AMP-activated protein kinase: the current landscape for drug development

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Abstract | Since the discovery of AMP-activated protein kinase (AMPK) as a central regulator of energy homeostasis, many exciting insights into its structure, regulation and physiological roles have been revealed. While exercise, caloric restriction, metformin and many natural products increase AMPK activity and exert a multitude of health benefits, developing direct activators of AMPK to elicit beneficial effects has been challenging. However, in recent years, direct AMPK activators have been identified and tested in preclinical models, and a small number have entered clinical trials. Despite these advances, which disease(s) represent the best indications for therapeutic AMPK activation and the long-term safety of such approaches remain to be established.

Cardiovascular disease
(CVD). A term encompassing diseases affecting the heart or circulatory system.

Non-alcoholic fatty liver disease
(NAFLD). A very common disease in humans in which there is an excessive accumulation of fat in the liver (steatosis) in individuals who are not alcoholic.

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Dramatic improvements in health care coupled with an increased standard of living, including better nutrition and education, have led to a remarkable increase in human lifespan[1]. Importantly, the number of years spent in good health is also increasing[2]. Despite these positive developments, there are substantial risks that challenge continued improvements in human health. Perhaps the greatest threat to future health is a chronic energy imbalance in which intake exceeds expenditure[3]. This energy imbalance contributes to a global epidemic of overweight and obesity[4] that accelerates ageing and also increases the risk of developing type 2 diabetes, cardiovascular disease (CVD), non-alcoholic fatty liver disease (NAFLD), chronic kidney disease and certain cancers[5]. And although lifestyle interventions that target this energy imbalance through caloric restriction and/or endurance exercise can reduce ageing, obesity and related diseases, implementing and maintaining these changes for prolonged periods is challenging given the pervasiveness of calorically dense foods and sedentary lifestyle-enabling technologies. Therefore, developing new pharmacological strategies that mimic a low energy state, such as that elicited by exercise and caloric restriction, could be potentially beneficial for treating chronic metabolic diseases.

At the cellular level, the balance between energy intake and demand can be inferred by the relative levels of the adenine nucleotides AMP, ADP and ATP that are continuously produced and consumed through numerous metabolic reactions[6]. Given their central role as a measure of cellular energy balance, a number of enzymes were known for many years to be regulated by alterations in adenine nucleotides[7]. However, a pivotal step in identifying a unifying mechanism that could link these changes to multiple branches of metabolism followed the discovery that the AMP-activated protein kinase (AMPK) provided a common regulatory mechanism for inhibiting both cholesterol (through phosphorylation of HMG-CoA reductase (HMGR)) and fatty acid (through phosphorylation of acetyl-CoA carboxylase (ACC)) synthesis[8] (BOX 1).

Over the last 30 years since formally naming AMPK[9], interest in the enzyme as a drug target has continued to grow. This interest has been stimulated in part by the discovery that AMPK is activated by physiological regulators that are associated with health and longevity such as caloric restriction[10] and exercise[11], hormones such as leptin[12] and adiponectin[13] and many natural products that have been used as traditional herbal medicines (reviewed in REF.[14]). Many of these herbal medicines such as resveratrol[15] and berberine[15–17] are being reformulated to enhance their bioavailability and efficacy. Two of the most widely used medicines in the world, metformin[18], the mostly commonly used drug for type 2 diabetes, and salicylate[19], the active ingredient in aspirin and salsalate, also activate AMPK, suggesting that some of their beneficial effects may be mediated in part by AMPK. The sodium/glucose cotransporter 2 (SGLT2) inhibitor canagliflozin, which has recently been approved for treating type 2 diabetes, also indirectly activates AMPK in a manner similar to metformin[20,21]. More recently, a number of small molecules have been identified that directly activate AMPK. Most of these compounds bind in a specific pocket formed by an interaction between the α and β subunits[22], termed the allosteric drug and

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metabolite (ADaM) site[23], and studies demonstrating preclinical and clinical efficacy of these activators are beginning to emerge[24–27]. For example, O304 has recently been shown to reduce fasting plasma glucose and blood pressure in individuals with type 2 diabetes taking metformin[28]. However, the most appropriate disease areas to be targeted and potential adverse effects of AMPK activation are still being clarified. Here, we assess the current state of play regarding the therapeutic potential of AMPK across distinct areas including metabolic diseases, cancer, neuromuscular disease, chronic kidney disease, pain and ageing, highlighting key drug development challenges.

Structure of AMPK

Mammalian AMPK is a heterotrimeric complex composed of three subunits: an α subunit (encoded by protein kinase AMP-activated-α (*PRKAA*)) harbouring a protein kinase catalytic domain and non-catalytic β (PRKAB) and γ (PRKAG) regulatory subunits. There are two isoforms of the α and β subunits and three isoforms of the γ subunit, giving rise to twelve possible combinations of the heterotrimeric αβγ AMPK complex.

The amino-terminal region of the α subunit contains a typical serine/threonine kinase domain. Within the kinase domain is a region known as the activation loop, or T-loop, which is conserved in many protein kinases and plays a pivotal role in their regulation. In AMPK, phosphorylation of Thr172 (pThr172) within the activation loop is required for maximal activity of AMPK[29,30]. Two upstream kinases account for the physiological phosphorylation of Thr172: liver kinase B1 (LKB1)[31–33] and calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2; sometimes referred to as CAMKKβ)[34–36]. Numerous studies have shown that Thr172 phosphorylation increases AMPK activity both in vitro and in vivo, and regulation of Thr172 phosphorylation is a central component of the mechanism of activation of AMPK by small molecules (see below). The kinase domain is followed by a flexible region, termed the α-linker[37]. Within the α-linker is a region of ~60 amino acids that has been named the auto-inhibitory domain (AID), so named because addition of this sequence to the isolated kinase domain reduces AMPK activity by approximately tenfold[38–40]. Closer to the carboxy-terminus than the AID is a short sequence that has been termed the α-hook[37], or α-regulatory subunit interacting motif 2 (α-RIM2)[41,42], which plays an important role in the nucleotide-dependent regulation of AMPK. Another region within the α subunit that may play a regulatory role is the serine/threonine-rich loop[43]. This region is not conserved in all eukaryotic species and so might represent a somewhat late evolutionary adaptation[43].

The β subunit isoforms undergo constitutive amino-terminal myristoylation, and this modification is required for AMP-stimulated and ADP-stimulated phosphorylation of Thr172 (REFS[44,45]). Within the β subunit is a region termed the carbohydrate binding module (CBM; sometimes referred to as the glycogen-binding domain), which shares primary sequence identity with sequences found in a number of proteins that metabolize glycogen or starch[46]. The CBM forms part of the binding pocket for small-molecule AMPK activators, underlining its significance as a key determinant of AMPK regulation. The carboxy-terminal region of the β subunit interacts with the α and γ subunits, and these interactions act as a scaffold for the formation of the heterotrimeric AMPK complex[47].

The γ subunit isoforms share highly conserved carboxy-terminal regions that harbour four cystathionine-β-synthase (CBS) domains of ~60 amino acids[48]. The γ2 and γ3 isoforms contain amino-terminal extensions that are not present in the γ1 isoform; there is no obvious sequence conservation between γ2 and γ3 or significant homology with other proteins[49]. In addition, variant forms of γ2 and γ3 transcripts have been identified that encode predicted proteins with truncated amino-terminal regions[50,51]. Recent studies demonstrate that the γ isoforms confer differential regulation of AMPK to both nucleotides and small-molecule activators[52–54]. To date, the only crystal structures available for AMPK are for γ1-containing complexes, and further work is required to elucidate the precise roles of the amino-terminal regions of γ2 and γ3 on AMPK function. Nonetheless, the finding that these regions affect the regulation of AMPK suggests that different γ isoform complexes could be differentially targeted by drugs.

Cystathionine-β-synthase (CBS) domains

Small protein domains (typically ~60 amino acids) that usually occur as tandem repeats, sometimes referred to as a Bateman domain, and that often bind to nucleotide or nucleotide-like molecules. AMP-activated protein kinase γ subunits contain four CBS domains, three of which bind adenine nucleotides.
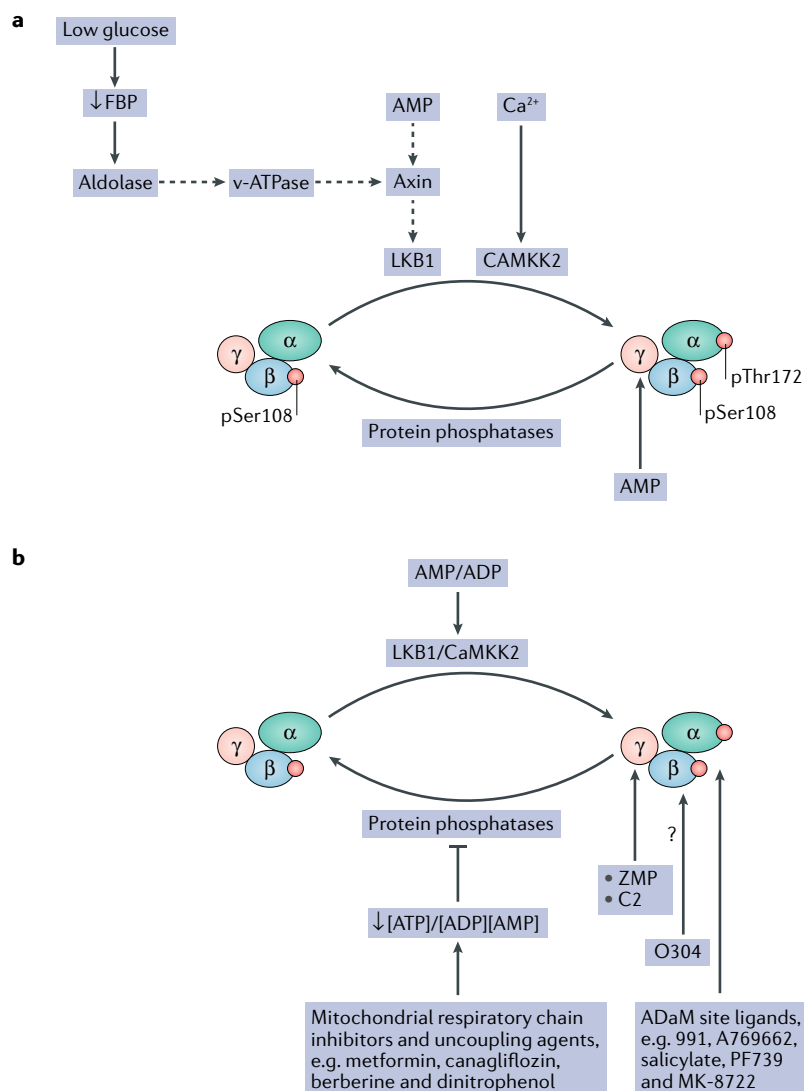
Fig. 1 | **Physiological and pharmacological AMPK regulation. a** | AMP-activated protein kinase (AMPK) is activated by phosphorylation of Thr172 (pThr172) within the activation loop of the α subunit, which is catalysed by liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2). An increase in intracellular calcium activates CAMKK2, whereas AMP increases phosphorylation by LKB1 by a mechanism involving axin. Low glucose levels can lead to an increase in Thr172 phosphorylation by LKB1 via a complex signalling pathway involving a decrease in fructose 1,6-bisphosphate (FBP) levels and the interaction between aldolase, vacuolar ATPase (v-ATPase) and axin. The molecular details of the interaction between these proteins remain unclear, and this is represented by the broken arrows. Binding of AMP to the γ subunit directly activates AMPK through an allosteric mechanism. **b** | AMPK activity can be modulated pharmacologically by compounds that deplete ATP. One mechanism by which this can occur is through inhibition of mitochondrial respiration, for example, complex I inhibition by metformin, canagliflozin, berberine and dinitrophenol. Compounds that bind to the γ subunit (5-aminoimidazole-4-carboxamide ribonucleotide (ZMP) and 5-(5-hydroxyl-isoxazol-3-yl)-furan-2-phosphonic acid (C2)) allosterically activate AMPK and protect against pThr172 dephosphorylation. A fall in intracellular ATP levels leads to a rise in ADP and AMP, decreasing the ATP:ADP/AMP ratio, and this protects against dephosphorylation of pThr172. In addition, AMP and ADP have been reported to increase phosphorylation of AMPK by the upstream kinases LKB1 and CAMKK2. Another class of activators (for example, 991, A769662, PF739 and MK-8722) bind at the allosteric drug and metabolite (ADaM) site, leading to allosteric activation and protection against dephosphorylation. Phosphorylation of Ser108 (pSer108) in the β1 subunit increases the affinity of binding of these ligands. Compound 0304 activates AMPK by protecting against dephosphorylation, but the binding site has not yet been determined.

