there is very little change in free energy in the adenylate kinase reaction, and the equilibrium constant is consequently close to 1. If the reaction is at equilibrium (which appears to be approximately the case in most cells), it is very easy to calculate that the AMP/ATP ratio will vary as the square of the ADP/ATP ratio [2]. Thus the AMP/ATP ratio is a more sensitive indicator of energy imbalance than the ADP/ATP ratio. Put another way, the high ATP/ADP ratio maintained by catabolism in a fully energized cell drives the adenylate kinase reaction towards ADP, thus keeping the AMP concentration very low (usually approx. 10-fold lower than ADP and 100-fold lower than ATP). However, if the ADP/ATP ratio rises due to an energy imbalance, the reaction will be displaced towards AMP. Because AMP starts at such a low concentration, even a small increase will represent a large change in concentration.

The first enzyme shown to respond to adenine nucleotides was the muscle isoform of phosphorylase (the enzyme that breaks down glycogen), which was shown as long ago as the 1930s to be activated by AMP, probably the first allosteric effect ever reported [3]. A second example was phosphofructokinase, one of the regulatory enzymes of glycolysis, with the cardiac muscle enzyme being shown in 1963 to be activated by AMP and inhibited by ATP [4]. Extrapolating from these early findings, Daniel Atkinson stated that "similar effects of AMP or ADP on enzymes involved at other branch points between biosynthesis and degradation seem likely" [5] and developed this into his 'energy charge hypothesis' [6]. However, rather few further examples of enzymes regulated by adenine nucleotides were found, and Atkinson's visionary idea became somewhat neglected. What he did not seem to have anticipated was that the function of sensing energy status would mainly be carried out not by the metabolic enzymes themselves, but by a signalling protein, AMPK (AMP-activated protein kinase), which forms the main subject of this review. By phosphorylating downstream targets (which are likely to run into many hundred) when activated by a rise in AMP/ATP, AMPK perfectly fulfils the function proposed by Atkinson, while obviating the need that every protein that is regulated by energy charge should contain binding sites for AMP and ATP. In fact, it is interesting to speculate as to why phosphorylase and phosphofructokinase should respond directly to these nucleotides, rather than simply being regulated by AMPK. In mammals it is primarily the muscle isoforms of these enzymes that are regulated by the nucleotides, and one possibility is that there has been selection for a particularly rapid activation of glycogenolysis and glycolysis in order to meet the huge demand for ATP during a sprinting or weightlifting type of exercise. It seems possible that the use of a protein kinase might introduce an unacceptable delay in this special case. Another point is that activation of phosphorylase is also brought about by phosphorylation by phosphorylase kinase, a protein kinase that is activated by the Ca^{2+} released during muscle contraction. This may normally be more important than its regulation by AMP, which can be regarded as a back-up mechanism. Interestingly, in cells other than skeletal muscle such as cardiac myocytes [7] or monocytes [8], phosphofructokinase is indeed regulated by AMPK, via phosphorylation of the enzyme that produces another allosteric activator, fructose 2,6-bisphosphate.

Early studies on AMPK

The first observations that can be ascribed, with hindsight, to AMPK were made in 1973. Carlson and Kim [9] reported that a crude preparation of rat liver acetyl-CoA carboxylase (involved in fatty acid synthesis) was inactivated in a time-dependent manner in the presence of ATP, whereas David Gibson and colleagues reported similar observations for HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase [10], the key regulatory enzyme of cholesterol synthesis. Both correctly surmised that the inactivation was due to phosphorylation catalysed by a protein kinase that contaminated their preparations. Although the identity of these kinase(s) remained elusive for several years, Kim and colleagues went on to show that acetyl-CoA carboxylase kinase was activated by AMP, and proposed that this was a mechanism by which energy charge regulated fatty acid synthesis [11]. Gibson and colleagues also made observations suggesting that the HMG-CoA reductase kinase was itself activated by phosphorylation by an upstream kinase [12]; this was only the second protein kinase cascade to have been discovered. It was subsequently reported by Hegardt's laboratory that HMG-CoA reductase kinase was also activated by AMP [13], but no-one seemed to connect the observations made with the acetyl-CoA carboxylase and HMG-CoA reductase kinases until 1987 when David Carling et al. [14] in my laboratory provided evidence that they were actually one and the same. Since it soon became clear that both acetyl-CoA carboxylase [15] and HMG-CoA reductase [16] were physiological substrates, it no longer seemed

appropriate to call it after only one of them, and we rather presumptuously changed the name to AMP-activated protein kinase, or AMPK [17].