The existence of multiple isoforms of AMPK sub-units has been known for many years[55], but the biological relevance of the different isoforms remains only partially understood. One area where there appears to be a substantial difference between the isoforms is in their relative tissue distribution. An important point to note here is the possibility of differences in AMPK isoform expression between species. Most of the in vivo studies on mammalian AMPK have used the mouse as the experimental model. However, there are significant differences in the expression of AMPK isoforms between mice and humans, which has important implications for extrapolating data between species. In rodent liver, α1 and α2 are expressed to similar levels, while β1 and γ1 are the major regulatory subunit isoforms, meaning that rodent liver expresses predominantly a mixture of α1β1γ1 and α2β1γ1 complexes[56,57]. However, in human liver, α1 is the predominant catalytic isoform, and β2 the major β isoform, such that α1β2γ1 is the predominantly expressed AMPK complex[57,58]. Similarly, the γ2 isoform is highly expressed in human heart, whereas the γ1 isoform appears to be the predominant γ subunit in rodent heart[57,59].

Regulation of AMPK activity

The activity of AMPK is tightly regulated in response to various hormonal and metabolic signals, involving several different mechanisms (FIG. 1). AMPK has been dubbed the fuel gauge of the cell, or nature's energy sensor[60,61], as it is activated in response to an increase in intracellular AMP and ADP levels that occurs in response to a fall in ATP, the immediate energy source for living cells. A consensus view for the regulation of AMPK by adenine nucleotides has now emerged, based in part on structural information identifying the nucleotide binding sites within AMPK. Binding of AMP, but not ADP, allosterically activates AMPK up to tenfold[29,37,53], while AMP and ADP increase phosphorylation of Thr172 and protect against dephosphorylation[29,37,53]. The effects of AMP and ADP are antagonized by ATP, such that regulation is dependent on the AMP/ADP:ATP ratio[62,63], and the isoform composition of AMPK, particularly that of the γ isoform, effects nucleotide-dependent regulation[52,53]. The crystal structure of the regulatory core of AMPK containing full-length γ1 revealed that three of the four CBS domains bind nucleotides[47]. CBS2 (also referred to as site 2 (REF.[64])) does not bind nucleotides. CBS4 (site 4) contains an AMP molecule that is permanently bound under physiological conditions[47], although AMP binding can be disrupted under non-physiological conditions[65]. The other two sites (CBS1 and CBS3) bind adenine nucleotides in an exchangeable manner, providing the basis for nucleotide sensing by AMPK[47]. Mutations in the γ isoforms can result in a constitutively active AMPK, and their physiological function has been explored using genetic models (BOX 2).

AMPK is also activated by an increase in intracellular calcium ions, which is mediated by CAMKK2 (REFS[34–36]). Activation by calcium appears to play an important role in regulating AMPK in some tissues in response to hormones, such as VEGF-induced activation of muscarinic receptors in endothelial cells[66–68] or ghrelin acting on

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the ghrelin receptor in neuronal cells[66,69]. An exciting recent development is the finding that fructose 1,6-bisphosphate (FBP), a glycolytic intermediate, mediates glucose sensing by AMPK[70]. Acute glucose starvation activates AMPK by a mechanism that involves decreased binding of FBP to aldolase. This promotes the interaction between AMPK and LKB1 via binding to axin on the lysosome, leading to phosphorylation and activation of AMPK independently of changes in adenine nucleotides[70]. This model is discussed in depth in a recent review[71].

Physiological functions of AMPK

AMPK activation in response to alterations in adenine nucleotides, calcium and substrate availability as detailed above leads to the phosphorylation of over 100 distinct proteins across a diverse array of metabolic pathways (FIG. 2). The optimal consensus motif of AMPK substrates has been established (BOX 3). As detailed below, the phosphorylation of numerous key metabolic proteins influences lipid, cholesterol, carbohydrate and amino acid metabolism as well as mitochondrial function, autophagy and cell growth. AMPK exerts these effects acutely through covalent modifications, which can influence metabolic activity, and chronically through phosphorylation of key transcriptional programmes, altering substrate utilization and availability and ultimately restoring cellular and whole organismal homeostasis.

Box 2 | Genetic models of AMPK activation

The vast majority of genetic studies exploring the role of AMP-activated protein kinase (AMPK) in vivo have relied on transgenic knockout mouse models. While these studies reveal substantial insights into the physiological role of AMPK, they do not provide direct information regarding the effects of AMPK activation. The first naturally occurring gain-of-function mutation to be identified was in the γ3 subunit in Hampshire pigs, which leads to a dramatic accumulation of glycogen in skeletal muscle[338]. In humans, naturally occurring mutations in the γ2 subunit cause severe cardiac abnormalities, including left ventricular hypertrophy, glycogen accumulation and ventricular pre-excitation (Wolff–Parkinson–White syndrome), often leading to sudden cardiac death or heart disease[339]. This difference in expression could account for the finding that knock-in mouse models of γ2 harbouring disease-causing mutations, while recapitulating earlier findings with human γ2 expression with respect to cardiac hypertrophy, do not lead to glycogen accumulation as observed with Wolf–Parkinson–White syndrome[191,195]. This was surprising, as previous studies in which human γ2 was overexpressed in mouse heart revealed that the human γ2 mutations produced a phenotype that was remarkably similar to the human disease[340]. These findings might be reconciled by differences in γ2 isoform expression in mouse versus human heart. In γ2 and γ3, these mutations occur in the cystathionine-β-synthase (CBS) domains, suggesting that the gain-of-function phenotype results from a disturbance in nucleotide sensing. Several subsequent studies have utilized genetic mouse models based on these mutations to investigate the effect of chronic AMPK activation in vivo, and these have confirmed that AMPK activation in skeletal muscle leads to increased glycogen accumulation[121,239,341]. A limitation of these models is posed by the restricted tissue distribution of the γ2 and γ3 isoforms. A more recent mouse model has been reported using a gain-of-function mutation in the γ1 subunit[213]. The γ1 isoform is widely expressed and accounts for the predominant γ isoform in most mammalian tissues, overcoming the limitations posed by the restricted expression of the other γ isoforms. Liver-specific expression of the γ1 gain-of-function mutant completely protected against hepatic steatosis in mice fed a high-fructose diet by decreasing de novo lipogenesis. This finding may have substantial implications for the therapeutic targeting of AMPK in the liver as a treatment for non-alcoholic fatty liver disease and its subsequent associated complications, such as non-alcoholic steatohepatitis.

Regulation of lipid metabolism

AMPK reduces lipid storage through phosphorylation of multiple substrates across distinct pathways that collectively act to promote fatty acid oxidation while suppressing fatty acid and cholesterol synthesis. The synthesis of both cholesterol and fatty acids is dependent on the same key intracellular metabolite, acetyl-CoA. With respect to cholesterol synthesis, acetyl-CoA becomes committed to the mevalonate pathway via a series of condensation reactions followed by reduction to mevalonic acid by HMGR[72]. AMPK inhibits HMGR and the synthesis of cholesterol through phosphorylation at Ser872 both in vitro[8] and in the liver of mice, leading to lower serum and liver cholesterol[73]. Acetyl-CoA is also converted to malonyl-CoA, the first committed step in fatty acid synthesis, by ACC, which exists as two distinct isoforms: ACC1, which is predominately expressed in lipogenic tissues such as liver and adipose tissue, and ACC2, which is more common in heart and skeletal muscle. The inhibitory effects of AMPK on fatty acid synthesis require phosphorylation of ACC1 and ACC2, as mice lacking these phosphorylation sites have elevated ACC activity, malonyl-CoA and fatty acid synthesis and are also insensitive to AMPK activators[25,74,75].

In addition to the acute regulation of fatty acid and cholesterol synthesis through phosphorylation of HMGR and ACC, AMPK may also play a role in inhibiting this pathway through repression of transcriptional programmes. Lipid synthesis is largely governed by the sterol-response element-binding proteins (SREBPs), which are synthesized as precursor forms that reside in the endoplasmic reticulum (ER) and must undergo subsequent processing before translocating to the nucleus. Although cholesterol and fatty acids are both synthesized from a common substrate (acetyl-CoA), their biosynthetic pathways are largely regulated by distinct SREBPs, with cholesterol metabolism (for example, HMGR and low-density lipoprotein (LDL) receptor) being regulated by the activity of SREBP2 and fatty acid metabolism (ATP-citrate lyase (ACLY), ACC and fatty acid synthase (FASN)) being regulated by SREBP1A and SREBP1C[76]. While the activity of the SREBP proteins is primarily regulated through intracellular concentrations of unsaturated fatty acids and cholesterol[76], AMPK phosphorylation of Ser372 and Ser374 on SREBP1C and SREBP2, respectively, may also inhibit their activity by preventing proteolytic processing and thus transcriptional activity[45]. However, the relative importance of AMPK phosphorylation of SREBP1C and SREBP2 for controlling fatty acid and cholesterol synthesis has not yet been established in vivo. The inhibitory effects of AMPK on SREBP may also occur through indirect mechanisms involving the regulation of p53, sirtuins and forkhead box protein (FOXO)[77,78]. In addition to SREBPs, the suppression of the lipogenic programme by AMPK can also occur through inhibition and phosphorylation of the carbohydrate-response element-binding protein (ChREBP) at Ser568 (REF.[79]). Thus, the inhibition of transcriptional programmes may further reinforce the effects of AMPK on ACC and HMGR to repress lipid synthesis.
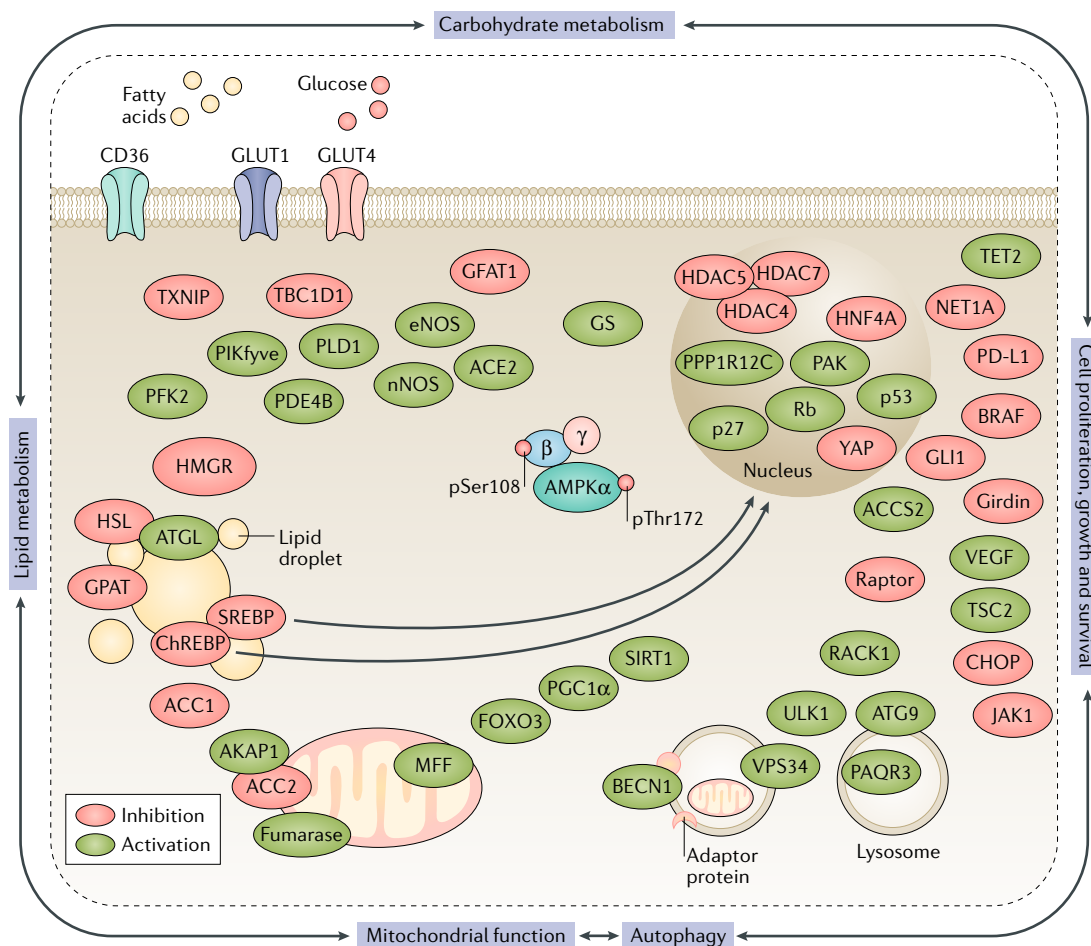
Fig. 2 | **Proteins and pathways regulated by AMPK.** AMP-activated protein kinase (AMPK) phosphorylates proteins critical for regulating fatty acid, cholesterol, carbohydrate and amino acid metabolism as well as autophagy, mitochondrial function (biogenesis, fission and mitophagy) and cell growth. AMPK phosphorylation of key substrates can modulate enzyme activities, cellular localization and the activation state of transcriptional programmes to elicit both acute and chronic effects in order to match energy demand with availability. ACC, acetyl-CoA carboxylase; ACE2, angiotensin-converting enzyme 2; AKAP1, A kinase anchor protein 1; ATG9, autophagy-related protein 9; ATGL, adipose triglyceride lipase; BECN1, beclin 1; CHOP, C/EBP homologous protein; ChREBP, carbohydrate-response element-binding protein; eNOS, endothelial nitric oxide synthase; FOXO3, forkhead box protein O3; GFAT1, glutamine fructose-6-phosphate aminotransferase 1; GLUT, glucose transporter; GPAT, glycerol-3-phosphate acyl transferase; GS, glycogen synthase; HDAC, histone deacetylase; HMGR, HMG-CoA reductase; HNF4A, hepatocyte nuclear factor 4α; HSL, hormone-sensitive lipase; JAK1, Janus kinase 1; MFF, mitochondrial fission factor; NET1A, neuroepithelial cell-transforming 1A; nNOS, neuronal NOS; PAK, p21-activated kinase; PAQR3, progestin and adipoQ receptor family member 3; PD-L1, programmed cell death ligand 1; PDE4B, phosphodiesterase 4B; PGC1α, peroxisome proliferator-activated receptor-γ co-activator 1α; PFK2, 6-phosphofructo-2-kinase; PIKfyve, 1-phosphatidylinositol 3-phosphate 5-kinase; PLD1, phospholipase D1; pSer108, phosphorylated Ser108; pThr172, phosphorylated Thr172; RACK1, receptor of activated protein C kinase 1; Rb, retinoblastoma protein; SIRT1, sirtuin 1; SREBP, sterol-response element-binding protein; TBC1D1, TBC1 domain family member 1; TSC2, tuberous sclerosis complex 2; TXNIP, thioredoxin-interacting protein; ULK1, unc-51-like autophagy activating kinase 1; VEGF, vascular endothelial growth factor.

As well as de novo lipogenesis, cellular lipid content is regulated through a balance of uptake, oxidation and lipolysis. The key rate-limiting enzymes regulating lipolysis are adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). Early studies indicated that the AMPK activator 5-aminoimidazole-4-carbox-amide ribonucleoside (AICAR, the pro-drug of ZMP) inhibited β-adrenergic-induced lipolysis and that this involved inhibitory phosphorylation of HSL at Ser565 (REFS[80,81]); however, more recent studies in mice lacking adipose tissue AMPK have shown that this is likely an off-target effect[82] potentially related to the inhibition of adenylyl cyclase, as described previously in the liver[83]. And while genetic reductions in adipose tissue AMPK are associated with increases in β-adrenergic-stimulated lipolysis in in some studies[84–86], this inhibitory role for AMPK on lipolysis is inconsistent with observations that AMPK is activated in adipose tissue under conditions of enhanced lipolytic flux, such as occurs with β-adrenergic stimuli, cold, exercise or fasting[82,88,89] and studies in other genetic models which show no effect of AMPK on lipolysis[82,87]. Interestingly, the activation

Lipogenesis
A metabolic pathway for the synthesis of fatty acids and triglycerides.

Box 3 | **AMPK consensus motif**

AMP-activated protein kinase (AMPK) regulation of cellular metabolism is dependent on the phosphorylation of distinct substrates. The optimal consensus motif of AMPK substrates has been established using a variety of methodologies (as recently reviewed[342]), and although minor modifications exist, relative to the serine/threonine phosphorylation site at P_0, hydrophobic residues (ϕ) in the $P+4$ and $P-5$ positions and a basic residue (B), typically arginine, in the $P-1$ to $P-4$ position, are important. Thus, the simple consensus motif can be written, where X is any residue, as $\phi X(B,X)XX(Ser/Thr)XXX\phi$. And while the presence of this motif can be queried using online resources such as Scansite, it is important to consider that consensus sequence requirements are only a guide to AMPK substrates owing to overriding structural and cellular localization constraints that may preclude phosphorylation of optimal substrates or promote phosphorylation of suboptimal motifs. The latter point may be especially relevant for AMPK, which seems to be more flexible in its recognition motif than many other protein kinases with respect to a basic residue at $P-3$ or $P-4$.

of AMPK by β-adrenergic stimuli is dependent on the lipase ATGL[88], which also becomes activated by AMPK under some conditions[90]. Further studies investigating the role of adipose tissue AMPK in regulating lipolysis under physiological conditions are required.

Non-esterified fatty acids released by adipose tissue lipolysis are an important substrate for many tissues and are taken up from the circulation by facilitative transporters such as fatty acid translocase (FAT; also known as CD36). Pharmacological and hormonal activation of AMPK can increase the translocation of FAT to the plasma membrane of muscle, leading to greater fatty acid uptake[91,92]. Once inside the cell, the entry of fatty acyl-CoA into mitochondria requires carnitine palmitoyl-transferase 1 (CPT1). Malonyl-CoA produced by ACC is an allosteric inhibitor of CPT1, and consistent with the inhibitory role of AMPK phosphorylation of ACC, pharmacological activators of AMPK increase fatty acid oxidation in liver and skeletal muscle through a pathway requiring ACC phosphorylation[74,75,93]. However, under conditions of elevated energetic demands such as occurs in contracting skeletal[94] or cardiac[95] muscle or in activated brown adipose tissue (BAT)[82], AMPK phosphorylation of ACC is not always essential for mediating increases in fatty acid oxidation, suggesting that alternative pathways may be important.

Regulation of mitochondrial function

AMPK may also control the rates of fatty acid oxidation indirectly by regulating mitochondrial function. Under conditions of heightened energetic demands, mitochondrial fusion occurs to maximize ATP production. A regulator of mitochondrial fusion is the A kinase anchor protein 1 (AKAP1), which is a mitochondrial scaffold protein that binds mitochondrial-targeted protein kinase A (PKA)[96]. In vitro, AMPK phosphorylates AKAP1 at Ser103, and mutation of this site blocks increases in fatty acid oxidation[96]. Although not fully understood, AKAP1-mediated increased fatty acid oxidation may involve the translocation of AKAP1 to the mitochondria, where it then provides a scaffold for PKA to promote the subsequent phosphorylation of dynamin-related protein 1 (DRP1), which is important for mitochondrial fission[96]. Consistent with this concept, both the circadian clock[97] and caloric restriction[98] enhance mitochondrial fusion through pathways

Mitophagy
Similar to autophagy, but refers specifically to the process by which cells turnover mitochondria.

involving AMPK, potentially through changes in DRP1 phosphorylation and activity. Future studies investigating the importance of AMPK phosphorylation of AKAP1 for regulating fatty acid oxidation under distinct physiological conditions will be important.