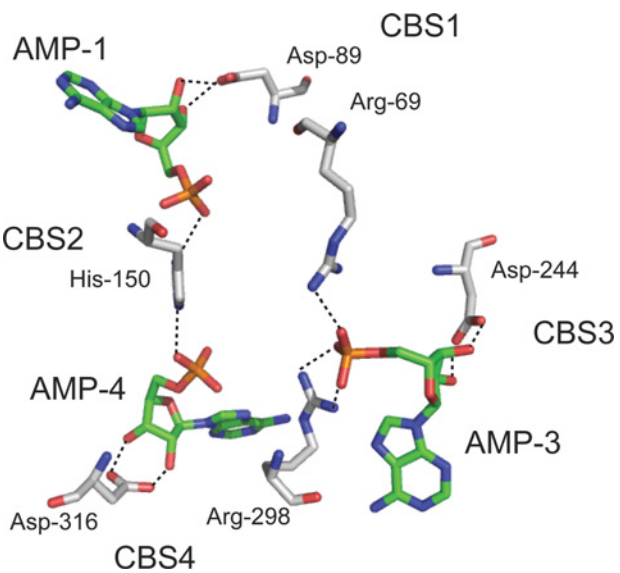
Structure and regulation of AMPK

AMPK is now known to exist as heterotrimers composed of catalytic α subunits and regulatory β and γ subunits [18]. The α subunits contain a typical serine/threonine kinase domain at the N-terminus [19], followed by an autoinhibitory domain that appears to maintain the kinase in an inactive conformation in the absence of AMP [20], and a C-terminal domain that contacts the β and γ subunits [21]. As first realized by Gibson and colleagues [12], AMPK is only active when phosphorylated by an upstream kinase, and we identified Thr^{172} on the α subunit as the site where this phosphorylation occurs [22]. Thr^{172} lies within the activation loop, where many protein kinases have to be phosphorylated to become active [23]. In 1994, my laboratory reported that, in a reconstituted cell-free system, phosphorylation of Thr^{172} by an upstream kinase (at that time not identified) was promoted by AMP [24]. We subsequently showed that AMP also protected against dephosphorylation by protein phosphatases, especially PP2Cα (protein phosphatase 2Cα); this was due to binding of AMP to AMPK rather than the phosphatase, because AMP had no effect on PP2Cα when assayed using other substrates [25]. At that time, we thought that AMP both promoted phosphorylation and inhibited dephosphorylation, although it was subsequently proposed that the apparent effect on phosphorylation might have been due to contamination of the preparation of upstream kinase used with PP2Cα [26]. The two activating effects of AMP (allosteric activation and protection against dephosphorylation) multiply together, producing up to 1000-fold activation overall. Both effects are also antagonized by ATP, allowing AMP to sense the AMP/ATP ratio and act as an energy sensor.

The β subunits of the complex contain a central CBM (carbohydrate-binding module) related to those in other proteins that bind to starch or glycogen, which is responsible for binding the complex to glycogen particles in intact cells [27–29] and *in vivo* [30]. We have reported recently that AMPK is inhibited by glycogen, and may therefore act as a glycogen sensor [31], although the physiological role of this regulation is still being elucidated. The C-terminus of the β subunit forms the core of the heterotrimeric complex and links the α and γ subunits [21]. Finally, the γ subunits contain four tandem repeats of a sequence element called a CBS motif (named after cystathionine β-synthase in which they are found). These always occur in tandem pairs, with one tandem pair being referred to as a Bateman domain [32]. Approx. 20 proteins carrying Bateman domains are found within the human genome, and they are also found in bacteria and archaea [33]. Aside from the AMPKγ subunits, most of these only contain a single Bateman domain comprising two CBS motifs. We found that the two Bateman domains in the AMPKγ subunits bind two molecules of the regulatory

Figure 1 | Representation of AMP-binding sites on the rat γ1 subunit of AMPK

Figure based on PDB code 2V8Q [21]. For clarity, only side chains that are important in nucleotide binding are shown, although the approximate positions of CBS1 to CBS4 are marked. The three molecules of AMP are marked with C atoms in green, N in blue, O in red and P in orange, but for amino acid side chains, C atoms are shown in white. Note that molecules of AMP interact with side chains from more than one CBS motif, e.g. AMP-3 interacts with Asp^{244} from CBS3 (hence its designation as AMP-3), but also with Arg^{69} from CBS1 and Arg^{298} from CBS4.



nucleotides, AMP or ATP, while the single Bateman domains in other proteins also bound adenosine-containing ligands, usually ATP, but in one case *S*-adenosylmethionine [34]. Those that bind ATP, which include IMP dehydrogenases and Cl^- channels, might act as additional energy sensors. Intriguingly, mutations at key residues within these domains in both AMPK and several other proteins cause a variety of hereditary diseases [34]. A crystal structure of the core of the AMPK complex [21] revealed that the two Bateman domains in the γ subunit associate in a pseudosymmetrical 'head-to-head' manner. The adenosine moieties of AMP and ATP bind in the hydrophobic clefts between CBS1 and CBS2, and between CBS3 and CBS4, with the phosphate groups clustered together in the middle (Figure 1). Because of the symmetrical arrangement of the four CBS motifs, there are four sites where nucleotides might bind, but only three are occupied. The 2′- and 3′-hydroxy groups of the ribose rings of the nucleotides form bidentate interactions with conserved aspartate groups from CBS1, CBS3 and CBS4, and the binding sites are correspondingly numbered 1, 3 and 4 (the fourth potential site, number 2, may be unoccupied because in CBS2 the aspartate residue has been replaced by arginine). An interesting feature of the nucleotide-binding sites is that the phosphate groups of the nucleotides in some cases form interactions with basic residues from the other Bateman domain. For example, AMP-3 bound to the Bateman

domain formed by CBS3 and CBS4 interacts with Arg^{69} from CBS1 (Figure 1). This is a subtle feature of nucleotide binding to AMPK that could not be displayed by other proteins that only have a single Bateman domain.

The finding that there were three AMP sites in the crystal structure [21] was a surprise, because previous binding studies had suggested only two, which were assumed to be formed by CBS1 and CBS2, and CBS3 and CBS4 [34]. However, AMP-4 appears to be very tightly bound, is present following bacterial expression and purification even though no AMP had been added, and does not exchange with ATP as does AMP in the other two sites. This explains why it was not observed in the original binding studies [34]. The function of this tightly bound non-exchangeable AMP site remains unclear. It is also not yet clear why there are two other exchangeable sites where AMP binds antagonistically with ATP, although one possibility is that one is responsible for allosteric activation by AMP, and the other for protection against dephosphorylation.

Genes encoding the α, β and γ subunits of AMPK have been found in almost all eukaryotes where genome sequences have been completed, including insects (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*), plants (*Arabidopsis thaliana*), fungi (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) and protozoa (*Giardia lamblia*). One interesting exception is the microsporidian parasite *Encephalitozoon cuniculi*, which has the smallest known eukaryotic genome, encoding only approx. 2000 proteins with an estimated 32 protein kinases (compared with 116 in *S. cerevisiae* and over 500 in humans). *E. cuniculi* lives as an obligate parasite inside mammalian cells and (like *G. lamblia*) lacks metabolically active mitochondria. Its genome does contain genes related to the ATP:ADP translocases of chloroplasts, and it seems likely that it simply takes up ATP made by the host cell [35]. It may therefore be able to exist without AMPK because the host cell already has it. In other non-mammalian eukaryotes, genetic studies have revealed interesting features of the roles of the AMPK orthologues. In *S. cerevisiae*, it is required for most responses to glucose starvation, including the slow growth and the switch from fermentative to oxidative metabolism (the diauxic shift) that occurs when glucose in the medium runs low [36]. In *C. elegans*, it is required for the extension of lifespan and the inhibition of germ cell development that occurs in response to caloric restriction in early life [37,38], suggesting that AMPK may be involved in the effects of caloric restriction in other eukaryotes. In the moss *Physcomitrella patens* (a primitive plant) it is not required for growth in continuous light, but is essential in more physiological alternate light–dark cycles [39]. Since darkness is the equivalent of starvation for a plant, this, taken together with the findings from other eukaryotes, suggests that the ancestral role of AMPK was in the response to starvation for a carbon source.

In mammals, there are two genes encoding the α subunit (*PRKAA1*/*PRKAA2*, encoding $\alpha1$/$\alpha2$), two for the β subunit (*PRKAB1*/*PRKAB2*, encoding $\beta1$/$\beta2$), and three for the γ subunit (*PRKAG1*/*PRKAG2*/*PRKAG3*, encoding $\gamma1$/$\gamma2$/$\gamma3$). These can give rise to at least 12 heterotrimeric combinations, with alternative splicing and/or translation start sites increasing the complexity further. Multiple genes encoding each subunit also occur in plants, although an interesting twist is that plants have unusual '$\beta\gamma$' subunits in which the CBM of β appears to have migrated to the γ subunit, as well as 'truncated' β subunits lacking a CBM [40]. However, they also have more conventional β and γ subunits.

Identification of the upstream kinases

Although preparations of an upstream kinase that phosphorylated Thr^{172} were purified from rat liver in the mid-1990s [22,24], identifying them as defined gene products took much effort. We showed in 1995 that a CaMKK (Ca^{2+}/calmodulin-dependent kinase kinase), which was purified via its ability to phosphorylate and activate CaMKI (Ca^{2+}/calmodulin-dependent kinase I), would also phosphorylate and activate AMPK [41]. At that time, we knew that the kinase kinase we were attempting to purify from rat liver was not Ca^{2+}/calmodulin-dependent, so we were not sure that phosphorylation of AMPK by CaMKK was physiologically relevant. The breakthrough came with various whole-genome screens that identified three kinases [Pak1 (now Sak1), Elm1, and Tos3] which can all act upstream of the *S. cerevisiae* orthologue of AMPK, the SNF1 complex [42–44]. Although there were no obvious orthologues of these kinases in the human genome, the kinases that were closest in sequence within the kinase domain were LKB1 (liver kinase B1), encoded by the *STK11* gene, and the two CaMKKs, CaMKKα and CaMKKβ. The latter finding was satisfying, given our earlier results with a partially purified CaMKK [41], but we were also able to rapidly show that the upstream kinase that we had been unsuccessfully trying to purify from rat liver was in fact a complex between LKB1 and two accessory subunits, STRADα and MO25α [45]. This was intriguing, because LKB1 had been identified previously as a tumour suppressor that is mutated in Peutz–Jeghers syndrome. The latter is an inherited human disorder in which the sufferers (who carry a heterozygous loss-of-function mutation in LKB1) develop numerous benign tumours in the intestine and also carry an increased risk of malignant tumours [46]. Somatic mutations in LKB1 also occur in many non-inherited cancers, including the cervical cancer suffered by Henrietta Lacks, from whom HeLa cells were cultured in 1951 [47]. Our finding that LKB1 was the major upstream kinase for AMPK, made at the same time by others [48,49], provided, for the first time, a link between AMPK and cancer, which is discussed further below.