A consistent finding across many different systems is that the activation of AMPK increases mitochondrial content, while genetic loss of function has the opposite effect (for reviews see REFS[99,100]). These alterations in mitochondrial content may be due to changes in either the synthesis (biogenesis) and/or degradation (mitophagy) of mitochondria, and it appears that AMPK plays a role in regulating both aspects. AMPK increases mitochondrial biogenesis through multiple complementary pathways. In skeletal muscle and adipose tissue, a key pathway regulating mitochondrial biogenesis is the transcriptional co-activator peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α), and consistent with changes in AMPK activity, there are often parallel changes in PGC1α and mitochondrial biogenesis[99,101,102], effects that are largely eliminated when AMPK is activated in the absence of PGC1α[103,104]. AMPK-induced upregulation of PGC1α likely involves multiple complementary mechanisms including the activation of p53, SIRT1 and histone deacetylase 4 (HDAC4)[99]. Collectively, this activation of AMPK enhances mitochondrial content and subsequently the capacity of the cell to respond to future energetic challenges[100].

Increases in mitochondrial biogenesis must be balanced by the removal of damaged mitochondria[100]. Severe energetic stress can be harmful to mitochondria; however, over time, all mitochondria become damaged and need to be replaced, a process that is initiated by mitochondrial fission. The fission of mitochondria is mediated by DRP1, which is recruited to the outer mitochondrial membrane by mitochondrial fission factor (MFF), fission mitochondrial 1 (FIS1) and MID49 and/or MID51. AMPK phosphorylates MFF (Ser129)[105,106], and this is essential for initiating mitochondrial fission[106]. Once fission occurs, AMPK further promotes the degradation of damaged mitochondria through a selective form of autophagy called mitophagy[65,66]. The activation of unc-51-like autophagy activating kinase 1 (ULK1) is a key signal for the formation of a mature phagophore, a newly formed membrane that encapsulates cytosolic constituents, delivering them to the lysosome[69]. AMPK binds to and directly phosphorylates ULK1 (REFS[107,108]) to promote mitophagy[109,110]. AMPK also phosphorylates authophagy-related protein 9 (ATG9)[111] and beclin 1 (REF.[112]), which activates the pro-autophagy Vps34 complex.

AMPK also indirectly regulates mitophagy through several mechanisms. The first involves the inhibition of mechanistic target of rapamycin complex 1 (mTORC1), which phosphorylates and inhibits ULK1 (REF.[100]). AMPK inhibition of mTORC1 involves phosphorylation of tuberous sclerosis complex 2 (TSC2)[113] and the mTOR subunit Raptor[114]. AMPK also phosphorylates acetyl-CoA synthetase (ACSS) at Ser656, which promotes nuclear acetate uptake and may result in the acetylation and activation of transcription factors such as TFEB,

which promotes lysosome biogenesis[115]. AMPK inhibition of mTORC1 may also contribute to TFEB activation[116]. Last, AMPK promotes autophagy by phosphorylating and increasing the transcriptional activity of FOXO3 (REF.[117]), which in turn increases the expression of several key autophagy genes including those encoding LC3, beclin 1, VPS34 and BNIP3 (REFS[118,119]). In addition, AMPK phosphorylation has recently been reported to promote import of FOXO3 into mitochondria, where it is cleaved, allowing it to regulate mitochondrial transcription[120]. Thus, AMPK promotes mitophagy both through direct phosphorylation and activation of ULK1, FOXO3 and ACSS and by antagonizing mTORC1.

Regulation of carbohydrate metabolism

In addition to regulating fatty acid metabolism, which in many cases may indirectly influence carbohydrate utilization owing to the Randle cycle, AMPK also directly regulates pathways controlling carbohydrate metabolism through multiple mechanisms, as detailed below.

In skeletal muscle of rodents, activation of AMPKα2β2γ3 heterotrimers increases glucose uptake by enhancing glucose transporter type 4 (GLUT4) translocation[121–125]. The mechanisms by which AMPK promotes skeletal muscle glucose uptake likely involve multiple pathways including the phosphorylation and inhibition of the RAB GTPase-activating protein (GAP) TBC1 domain family member 1 (TBC1D1), which normally sequesters GLUT4 to the Golgi[126,127]. AMPK also phosphorylates 1-phosphatidylinositol 3-phosphate 5-kinase (PIKfyve)[128], a phosphoinositide phosphate kinase, as well as phospholipase D1 (REF.[129]), which activates ERK, and this further promotes GLUT4 translocation to the plasma membrane. In addition to stimulating the acute translocation of GLUT4 to the plasma membrane, AMPK phosphorylation of HDAC4 results in subsequent nuclear translocation that increases myocyte enhancer factor and GLUT4 expression[130,131]. However, many cells do not express GLUT4 and instead rely on GLUT1 to control glucose uptake. AMPK has been reported to increase GLUT1-dependent glucose uptake through activation of GLUT1 transporters at the plasma membrane[132] and increasing GLUT1 expression[133]. In addition, AMPK phosphorylation of thioredoxin-interacting protein (TXNIP) promotes rapid degradation of TXNIP, which increases both GLUT1 plasma membrane localization and mRNA expression[134]. TXNIP expression is also reduced in response to AMPK phosphorylation and inhibition of ChREBP[135]. Collectively, this activation of AMPK promotes cellular glucose uptake by enhancing the translocation and expression of GLUT proteins.

Once glucose enters the cell, it is rapidly converted to glucose-6-phosphate (G6P), which is then directed towards glycolysis or glycogen synthesis depending on energetic demands. In some cells, activation of AMPK stimulates glycolysis through phosphorylation and activation of 6-phosphofructo-2-kinase (PFK2), which leads to a decrease in fructose 2,6-bisphosphate, an *allosteric activator* of 6-phosphofructo-1-kinase (PFK1)[136]. PFK2 exists in several isoforms, but only the cardiac[136] and inducible[137] isoforms (present in haemopoietic cells and in some tumour cells) are regulated

by AMPK, accounting for the cell-type-specific effect of AMPK on glycolysis. One of the initial substrates of AMPK to be identified was glycogen synthase. AMPK inhibited glycogen synthase activity in vitro through phosphorylation of Ser7 (REF.[138]); however, this inhibitory effect was later found to be inconsistent with observations that both genetic and pharmacological activation of AMPK enhanced glycogen storage[139]. Subsequent studies using genetic knock-in mice established that allosteric activation of glycogen synthase by G6P could override the inhibitory effects of AMPK phosphorylation in response to pharmacological AMPK activators[140]. AMPK also promotes inhibitory phosphorylation of glycogen synthase in liver, which is important for mediating the effects of pharmacological AMPK activators, but not glucagon, to reduce glycogen synthesis[141]. In addition to glycogen synthesis, elevations in G6P can result in hexosamine biosynthesis. Glutamine fructose-6-phosphate aminotransferase 1 (GFAT1) is the rate-limiting enzyme in the hexosamine biosynthesis pathway, and AMPK phosphorylates GFAT1 (Ser243) to inhibit its activity[142]. This inhibition of glycogen synthase and GFAT1 helps to ensure that increases in G6P are directed towards glycolysis rather than storage.

Early studies using nonspecific AMPK activators such as AICAR[143,144] indicated that AMPK repressed key gluconeogenic genes such as those encoding phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), both of which have been shown to contribute to aberrant hepatic glucose production in metabolic disorders[145]. However, AMPK becomes activated in the liver during exercise, an effect associated with increased, not lower, hepatic glucose output[146]. Studies using a combination of genetic mouse models lacking AMPK and direct AMPK activators (see below) have found that acute AMPK activation does not inhibit hepatic gluconeogenesis and that previous observations using nonspecific activators such as AICAR or metformin may have been related to the inhibition of adenylyl cyclase or alterations in mitochondrial redox status[25,83,147,148]. Despite not directly inhibiting gluconeogenic gene expression, the activation of AMPK in hepatocytes by metformin and A769662 indirectly suppresses gluconeogenesis by reducing lipid-induced insulin resistance, thereby allowing insulin to lower hepatic glucose production more effectively[74]. Recently, AMPK has been shown to phosphorylate phosphodiesterase 4B, leading to its activation[149], and this antagonizes glucagon-stimulated cAMP accumulation, an effect that would lead to decreased hepatic gluconeogenesis and may also have implications for the regulation of adipose tissue lipolysis discussed above. Mice lacking liver AMPK also have lower levels of liver glycogen, which reduces glycogenic flux and blood glucose during exercise[146]. Similarly, during prolonged fasting, AMPK activation in muscle is important for promoting autophagy, muscle breakdown and the release of essential gluconeogenic precursors such as alanine[150]. Thus, while AMPK in the liver does not directly inhibit liver gluconeogenesis, AMPK in liver and muscle is important for maintaining glucose homeostasis during energetic challenges such as exercise and caloric

Allosteric activator
A molecule that activates an enzyme by binding at a site distinct from the active site.

Gluconeogenesis
A metabolic pathway for the synthesis of glucose from precursor substrates such as lactate and amino acids.

restriction and regulating liver insulin sensitivity by suppressing lipogenesis.

Pharmacological activation of AMPK

A number of pharmacological compounds that increase AMPK activity indirectly (TABLE 1) or through direct binding (TABLE 2) have been identified, and in many cases, their mode of action has been elucidated (FIG. 1). A small number of these compounds have also been approved for use or are in clinical trials.

Indirect AMPK activators

AMPK is activated in response to an increase in AMP and ADP levels, and this underpins the mechanism of action of a wide number of compounds that indirectly activate AMPK (FIG. 1). Essentially, any treatment that leads to a rise in the AMP/ADP:ATP ratio leads to activation of AMPK. It is not surprising, therefore, that many compounds that activate AMPK in cells do so by inhibition of the mitochondrial electron transport chain or by causing mitochondrial uncoupling, both of which would be expected to reduce mitochondrial ATP synthesis.

The biguanide metformin (TABLE 1), a widely used oral anti-diabetic agent, inhibits complex I in the mitochondria, leading to a reduction in mitochondrial respiration and ATP production[151], and has been shown to activate AMPK[18]. Metformin-induced activation of AMPK is dependent on uptake from the circulation by organic cation transporters, and consistent with this response, the activity of these transporters is important for dictating therapeutic effects between individuals[152]. Organic cation transporters are highly expressed in the liver[152], where the activation of AMPK by metformin requires the upstream kinase LKB1 (REF.[153]). However, the closely related and lipophilic biguanide phenformin has a much greater propensity to enter cells and activate AMPK outside of hepatocytes, but this compound was withdrawn from most countries owing to increased risk of lactic acidosis. To promote AMPK activation outside the liver, a number of groups have embarked on developing metformin-like complex I inhibitors such as R419 (REF.[154]) (TABLE 1).