Part of the evidence that LKB1 was the critical upstream kinase for AMPK came from the use of HeLa cells, in which AMPK was no longer activated by classical metabolic stresses or AMPK activators [45]. However, we and others also realized that some Thr^{172} phosphorylation still occurred in HeLa cells, and were rapidly able to trace this to the CaMKKs, primarily CaMKKβ [50–52]. This provides an alternative pathway in which AMPK is activated by a rise

in cell Ca^{2+} independently of AMP, although, because the effect of AMP is primarily on dephosphorylation, the two stimuli can act synergistically [53]. This Ca^{2+}-mediated mechanism is responsible for activation of AMPK in response to depolarization in neurons [50], activation of the T-cell receptor in T-lymphocytes [54] and activation of endothelial cells by thrombin [55]. Since increases in intracellular Ca^{2+} often trigger energy-requiring processes (such as secretion or contraction), we view the Ca^{2+}-mediated activation of AMPK as a feedforward mechanism to anticipate a demand for ATP before it has happened. Others have shown that the kinase TAK1 [TGF (transforming growth factor)-β-activated kinase 1], usually considered to act upstream in MAPK (mitogen-activated protein kinase) pathways, can also phosphorylate and activate AMPK at Thr^{172} [56]. The physiological role of this remains unclear, although it has been proposed to underlie the activation of AMPK by TRAIL [TNF (tumour necrosis factor)-related apoptosis-inducing ligand] in human epithelial cells derived from breast cancers [57].

Downstream targets of AMPK

With the findings that AMPK phosphorylated not one but two substrates *in vivo* (acetyl-CoA carboxylase and HMG-CoA reductase) [17], there seemed no reason for the number of substrates to stop there. The number of well-characterized targets is now approx. 30, and there seems little doubt that it will eventually run into three figures. A full description of these is beyond the scope of the present review, and the reader is referred elsewhere [18]. However, in line with the discussion in the Introduction, in general, AMPK phosphorylates targets that switch on catabolic pathways while switching off anabolic pathways. In many cases, these effects are mediated by multiple mechanisms and occur via both short- and long-term effects: for example, activation of glucose uptake [58] can occur acutely via translocation of GLUT (glucose transporter) 4 to the membrane [59] or activation of pre-existing GLUT1 at the membrane [60], or in the longer term via increased transcription of the *GLUT4* gene [61]. Similarly, fatty acid oxidation is activated acutely by phosphorylation and inactivation of ACC2, the isoform of acetyl-CoA carboxylase that generates malonyl-CoA at the outer surface of the mitochondrion and thus causes inhibition of fatty acid uptake into mitochondria via the carnitine palmitoyl-CoA transferase I system [58]. However, in the longer term, AMPK activation also increases mitochondrial biogenesis by up-regulating the transcriptional co-activator PGC-1α [PPARγ (peroxisome-proliferator-activated receptor γ) co-activator 1α] [62], which in turn increases expression of mitochondrial genes encoded in the nucleus (including enzymes of fatty acid oxidation) as well as replication of mitochondrial DNA. AMPK has been reported to phosphorylate PGC-1α and it has proposed that this up-regulates its own transcription [63]. Alternatively, AMPK activates the NAD^+-dependent deacetylase, SIRT1, and thus triggers deacetylation of PGC-1α [64].

Another interesting target for AMPK is TORC1 [TOR (target of rapamycin) complex 1], a stimulator of protein synthesis and cell growth that is activated by growth factors and that is hyperactivated in many tumours. AMPK inactivates TORC1 via phosphorylation both of its upstream regulator TSC2 (tuberous sclerosis complex 2) [65], and its regulator subunit raptor [regulatory associated protein of TOR] [66].

Activation of AMPK by metabolic stresses, cytokines, drugs and xenobiotics

The classical stimuli that activate mammalian AMPK are metabolic stresses that either accelerate ATP consumption (e.g. muscle contraction [67]) or that interfere with the catabolic production of ATP (e.g. mitochondrial poisons [68], glucose deprivation [69], hypoxia [7] or ischaemia [70]). AMPK activation is required for the increased glucose uptake that occurs during muscle contraction [71] and may be responsible for many of the health benefits of regular exercise [72], including increasing insulin sensitivity and protection against development of Type 2 diabetes. Although AMPK is activated by glucose deprivation, in most mammalian cells (which express low-K_m isoforms of hexokinase and glucose transporters such as GLUT1 and GLUT4), glucose has to be reduced to very low levels (which would not be tolerated *in vivo*) before AMPK becomes activated. However, in specialized 'glucose-sensing' cells, which express the high-K_m forms glucokinase and GLUT2, AMPK is activated by more physiological variations in glucose. Such cells include pancreatic β-cells, which secrete insulin, and glucose-sensing neurons in the hypothalamus [69,73]. Activation of AMPK in the hypothalamus by hypoglycaemia has the potential to trigger corrective responses, including increased appetite [74,75] and release of adrenaline and glucagon by the adrenal gland and pancreatic α-cells [76], which in turn promotes glucose production by the liver. I find it fascinating that a system that may have evolved to allow single-celled eukaryotes to respond to glucose starvation (see above) still has this function in metazoans, but is now controlling complex physiological responses such as hormone secretion and feeding.