A number of plant-derived natural products, which are used in traditional herbal medicine, also activate AMPK indirectly by inhibition of mitochondrial respiration[14] (for example, berberine and quercetin (see TABLE 1)). Most widely studied is the polyphenol resveratrol, which activates AMPK through multiple mechanisms including the inhibition of mitochondrial function[155] and phosphodiesterases[156], as well as through activation of SIRT1 (REF.[157]). PT-1, a small-molecule AMPK activator, was initially reported to activate AMPK by binding between the kinase domain and AID region of the α subunit, relieving auto-inhibition[158]. A subsequent study, however, showed that in cells, PT-1 activates AMPK by inhibiting the mitochondrial respiratory chain[159]. More recently, the sodium/glucose cotransporter inhibitor canagliflozin, but not the closely related compounds dapagliflozin and empagliflozin, was shown to activate AMPK via inhibition of complex I[20,21]. In addition to electron transport chain inhibition, compounds that cause mitochondrial uncoupling, such as dinitrophenol, increase the AMP/ADP:ATP ratio and activate AMPK[160]. However, as these compounds activate AMPK indirectly, it is likely that some of their effects are not mediated by AMPK[83,147,161,162].

Direct AMPK activators

The initial efforts to identify direct activators of AMPK focused on compounds that would mimic nucleotide-dependent activation, although at the time of these studies, the nature of the nucleotide binding sites was not known. The first such activator to be identified was 5-aminoimidazole-4-carboxamide ribonucleotide (also known as ZMP), the monophosphate derivative of the cell permeable precursor AICAR[80,81,163] (TABLE 2). This finding led to the wide use of AICAR as a pharmacological tool to investigate the effect of activation of AMPK in cells. The levels of ZMP in the cell can also be increased by methotrexate, which inhibits the folate-dependent enzyme AICA ribotide transformylase/IMP cyclohydrolase (ATIC), which is responsible for converting ZMP to IMP[164]. However, a problem with approaches that increase ZMP is that this affects other AMP-sensitive enzymes, such as fructose 1,6-bisphosphatase and glycogen phosphorylase[165,166]. It is now clear that some of the effects of AICAR on metabolic pathways are AMPK-independent[82,147,167], cautioning the use of AICAR as a sole means for demonstrating the involvement of AMPK in a particular pathway.

More recently, a screen of AMP mimetics identified 5-(5-hydroxy-isoxazol-3-yl)-furan-2-phosphonic acid (compound 2 (C2)) as a potent allosteric AMPK activator[168]. C2 appears to selectively activate α1-containing AMPK complexes and has no effect on γ3-containing complexes[169,170]. The crystal structure of AMPK bound to C2 has been resolved, revealing two molecules of C2 bound to the γ subunit[170]. Intriguingly, these binding sites differ from the nucleotide binding sites, suggesting that the γ subunit could be exploited for drug targeting independently of nucleotide binding[170]. In this regard, a recent study identified 2-(2-(4-(trifluoromethyl) phenylamino)thiazol-4-yl)acetic acid as a potent AMPK activator that acts as an AMP mimetic[171]. Another small molecule, O304, has recently been shown to protect against pThr172 dephosphorylation by protein phosphatase 2C without allosteric activation of AMPK, thus mimicking the effect of ADP but not AMP[28]. The exact site of binding of O304 has not been reported.

In 2006, Abbott Laboratories identified a small-molecule activator of AMPK from a screen of ~700,000 compounds[172]. Subsequent optimization led to A-769662 (TABLE 2), which was shown to allosterically activate purified AMPK and to activate AMPK in primary hepatocytes[172]. In contrast to AMP, A-769662 had no direct effect on fructose 1,6-bisphosphatase or glycogen phosphorylase. Activation of AMPK by A-769662 was additive with AMP, suggesting that A-769662 bound at a site distinct from the nucleotide binding sites[172]. For several years, A-769662 remained the only direct AMPK activator of its type. In 2012, a paper reviewed the existing patent databases for AMPK activators, reporting 26 patents that disclosed 10 classes of direct AMPK

activators[173]. Strikingly, many of the compounds listed were closely related to A-769662, suggesting a common mode of action. Indeed, further studies indicated that many of the listed compounds appeared to activate AMPK by binding to a single site within AMPK. The crystal structure of full-length AMPK (α2β1γ1) in complex with one of these small-molecule activators (termed 991, see TABLE 2) revealed that the compound binds in a pocket formed between the amino-terminal lobe of the kinase domain and the CBM of the β subunit[22]. Several structures of AMPK bound to different activators have now been published[24,27,174], allowing the molecular basis for activation to be determined. The binding pocket is formed by the interface generated by one end of the five stranded β-sheet of the amino-terminal lobe of the kinase packing against a pair of anti-parallel β-strands from the CBM. A key feature of the structure was the finding that phosphorylated Ser108 in β1 is involved in a network of electrostatic interactions with Lys31 and Thr21 (both from the kinase domain) and Asn111 (from the CBM)[22]. Mutation of Ser108 to Ala decreased binding of either 991 or A769662 by 50-fold to 100-fold[22], providing an explanation for an earlier study showing that phosphorylation of Ser108 in β1 was important for the activation of AMPK by A-769662 (REF.[175]). The nature of the binding pocket suggests that, in addition to drugs, a natural ligand could bind at the ADaM site, and while an endogenous metabolite has not been identified, salicylate, a natural product derived from willow bark, activates AMPK by directly binding to the ADaM site[19]. In vivo, aspirin (acetylsalicylate) and salsalate (a dimer of salicylate) are converted to salicylate, and so activation of AMPK may play a role in mediating some of the beneficial effects of these compounds in tissues where concentrations of salicylate reach sufficiently high levels.

Ligand binding at the ADaM site allosterically activates AMPK and protects against dephosphorylation[39,175]. Protection against dephosphorylation is mediated by stabilization of the interaction of the CBM with the kinase domain in the ligand-bound state, promoting interaction of the kinase domain with the regulatory core of the enzyme[22]. Most of the contacts with the regulatory core are formed by residues in the activation loop of the kinase, stabilizing the activation loop structure. In this conformation, although the phosphate group of pThr172 is partially exposed to the solvent, it is not accessible to protein phosphatases. Allosteric activation following ligand binding at the ADaM site is dependent on the isoform composition of the AMPK complex[22,52,54] and involves the interaction between an α-helix immediately carboxy-terminal to the CBM (referred to as the C-interacting helix) and the αC helix of the kinase domain[22]. Mutation of Leu166 to Glu within the C-interacting helix, which is predicted to block interaction with the αC helix, significantly reduces allosteric activation of AMPK by 991 but has no effect on allosteric activation by AMP[22]. The amino acid sequence of the C-interacting helix differs between β1 and β2, and the sequence of the αC helix differs between α1 and α2 (REF.[22]). It is possible, therefore, that these isoform-specific sequence variations could account for the differences in allosteric activation of the AMPK complexes by binding at the ADaM site.

All the ADaM site activators identified to date bind more tightly to β1-containing than to β2-containing AMPK complexes. The difference in β-isoform binding is compound-dependent, varying from less than 10-fold (for example, 991 (REF.[22])) to over 1,000-fold (for example, PF249 and PF06409577 (REF.[27])). In some cases, binding to β2-containing complexes is so weak that the compound is effectively a β1-specific activator, for example, PF249 and PF06409577 (REF.[27]), whereas in other cases, the compound binds sufficiently strongly to both β isoforms to activate β1 and β2 AMPK complexes, for example, PF739 (REF.[24]) and MK-8722 (REF.[26]). Using a recently identified activator, SC4, it was shown that Asp111 in β2 interacts with the imidazopyridine 4′-nitrogen of the compound, which could provide increased stabilization of binding to β2-containing AMPK complexes[176]. The availability of pan-β activators in addition to highly selective β1 activators has provided a useful tool for investigating β isoform-specific effects in vivo.

A recent study reported that in contrast to β1, Ser108 phosphorylation is not involved in binding of 991 to β2-containing AMPK complexes[54]. There is no high-resolution structural information available for AMPKβ2 complexes bound to ADaM site ligands, and so the molecular basis for the difference in requirement for Ser108 phosphorylation between the β-isoforms remains unknown. Nonetheless, the dramatic increase in binding affinity for ADaM site ligands following phosphorylation of Ser108 in β1 raises the possibility that the modulation of Ser108 phosphorylation could provide a potential strategy for targeting drugs to β1-containing AMPK complexes. The recent finding that ULK1 phosphorylates Ser108 in β1 under conditions that favour an increase in the AMP/ADP:ATP ratio provides a potential way to exploit this possibility[177]. By increasing Ser108 phosphorylation, through ULK1, it might be possible to increase the potency of AMPK β1 activation. However, Ser108 is an autophosphorylation site[178,179], and in recombinant AMPK preparations expressed in *Escherichia coli*, Ser108 is efficiently phosphorylated even in the absence of Thr172 phosphorylation[180], so whether it would be possible to exploit Ser108 phosphorylation in vivo remains speculative.