Just as mammals have specialized glucose-sensing cells, they also have specialized oxygen-sensing cells such as the Type 1 cells in the carotid body (which sense oxygen in the blood flow to the brain and trigger corrective changes in breathing) and the smooth muscle in the pulmonary vasculature (which, unlike that in other blood vessels, contracts in response to hypoxia and thus diverts blood flow to better oxygenated areas of the lung). Exactly why ATP production should be more sensitive to hypoxia in these rather than other cells remains unclear, but, with Mark Evans and Chris Peers, we have provided evidence that their responses to hypoxia work in part via activation of AMPK, leading in carotid body Type 1 cells to inactivation of the voltage-gated Ca^{2+}-activated K^+ channel, BK_{Ca}, and consequent secretion of neurotransmitters on to afferent nerves that transmit the hypoxic signal to the brain [77–80].

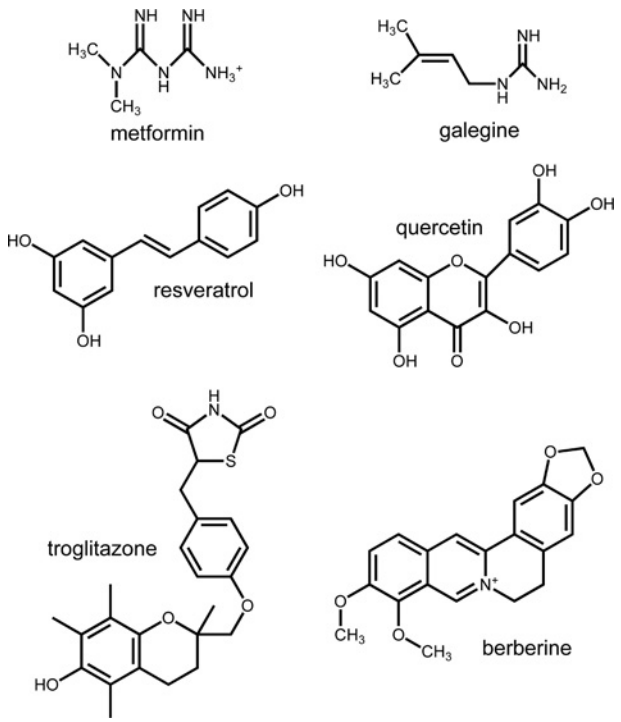
Whereas AMPK evolved in unicellular eukaryotes and can regulate energy balance in a cell-autonomous manner, it is perhaps not surprising that cytokines that arose later to regulate energy balance at the whole-body level have adapted to interact with it. Thus leptin, which is released from adipocytes and whose plasma concentration increases with obesity, activates AMPK in skeletal muscle, accounting for its ability to promote fatty acid oxidation and hence energy expenditure [81]. A more well-known function of leptin is to inhibit food intake via effects on the hypothalamus, thus sending a signal to the brain that fat stores are adequate. Evidence has been reported suggesting that the $\alpha 2$ isoform of AMPK is inhibited by leptin in the hypothalamus, and that this is responsible for its ability to suppress appetite [74], although this was not supported by experiments in which $\alpha 2$ was knocked out in specific hypothalamic neurons [73]. However, there is general agreement that agents that increase food intake when administered directly to the hypothalamus, including AICAR (5-amino-4-imidazolecarboxamide riboside) (see below), ghrelin, cannabinoids and adiponectin, all activate AMPK [75,82,83]. Adiponectin, like leptin, is an adipokine, i.e. a cytokine released by adipocytes. Paradoxically, and in contrast with leptin, its plasma concentration is low in obese individuals and high in lean individuals [84]. Adiponectin, acting via the AdipoRI receptor, activates AMPK by an unknown mechanism, and this is responsible for its ability to stimulate fatty acid oxidation and down-regulate gluconeogenic enzymes in the liver [85,86]. Finally, a number of other cytokines or hormones have been reported to modulate AMPK activity, accounting for diverse effects in different cell types, including IL-6 (interleukin 6) [87], ciliary neurotrophic factor [88,89] and MIF (macrophage-inhibitory factor) [90] and even the vitamin-like agent lipoic acid [91]. In most of these cases, the exact mechanisms by which they activate AMPK, whether through changes in AMP/ATP or some other mechanism, remain unclear.

Type 2 diabetes occurs when insulin resistance is no longer adequately compensated for by increased insulin secretion. Insulin resistance is strongly correlated with excessive storage of triacylglycerols in liver and skeletal muscle, and can be mimicked with cells in culture by incubation in high concentrations of glucose or fatty acids, suggesting that it might be in part a cellular response to excessive nutrient storage. By 1999, the idea that AMPK was a 'metabolic master switch' that transformed cells from anabolic mode (in which they would store nutrients) to catabolic mode (in which they would oxidize them instead) was well established. This led us to predict that drugs that activated AMPK might be useful for treatment of insulin resistance and Type 2 diabetes [92]. In fulfilment of this, it was almost immediately shown that the nucleoside AICAR, which is taken up into cells and converted into ZMP (AICAR monophosphate), an analogue of AMP that activates AMPK [93], would reverse the metabolic abnormalities in animal models of insulin resistance such as the *ob*/*ob* mouse [94], the *fa*/*fa* rat [95,96], and the fat-fed rat [97]. More surprisingly, it was shown that two of the major classes of drugs already in use for treatment of Type 2 diabetes, i.e. metformin and the thiazolidinediones, activated AMPK in intact cells and *in vivo* [98–100]. Metformin is currently the first choice drug for treatment of Type 2 diabetes, and is prescribed to >100 million people worldwide. In mice in which LKB1 is knocked down specifically in the liver, metformin is no longer able to activate AMPK [101]. These mice have much higher plasma glucose than wild-type mice (even when the latter are fed on a high-fat diet) and the glucose-lowering effects of metformin seen in the fat-fed wild-type mice are lost [101]. This provides strong evidence that AMPK is the therapeutic target for metformin. The thiazolidinediones activate AMPK in intact cells at relatively high concentrations [99,102], which is due to their ability to inhibit mitochondrial function (see below). However, they also have a more direct target, i.e. the adipocyte transcription factor PPARγ [103]. Studies with adiponectin-deficient mice suggest that a major part of the glucose-lowering action of thiazolidinediones comes from their ability to promote release of adiponectin from adipocytes [104], and the ultimate target of adiponectin is, of course, AMPK.