Dual activation of AMPK (nucleotide and ADaM site)

The fact that AMPK can be activated by ligands that bind to distinct sites within the enzyme provides an attractive rationale for using dual treatments to activate AMPK. In cell-free assays, there is synergistic activation of AMPK when AMP and salicylate or A769662 are provided in combination, effects that are dependent on phosphorylation of Ser108 within the β1 subunit[181,182]. Surprisingly, AMP and A-769662 synergistically activate recombinant AMPK in the absence of Thr172 phosphorylation[182]. Subsequent studies confirmed these findings using different AMPK activators[170,180]. One study resolved the crystal structure of non-phosphorylated Thr172 AMPK complex (α2β1γ1) bound to AMP and 991, showing that the activation loop adopts a similar conformation to that seen in the phosphorylated AMPK
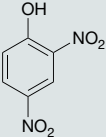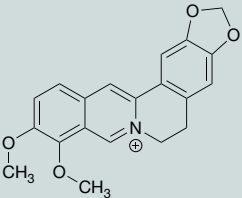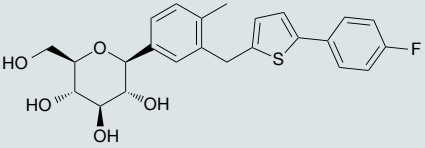
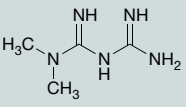Table 1 | **Indirect pharmacological AMPK activators**
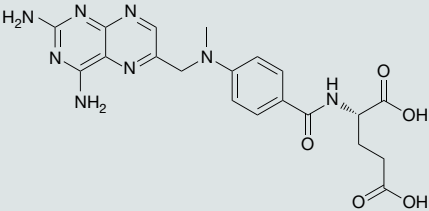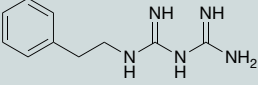
Compound (company)	Structure	Isoform specificity	Binding site	Comments	Development stage	Refs
2,4-Dinitrophenol		No report of isoform specificity	Mitochondrial inner membrane	Mitochondrial uncoupler that causes weight loss but with potentially fatal consequences	Withdrawn, but new slow-release agents are under development for obesity and NAFLD	160,161
Berberine		No report of isoform specificity	Complex I	Plant product used in traditional Chinese herbal medicine. Mitochondrial respiratory chain inhibitor (complex I) but has poor oral bioavailability	Berberine derivatives with greater bioavailability are being developed for multiple indications	16,17
Canagliflozin (Janssen)		No report of isoform specificity	Complex I	Sodium/glucose cotransporter inhibitor used to treat type 2 diabetes	Approved for type 2 diabetes	20,21
Metformin		No report of isoform specificity	Complex I	Widely used oral type 2 diabetes medication. Mitochondrial respiratory chain inhibitor (complex I), although other mitochondrial targets may also be important	• Approved for type 2 diabetes • Clinical testing underway for numerous other diseases including CVD and many cancers	18
Methotrexate (4-amino-10-methyl-pteroylglutamic acid)		No report of isoform specificity	–	Prevents breakdown of ZMP to IMP resulting in ZMP accumulation and activation of AMPK	Approved for rheumatoid arthritis and cancer	164
Phenformin		No report of isoform specificity	Complex I	Increased cellular penetrance compared with metformin	• Approved for type 2 diabetes in 1952, but withdrawn owing to lactic acidosis • Clinical testing underway for cancer	31
PT-1		Pan-AMPK activator in cultured cells, but does not activate γ3 complexes in skeletal muscle	–	Originally thought to activate AMPK directly, but later shown to activate indirectly by inhibiting mitochondrial respiration	Preclinical only	159
Quercetin		No report of isoform specificity	Complex I	Abundant flavonoid	Derivatives with greater bioavailability may be developed	343

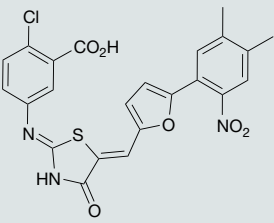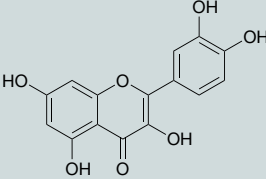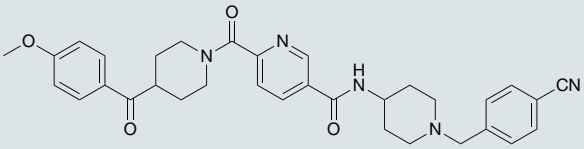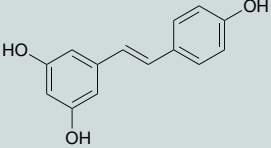Table 1 (cont.) | **Indirect pharmacological AMPK activators**

Compound (company)	Structure	Isoform specificity	Binding site	Comments	Development stage	Refs
R419 (Rigel Pharmaceuticals)		No report of isoform specificity	Complex I	Increases glucose uptake and fatty acid oxidation in cultured cells. Increases exercise capacity, muscle mitochondrial content and insulin sensitivity in mice fed a high-fat diet	Preclinical only	154,238
Resveratrol		No report of isoform specificity	Multiple mechanisms, including inhibition of ATP synthase and activation of SIRT1 and PDE4	Abundant polyphenol found in the skin of red grapes	Clinical testing for multiple indications underway	15

AMPK, AMP-activated protein kinase; CVD, cardiovascular disease; NAFLD, non-alcoholic fatty liver disease; SIRT1, sirtuin 1; ZMP, 5-aminoimidazole-4-carboxamide ribonucleotide.

complex[180]. The structure also revealed that Ser108 in β1 was phosphorylated, explaining the finding that 991 binding was the same with both the phosphorylated and non-phosphorylated Thr172 complexes[180]. These studies indicate that the binding energies of 991 (or A-769662) and AMP are capable of inducing an active-like conformation in the non-phosphorylated activation loop, and this accounts for the activity of the non-phosphorylated complex in vitro. The possibility of synergistically activating AMPK, while bypassing the requirement for upstream kinase activity, has major implications for potential therapeutic strategies targeting AMPK where the upstream kinase may be lacking (for example, LKB1-deficient tumours). However, in vitro studies indicate that this strategy is unlikely to be feasible. In human cells engineered to lack expression of both LKB1 and CAMKK2, Thr172 phosphorylation was almost completely abolished. Treatment of these cells with 991 and conditions that increased AMP failed to lead to increased phosphorylation of ACC, suggesting that in human cells Thr172 phosphorylation is essential for AMPK function[180]. However, outside of tumours, under most conditions, upstream kinase activity does not appear to be altered, suggesting that combination therapies may be an effective means to enhance AMPK activity in tissues. For example, combined treatment of hepatocytes and skeletal or cardiac muscle with AMPKβ-directed activators (A769662, 991 and salicylate) and AICAR (where ZMP binds to the γ isoform) or metformin (where metformin increases AMP and/or ADP) leads to a synergistic increase in Thr172 phosphorylation and activation of AMPK[183–186]. These findings support the hypothesis that dual activation of AMPK through the nucleotide-binding and ADaM-binding sites may be an effective means for maximally activating AMPK.

Therapeutically targeting AMPK

The role of AMPK in regulating a diverse range of cellular processes has revealed the potential of AMPK-activating therapies to treat a number of different diseases (FIG. 3).

Non-alcoholic steatohepatitis (NASH). A severe form of non-alcoholic fatty liver disease in which the liver becomes inflamed.

Metabolic diseases

The epidemics of obesity and type 2 diabetes have prompted the need to develop new therapies that can reduce their incidence and related comorbidities including NAFLD, non-alcoholic steatohepatitis (NASH) and CVD. The findings that AMPK activation promotes fatty acid oxidation, mitochondrial function, autophagy and skeletal muscle glucose uptake, while suppressing inflammation as well as fatty acid and cholesterol synthesis, provides a strong rationale for therapeutic targeting. Notably, AMPK activity is lower in mesenchymal stem cells from human infants born from obese mothers[187], an effect that is also observed in multiple tissues of individuals with obesity and insulin resistance[188]. Importantly, these reductions in AMPK are largely restored with treatments that exert beneficial metabolic effects such as endurance exercise, caloric restriction and bariatric surgery, suggesting that AMPK may play an important role in mediating these effects[188].

Obesity. Reducing obesity through a combination of lowering caloric intake and increasing energy expenditure, both of which are regulated by AMPK, is one means to effectively treat multiple aspects of cardiometabolic disease. Consistent with its role in increasing energy supply, hypothalamic AMPK activation in response to ghrelin or low glucose stimulates appetite, while reduced AMPK activity in response to leptin and insulin suppresses appetite[69,189]. Similarly, genetic inactivation of AMPK in the hypothalamus reduces appetite in mice[87,190], while a constitutively active AMPK mutation is associated with increased food intake and adiposity in both mice and humans[191]. The ability of AMPK to increase appetite, at least with respect to ghrelin, is dependent on the phosphorylation and inhibition of ACC, which reduces malonyl-CoA, a known regulator of food intake and neuropeptide expression[192], while activation of autophagy[193] and p21-activated kinase (PAK) may be important in the response to fasting[194]. Despite substantial evidence suggesting that activation of hypothalamic AMPK increases

Table 2 | **Direct AMPK activators**
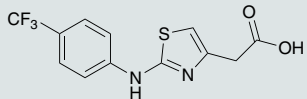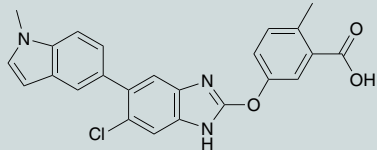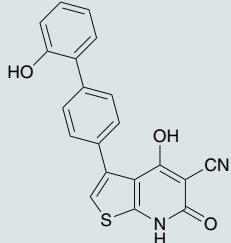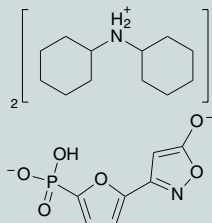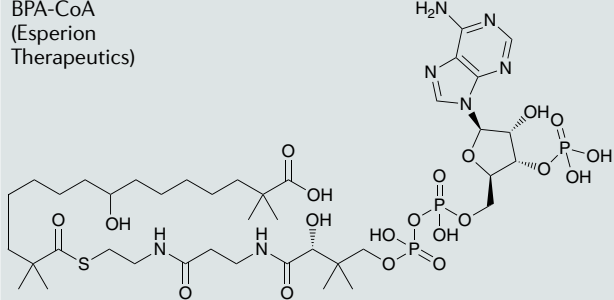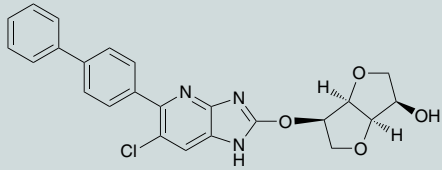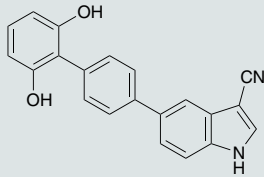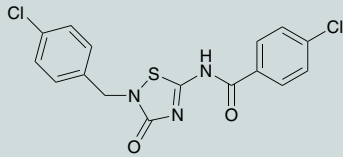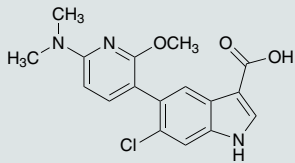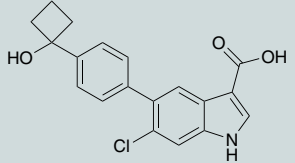
Compound (company)	Structure	Isoform specificity (K_d β1; K_d β2)	Binding site	Comments	Development stage and/or indication	Refs
Activator 3 (2-(2-(4-(trifluoromethyl)phenylamino)thiazol-4-yl)acetic acid; Dr. Reddy's Laboratories Ltd)		Pan-AMPK activator (0.04 μM; 0.02 μM)	Predicted to bind to CBS1 within the γ subunit	Improves metabolic profile in rats fed a high-sucrose diet	Currently limited to rodent models	171
991 (Merck Sharpe & Dohme–Metabasis Therapeutics)		Pan-β (0.05 μM; 0.5 μM)	ADaM site	Used in co-crystallization of full-length AMPK	In vitro and/or ex vivo use	22
A769662 (Abbott)		β1>>β2 (0.5 μM; 15 μM)	ADaM site	First ADaM site activator identified; poor bioavailability	In vitro, ex vivo and in vivo preclinical rodent models	172
C2 (Metabasis Therapeutics)		Pan-β; α1>>α2 activation; does not activate γ3 complexes (0.011 μM; 0.015 μM)	γ subunit (but distinct from nucleotide binding sites)	Cell-permeable prodrug: C13	In vitro and/or ex vivo use	168–170
BPA-CoA (Esperion Therapeutics)		β1 subunit	Unknown	• Cell-permeable prodrug BPA is converted to a BPA-CoA by acyl-CoA synthetase 4 in hepatocytes • BPA-CoA allosterically activates AMPK and also inhibits ACLY	Phase III clinical trials for CVD	248
MK-8722 (Merck)		Pan-β (0.001 μM; 0.05 μM)	ADaM site	Improves glucose homeostasis in vivo in mouse and primate models; mild cardiac hypertrophy reported	Non-human primates	26
MT 63–78 (Mercury)		Pan-β? Full isoform specificity not reported (20 μM; not determined)	ADaM site	Inhibits prostate cancer cell growth in vitro and in a xenograft model	In vitro, ex vivo and/or in vivo preclinical rodent models	281
O304 (Betagenon)		Full isoform specificity not reported	Unknown	Protects against AMPK pThr172 dephosphorylation	• Phase IIa trials • Type 2 diabetes	28