A rapidly developing area is the regulation of AMPK by 'nutraceuticals' (natural products present in foods that are claimed to have health benefits) as well as by phytochemicals used in traditional medicines. Thus AMPK is activated by resveratrol from red wine, epigallocatechin gallate from green tea, capsaicin from peppers, the widespread phytochemicals genistein and quercetin, and berberine, a yellow dye extracted from plants of the genus *Berberis* and from Chinese goldthread (*Coptis chinensis*), which has been used in Chinese medicine for centuries [105–108].

A puzzling question was how such a wide variety of drugs and xenobiotics, with very varied structures (Figure 2), could all cause AMPK activation. We noticed that some of them, including metformin [109,110], thiazolidinediones [111] and berberine [112], had been reported to be inhibitors of the respiratory chain, whereas others, such as resveratrol, were reported to be inhibitors of the mitochondrial ATP synthase [113]. This suggested the hypothesis that these compounds inhibited mitochondrial ATP production and therefore activated AMPK by increasing cellular AMP/ATP. To test this, we made use of mutations in the $\gamma 2$ subunit of AMPK that has been shown to cause heart disease by interfering with the binding of AMP [34]. One of these mutations, R531G, affects the residue equivalent to Arg^{298} in $\gamma 1$ (Figure 1), and completely abolishes activation by AMP. We introduced into HEK (human embryonic kidney)-293 cells, by homologous recombination at a previously inserted Flp recombinase target site, DNAs encoding either wild-type $\gamma 2$ or the R531G mutant, generating isogenic cell lines in which the wild-type or mutant had replaced the endogenous $\gamma 1$ subunit [114]. In these cells, most of the AMPK-activating compounds tested activated the wild-type AMPK, but not the R531G mutant. These included metformin and a related biguanide, phenformin, galegine [the natural product from French lilac (*Galega officinalis*),

Figure 2 | **Structures of phytochemicals and drugs that activate AMPK by inhibiting mitochondrial ATP production**



from which metformin was originally derived], the thiazolidinedione troglitazone, resveratrol, quercetin and berberine. Other compounds did activate the R531G mutant, including A23187 and A-769662. This makes sense, because A23187 is an ionophore that increases intracellular Ca^{2+} and activates AMPK via the CaMKK pathway [50], whereas A-769662 is a novel activator developed by Abbott Laboratories [115], which binds directly to AMPK at a site distinct from the AMP site [116,117]. These positive controls show that the R531G mutant was fully functional, apart from its AMP-insensitivity. We also showed by measuring cellular oxygen uptake that all of the compounds that failed to activate the R531G mutant also inhibited mitochondrial function in the intact cells [114]. Thus AMPK is activated by many xenobiotic compounds because they are inhibitors of mitochondrial function. This raises another question: why should so many phytochemicals inhibit mitochondria? Most of them are products of secondary metabolism, and it is interesting to speculate that plants make them to deter grazing by herbivores and/or attack by pathogenic bacteria or fungi. Consistent with this, *G. officinalis*, which produces galegine (a potent AMPK activator [118] from which metformin was derived) has as one of its common names goat's rue, presumably because goats regret eating it! It is classed as a noxious weed in the U.S.A. because it is toxic to herbivores. Also, the resveratrol content of red wines varies because it is produced in response to fungal infection and has antifungal effects [119].

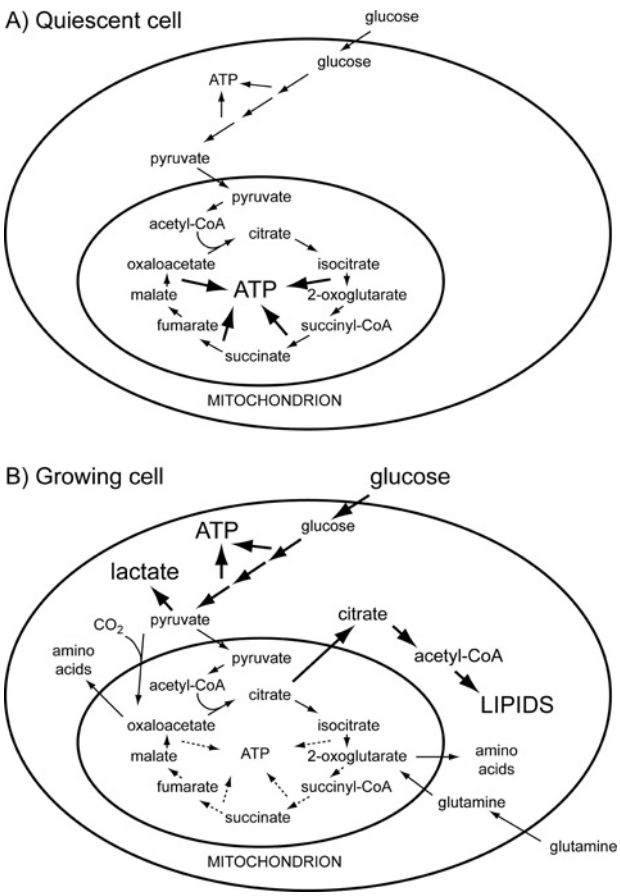
AMPK and cancer

Our finding that the tumour suppressor LKB1 was the key upstream kinase for AMPK introduced the possibility that AMPK might exert the tumour-suppressor actions of LKB1 and provided a link between AMPK and cancer. Although LKB1 also acts upstream of a small family of AMPK-related kinases [120], AMPK is the only member of this family that is known to inhibit cellular biosynthesis and growth (in part by inhibiting TORC1, see above) and to cause a cell-cycle arrest in tumour cells [121]. AMPK can also potentially reverse the changes in metabolism that occur in most tumour cells. In quiescent cells, the TCA (tricarboxylic acid) cycle can be regarded as a catabolic pathway that generates most of the ATP, with the amount of ATP being generated per molecule of glucose via oxidative phosphorylation being almost 20 times greater than that produced by glycolysis alone. However, in rapidly growing cells the TCA cycle becomes in part an anabolic pathway, providing precursors for biosynthesis, especially citrate for lipid synthesis and 2-oxoglutarate and oxaloacetate for amino acid synthesis (Figure 3). These TCA cycle intermediates must be replenished by anaplerotic pathways, such as the conversion of pyruvate into oxaloacetate by pyruvate carboxylase, and glutaminolysis (which is also a source of amine groups, and is why cultured cells are usually grown in high glutamine). This use of the TCA cycle as an anabolic rather than a purely catabolic pathway may mean that it can generate less ATP and, to compensate for this, glucose uptake and glycolytic breakdown of glucose to lactate must increase. This is the famous Warburg effect, an increased reliance on glycolysis in tumour cells first observed by Otto Warburg in the 1920s [122]. The need for rapid glucose uptake may also explain why cultured mammalian cells, mostly derived from tumours, are generally grown in a high non-physiological glucose concentration (25 mM, as in Eagle's medium).

By up-regulating mitochondrial biogenesis [62] and TCA cycle enzymes such as succinate dehydrogenase [123] (which, intriguingly, can be a tumour suppressor [124]), AMPK can potentially reverse the Warburg effect. In *S. cerevisiae*, the diauxic shift caused by exhaustion of glucose in the medium (see above), in which growth slows and the organism switches from fermentative to oxidative metabolism, is equivalent to a reversal of the Warburg effect, and, interestingly, the AMPK orthologue is required for this switch [36]. In addition, it now appears that activation of TORC1, for example owing to oncogenic mutations that activate the Ras/Raf/ERK (extracellular-signal-regulated kinase) or the PI3K (phosphoinositide 3-kinase)/Akt pathways, is also important in the Warburg effect. TORC1 specifically increases the translation of mRNAs containing so-called 5′-TOP (5′ terminal oligopyrimidine tract) sequences. These mRNAs encode mainly ribosomal proteins and elongation factors, but also include the mRNA encoding HIF-1α (hypoxia-inducible factor 1α). HIF-1α expression is induced in hypoxic cells owing to decreased protein degradation, and it promotes transcription of glycolytic enzymes and GLUT1, thus allowing increased ATP production by glycolysis during

Figure 3 | Simplified comparison of energy metabolism in quiescent and in rapidly growing cells

In a quiescent cell (**A**), the TCA cycle acts as a catabolic pathway and generates most of the ATP. In a rapidly growing cell (**B**), the TCA cycle has adopted a more anabolic role, providing precursors for biosynthesis of lipids and proteins. TCA cycle intermediates must be replenished by anaplerotic reactions, such as the conversion of pyruvate into oxaloacetate by pyruvate carboxylase, and glutaminolysis. The TCA cycle may be less capable of generating ATP, so glucose uptake and glycolysis are up-regulated, generating lactate (the Warburg effect).



hypoxia. However, in many tumour cells, translation of HIF-1α mRNA, because of its 5′-TOP sequence, is increased by hyperactivated TORC1 even under normoxic conditions [125], accounting in part for the Warburg effect. Since AMPK inhibits TORC1 [65,66], it has the potential to reverse this.

The findings that LKB1 acted upstream of AMPK [45] and that AMPK was the probable target for metformin [98] led a colleague, Andrew Morris, to examine whether patients with Type 2 diabetes who are treated with metformin have a different incidence of cancer from those treated with other medications. Intriguingly, cancer incidence in those on metformin was approx. one-third lower, a finding that has been reproduced in at least seven subsequent epidemiological studies [126–134]. Although these are all retrospective studies and do not prove a causal relationship, metformin has been shown to delay onset of tumours in mouse models [135,136], and, in one of these studies, the same effect was seen with A-769662 [136]. Since metformin and A-769662 activate AMPK by different mechanisms, it seems unlikely that their ability to delay tumour formation would both be via 'off-target', i.e. AMPK-independent, effects.