Table 2 (cont.) | **Direct AMPK activators**

Compound (company)	Structure	Isoform specificity (K_d β1; K_d β2)	Binding site	Comments	Development stage and/or indication	Refs
PF249 (Pfizer)		β1>>β2 (0.01 μM; 40 μM)	ADaM site	Improves kidney function in a rat model of diabetic nephropathy; no effect on glucose lowering in vivo	Preclinical mouse models	27
PF06409577 (Pfizer)		β1>>β2 (0.005 μM; 40 μM)	ADaM site	• Improves kidney function in a rat model of diabetic nephropathy • Reduces NAFLD and LDL cholesterol	Non-human primates	25,27
PF739 (Pfizer)		Pan-β (0.01 μM; 0.1 μM)	ADaM site	Improves glucose homeostasis in vivo in mouse and primate models	Non-human primates	24
Salicylate		β1>>β2 (1 mM; >10 mM)	ADaM site	• Weak affinity • Prodrug: aspirin or salsalate	Clinical concentrations of salicylate achieved with salsalate but not aspirin are likely to activate AMPK. Approved for arthritis. In clinical trials for type 2 diabetes and CVD	19
SC4 (Shionogi and Co. Ltd)		Pan-β (0.005 μM; 0.02 μM)	ADaM site	Increases glucose uptake in skeletal muscle cells	Ex vivo use	176
ZMP		No report of isoform specificity (1.5 mM measured at 4 mM ATP)	CBS3 (γ subunit)	Cell-permeable prodrug: AICA riboside	In vitro and preclinical use	80,81,163

ACLY, ATP-citrate lyase; ADaM, allosteric drug and metabolite; AICA, 5-aminoimidazole-4-carboxamide; AMPK, AMP-activated protein kinase; BPA, bempedoic acid; BPA-CoA, bempedoic acid-CoA; CBS, cystathionine-β-synthase; CVD, cardiovascular disease; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease; pThr172, phosphorylated Thr172; ZMP, 5-aminoimidazole-4-carboxamide ribonucleotide.
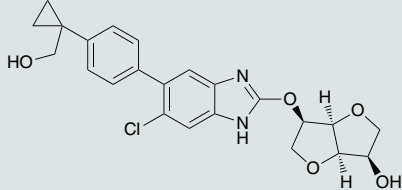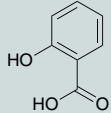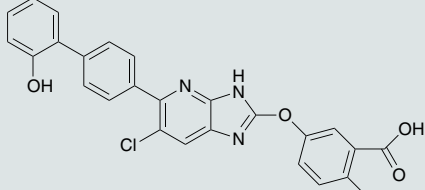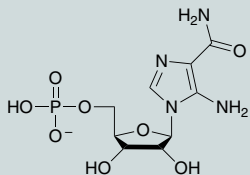
appetite, it should be noted this is not always observed, as mice expressing a germline constitutively active AMPK γ2 mutation have reduced food intake[195], suggesting that there may be differential responses to AMPK between distinct neuronal populations. Further studies examining the specific neuronal populations that are important for AMPK regulation of appetite and the mechanisms mediating these effects will be important to establish whether this pathway may be pharmacologically manipulated for the treatment of obesity.

In addition to suppressing appetite, reductions in hypothalamic AMPK may also promote weight loss by enhancing energy expenditure. An important regulator of energy expenditure is BAT. The hypothalamus controls sympathetic outflows to BAT, which enhances the activity of uncoupling protein 1 (UCP1), decoupling oxidative phosphorylation from ATP synthesis, thereby generating a futile cycle that enhances ATP turnover. Importantly, in humans with obesity and type 2 diabetes, the metabolic activity of BAT is reduced, suggesting that finding ways to enhance the metabolic capacity of this tissue may increase energy expenditure. Interestingly, factors that enhance BAT thermogenesis and energy expenditure such as 3,3′,5-triiodothyronine (T3),

Thermogenesis
A process by which cells generate heat.
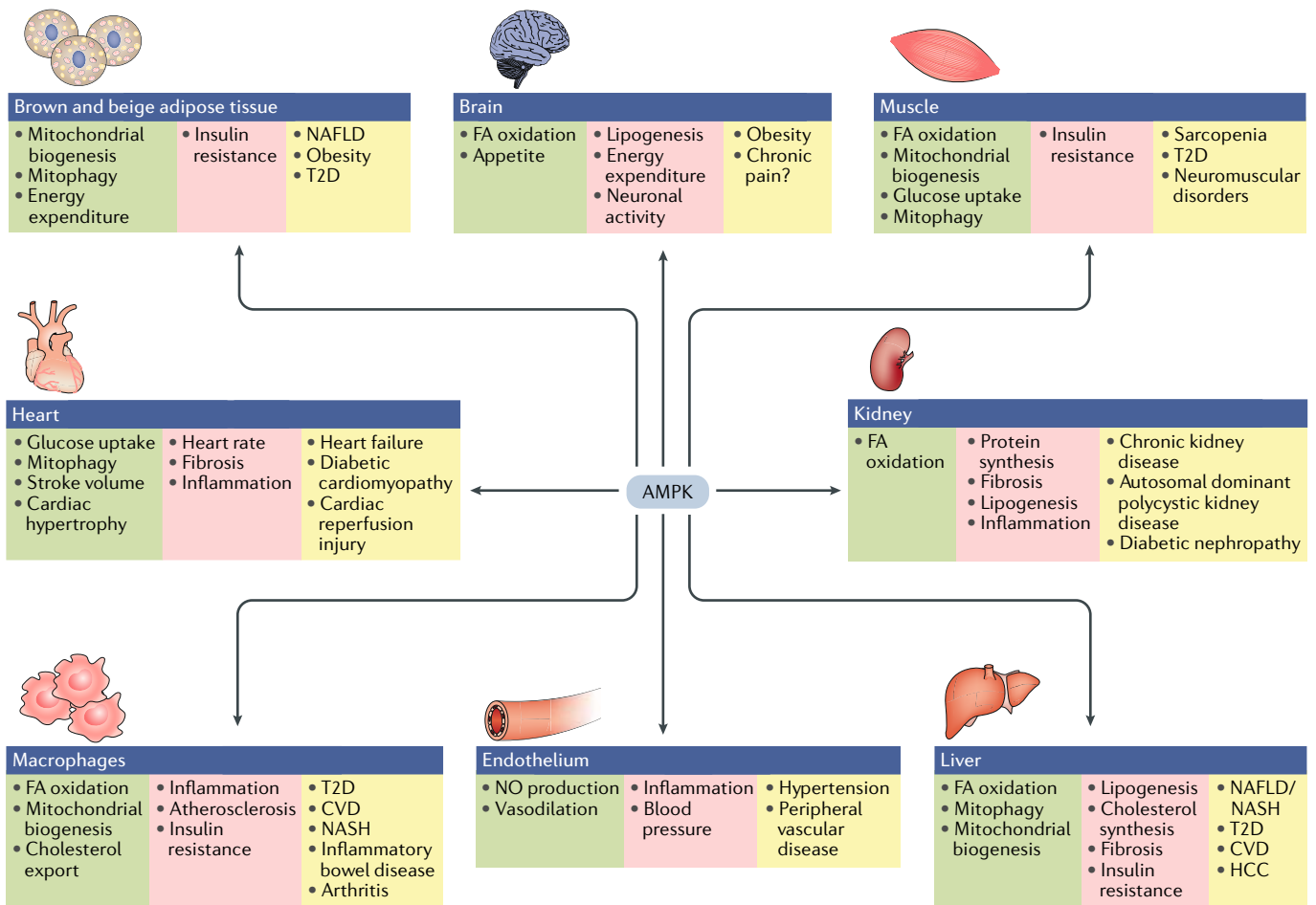
Fig. 3 | **Tissue-specific roles of AMPK in the metabolic syndrome.** AMP-activated protein kinase (AMPK) regulates a diverse range of cellular processes that are linked to human disease. The major pathways known to be regulated by AMPK in different tissues are highlighted for different organs and cell types within the body. Those that are positively impacted by AMPK are shown in green boxes, while those that are inhibited by AMPK are in red boxes. Diseases that have the potential to be treated by AMPK modulation are shown in yellow boxes. CVD, cardiovascular disease; FA, fatty acid; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NO, nitric oxide; T2D, type 2 diabetes.

nicotine, oestradiol and bone morphogenetic protein 8 all suppress hypothalamic AMPK[196,197]. Some studies have used compound C as a pharmacological inhibitor of AMPK, but it is now well established that this compound inhibits a wide variety of kinases[198] and so the results of these studies need to be interpreted with caution. With the recent identification of more specific AMPK inhibitors, such as SBI-0206965 (REF.[199]), it will be interesting to examine whether delivery of AMPK inhibitors into the central nervous system might suppress appetite and promote weight loss.