These results suggest that AMPK activation provides protection against the onset of cancer, but could AMPK activators also be used to treat pre-existing cancer? Large-scale trials of metformin as an adjuvant therapy (i.e. following surgery and other standard therapies) in breast cancer are now under way. However, one caveat is that there are increasing indications that AMPK activation may be down-regulated in many cancers. My colleague Alastair Thompson examined 350 breast tumours by immunohistochemistry using a phosphospecific antibody that recognizes the active form of AMPK phosphorylated on Thr^{172}, and found that the staining was reduced, compared with normal epithelium in the same biopsy, in 90% [137]. This correlated with reduced staining using an antibody recognizing acetyl-CoA carboxylase phosphorylated at the AMPK site, an excellent marker for AMPK activation. Moreover, the tumours that had reduced AMPK were also those where the prognostic indicators were the poorest, suggesting that loss of AMPK activation makes tumours more aggressive.

Although we do not know the cause of loss of AMPK activation in these cases of breast cancer, in other cancers it may be caused by mutations in the LKB1 gene, *STK11*. Loss-of-function mutations in *STK11* are estimated to occur in ~30% of non-small-cell lung cancers [138,139] and ~20% of cervical cancers [47]. According to the COSMIC (Catalogue of Somatic Mutations in Cancer) database (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic), LKB1 mutations are quite frequent in cervical (14%), skin (11%) and lung cancers (10%), although rare in breast cancers. It has been pointed out that these may be underestimates because conventional methods for detecting mutations may miss large deletions [47]. Mice in which LKB1 is specifically knocked out in skin [140], prostate [141] and endometrium [142] develop highly invasive tumours, and (although anecdotal) the cervical cancer that killed Henrietta Lacks in 1951 (which carried a large deletion in *STK11* [47]) caused a peculiarly aggressive form of the disease [143].

Given that AMPK has the potential to cause a cell-cycle arrest and to reverse the metabolic changes that occur in tumour cells, it seems almost inevitable that there should be an intense selection pressure for tumours to lose the function of AMPK. As well as loss of LKB1, there may be other mechanisms by which AMPK activation is down-regulated. It has been reported that in melanoma cells that carry the activating V600E in B-Raf (which account for about half of all malignant melanomas in humans), LKB1 is phosphorylated at two sites in the C-terminus, and that this may reduce its ability to activate AMPK [144]. In many tumours, the PI3K/Akt pathway is hyperactivated [e.g. due to loss of PTEN (phosphatase and tensin homologue deleted on chromosome 10)], and Akt (also known as protein kinase B)

has been found to phosphorylate the α1 subunit of AMPK at Ser^{485}. This has been proposed to inhibit phosphorylation at Thr^{172} by LKB1 [145]. Although it remains unclear whether this occurs in tumours, it does represent another potential mechanism via which AMPK activation could be reduced.

In some ways, viral infection of cells bears similarities to tumour formation. In both cases, the normal function of the cell has been taken over by an interloper (a virus or a mutation) that converts a quiescent cell into one with a high biosynthetic capacity. Given that virus replication requires energy, one might imagine that it would trigger AMPK activation, which would then oppose the switch to rapid biosynthesis. Mark Harris at the University of Leeds has shown recently that, as in many tumour cells, AMPK activation is down-regulated in human hepatocytes infected with HCV (hepatitis C virus) [146]. HCV is an RNA virus that makes a polyprotein that is cleaved into several structural and non-structural proteins, and also has a lipid envelope. It may have to switch off AMPK in order to prevent inhibition of protein and lipid biosynthesis, and it appears to do this by activating Akt and phosphorylating Ser^{485} on AMPK-α1. Consistent with this hypothesis, overexpression of an S485A mutant in human hepatocyes greatly reduced viral replication. Another exciting finding was that AMPK activators such as metformin, AICAR and A-768662 could overcome the down-regulation of AMPK and thus inhibit viral replication [146]. This suggests that metformin might represent a new treatment for HCV infection, and it seems possible that this might extend to other viruses as well. Another point is that chronic HCV infection greatly increases the risk of fatty liver disease and also liver cancer. Reduced AMPK activation could help to account for both of these.

One of my own major research interests at the moment concerns these mechanisms by which cancer mutations and viruses down-regulate AMPK activation. It seems to me that if we can understand how this happens, we may be able to develop ways to overcome it. The ability of metformin to overcome inhibition of AMPK by HCV is a case in point, and metformin would be predicted to be effective in those tumours in which AMPK is down-regulated due to phosphorylation at Ser^{485} by Akt. As another example of this kind of approach, we have recently been studying tumour cell lines derived from cervical cancers (HeLa), lung cancers and melanomas that lack LKB1, and have shown that activating AMPK via the Ca^{2+}/CaMKK pathway (which still exists in these cells) can cause a cell-cycle arrest. Although the AMPK field is clearly maturing, exciting opportunities for exploiting our increased insights into it are beginning to appear.

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