While reductions in hypothalamic AMPK activity may enhance sympathetic drive to BAT, AMPK expression is very high within BAT itself compared with other tissues and is further activated in response to cold or β-adrenergic stimuli[89,200], suggesting an important role for the kinase in controlling BAT function. Supporting this idea, when either the AMPK α or β subunits are genetically removed in adipose tissue of adult mice, defects in BAT mitochondrial function rapidly develop, resulting in an impaired ability to enhance energy expenditure in response to cold or β-adrenergic stimuli[82,201]. Impaired BAT thermogenesis in mice lacking AMPK has been linked to reduced BAT development[104,202], as well as reductions in mitophagy that result in impaired mitochondrial function[82]. Reductions in adipose tissue AMPK also limit the browning of white adipose tissue and the generation of beige and brite adipose tissue in response to β-adrenergic stimuli[82] or direct AMPK activators such as A769662 (REF.[201]) through mechanisms that are not yet fully understood. Importantly, these defects in brown and beige adipose tissue in the absence of AMPK translate into reductions in whole body energy expenditure and modest increases in obesity when mice are fed a high-fat diet[82,201]. These findings are consistent with studies showing that pharmacological and indirect genetic activation of AMPK promotes the browning of white fat[102,204–207]. Intriguingly, activation of AMPK by 0304 also increases adipose tissue energy expenditure, independently of changes in UCP1 expression, when mice are housed under thermoneutral conditions[28]. Genetic activation of AMPK also protects

against diet-induced obesity through a mechanism that involves increased UCP1-independent thermogenesis in subcutaneous white adipose tissue[208]. Although the precise molecular details of these effects remain to be fully established, these studies provide evidence of new pathways that might be exploited for therapeutic strategies for treating obesity. And while it is known that AMPK activity is reduced in several different adipose tissue depots of individuals with obesity and insulin resistance[209], potentially owing to inflammation[210], ubiquitylation[211] or microRNAs[104], future studies are needed to determine whether activating adipose tissue AMPK under thermoneutral conditions in humans is sufficient for increasing adipose tissue energy expenditure. It will also be interesting to evaluate whether the activation of AMPK in adipose tissue in combination with appetite-suppressing therapies, such as GLP1 receptor agonists, may yield synergies towards weight loss and the treatment of obesity.

Non-alcoholic fatty liver disease, non-alcoholic steato-hepatitis and type 2 diabetes. NAFLD is defined as hepatic fat accumulation (>5% of liver weight) and includes a wide spectrum of liver pathology ranging from steatosis without inflammation to NASH, which increases the risk of type 2 diabetes, CVD, liver failure, cirrhosis and hepatocellular carcinoma[212]. There are currently limited pharmacotherapies that are effective for treating NAFLD and NASH[212]. An important and defining feature of NAFLD is elevated rates of liver de novo lipogenesis (DNL) and adipose tissue insulin resistance. Consistent with the role for AMPK in inhibiting DNL, activation of liver AMPK using A-769662 reduces NAFLD in rodent models[172]; however, the poor solubility, limited bioavailability and modest potency of A-769662 limited its use to preclinical studies. Since this time, multiple studies have established that genetically or pharmacologically activating AMPK reduces NAFLD, liver insulin resistance and markers of inflammation and liver fibrosis[74,186,213], effects that are primarily mediated through the phosphorylation of ACC and subsequent reductions in malonyl-CoA and liver DNL[74]. Consistent with their different mechanisms of AMPK activation discussed above, metformin and salicylate synergistically activate AMPK in vitro and in vivo, causing a greater suppression of DNL, liver triglycerides and insulin resistance than either treatment alone[181]. Whether these beneficial effects are also observed in humans remains to be determined.

While numerous AMPK activators exert positive effects in mouse models of NAFLD (reviewed in REF.[214]), a key challenge with respect to the development of direct AMPK activators for treating NAFLD in humans has been that, in contrast to mice[87], human liver consists of predominately the AMPK β2 isoform[57,58,181]. Recent studies testing an AMPK activator (PF-06409577) that has greater selectivity towards β1-containing complexes but also activates β2-containing complexes has shown positive effects on NAFLD and NASH in non-human primates, effects that were also observed in mice and shown to be completely dependent on liver AMPK and the phosphorylation of ACC[25]. Furthermore, novel

small-molecule inhibitors of ACC, which mimic the effects of AMPK phosphorylation, are currently in clinical development for the treatment of NASH[215], non-small-cell lung carcinoma[216] and hepatocellular carcinoma[217]. Interestingly, this activation of liver AMPK promoted a dramatic increase in SREBP1C, consistent with previous observations obtained with small-molecule inhibitors of ACC[218]. However, in contrast to ACC inhibition[218], AMPK activation did not increase triglyceride levels (hypertriglyceridaemia)[25]. These data suggest that AMPK regulation of additional substrates involved in triglyceride synthesis or packaging, such as glycerol-3-phosphate acyl transferase (GPAT)[219], may be important for restraining this therapeutic liability that has been observed in response to ACC inhibition. Future studies examining additional mechanisms by which AMPK reduces DNL without causing hypertriglyceridaemia and whether AMPK activation is also effective for reducing liver DNL in humans, will be important for determining whether AMPK activation is effective for treating NAFLD.

As well as directly phosphorylating ACC, reductions in liver DNL can also be achieved by starving ACC of its key substrate acetyl-CoA through several mechanisms[214]. Both salicylate, when delivered to mice as salsalate, and dinitrophenol cause mitochondrial uncoupling and reduce acetyl-CoA and NAFLD in mouse models, effects that are likely largely independent of AMPK activation[161,162]. Fatty acids derived from adipose tissue lipolysis also contribute to liver acetyl-CoA and can be reduced by promoting fatty acid oxidation within adipocytes through the browning of white fat (increasing the amount of beige and brite adipose tissue)[212,214]. Consistent with an important role for AMPK in regulating this process, mice lacking AMPK in adipose tissue have decreased beige adipose tissue in response to a β3 agonist and develop NAFLD independently of substantial alterations in adiposity[82]. Activation of AMPK with O304 also protects mice from developing NAFLD, effects that are associated with increases in white adipose tissue energy expenditure and reductions in lipolysis[28]. However, further studies with adipose tissue-targeted AMPK activators will be important to establish the therapeutic importance of adipose tissue AMPK for potentially treating NAFLD.

Improving adipose tissue insulin sensitivity by suppressing macrophage inflammation also reduces lipolysis and liver acetyl-CoA[212]. Genetic and pharmacological studies have established that the activation of macrophage AMPK is associated with the suppression of inflammation[220–222]. In addition, anti-inflammatory agents, such as salicylate and methotrexate, activate AMPK[19,223], while pro-inflammatory stimuli (for example, lipopolysaccharide and TNF) suppress AMPK activity by increasing protein phosphatase activity and through inhibitory phosphorylation[210,224–226], collectively supporting the concept that AMPK plays a vital role in mediating the balance between pro-inflammatory and anti-inflammatory stimuli[227,228]. The ability of AMPK to suppress multiple inflammatory pathways (for example, NF-κB, the NLRP3 inflammasome and ER stress) in response to numerous distinct stimuli suggests that

AMPK suppression of inflammatory programmes may be mediated in part through regulation of key tenets controlling cellular metabolism such as mitochondrial function, mitophagy and/or autophagy and fatty acid oxidation, all of which have been shown to regulate inflammatory pathways[220–222,229]. Additional levels of control may also involve the circadian clock, which is vital for regulating immune cell function[203] and is controlled through AMPK phosphorylation of cryptochrome 1 (REF.[230]). In addition, AMPK suppression of inflammatory pathways may also involve phosphorylation of C/EBP homologous protein (CHOP; also known as DDIT3)[231], a critical component of the ER stress response, as well as Janus kinase (JAK), which is required for activation of the signal transducer and activator of transcription (STAT) pathway[232]. Further studies examining whether AMPK also directly phosphorylates and inhibits key components of the NF-κB and NLRP3 inflammasome pathway are warranted. Importantly, mice lacking AMPK in macrophages have greater inflammation in liver and adipose tissue and elevated free fatty acids when fed a high-fat diet[220], supporting a potentially important role for macrophage AMPK in reducing the progression of NAFLD to NASH. However, further studies in more advanced models of NASH are required to confirm that activation of macrophage AMPK may be effective for reversing established disease, especially where substantial cirrhosis may have developed. Importantly, inflamed M1 macrophages can reduce liver fibrosis[233], an effect that might be impaired because of AMPK promoting a switch to anti-inflammatory M2 macrophages[227].

Another approach to help lower blood glucose and free fatty acids and potentially divert these substrates away from liver DNL involves enhancing skeletal muscle glucose uptake and fatty acid oxidation[214]. The activation of skeletal muscle AMPK using indirect AMPK activators such as AICAR or genetic mutations in the γ isoform enhances glucose uptake, glycogen synthesis, mitochondrial biogenesis, fatty acid oxidation and exercise capacity in rodent models[234]. With respect to glucose uptake, fatty acid oxidation and mitochondrial biogenesis, TBC1D1[126,127], acetyl-CoA carboxylase 2 (ACC2)[75] and PGC1α[103] appear to be important for the effects of AICAR in mice. AICAR also acutely increases skeletal muscle glucose uptake in healthy participants[235]; however, these effects are blunted in older individuals[236] and those with hyperinsulinaemia[237], suggesting that it may not be of substantial utility for increasing glucose uptake in many patients with insulin resistance. Other AMPK activators such as R419, a complex I inhibitor (TABLE 1), enhanced muscle mitochondrial content, exercise capacity and insulin sensitivity in control but not AMPK-muscle-null mice[238]; however, the potential for development of lactic acidosis precluded clinical development.

Given the positive effects of AMPK activators on glucose uptake, fatty acid oxidation, mitochondrial biogenesis and insulin sensitivity in preclinical models, more specific AMPK agonists targeting both the β1 and importantly the β2 isoform, which is highly expressed in skeletal muscle and important for stimulating glucose uptake[124], have recently been developed.

These pan-AMPK activators, 991 (REF.[183]), PF739 (REF.[24]), MK-8722 (REF.[26]) and O304 (REF.[28]), all enhance skeletal muscle glucose uptake and lower blood glucose in a variety of models including obese mice, dogs and non-human primates. Although speculative, this reduction in blood glucose independent of insulin might be anticipated to allow pancreatic β-cells to recover, potentially alleviating or reversing type 2 diabetes[24]. Despite these positive effects, mice and non-human primates treated with MK-8722 also developed cardiac hypertrophy with glycogen accumulation, but without cardiac dysfunction[26]. This led to the speculation that the cardiac adaptations with MK-8722 may be similar to those observed with chronic endurance exercise[26]. Given the known effects of AMPK activators to improve exercise capacity and spontaneous activity[238–240], it is interesting to speculate that perhaps this may have been a contributing factor to the cardiac hypertrophy observed with AMPK activation. Importantly, a recent phase IIa trial with O304 in individuals with type 2 diabetes treated with metformin has also shown glucose-lowering effects, however, without cardiac hypertrophy[28]. Future studies examining the mechanisms by which these new-generation AMPK activators reduce blood glucose and in some cases cause cardiac hypertrophy and whether they may also be effective for treating type 2 diabetes, NAFLD and/or NASH will be important.

Cardiovascular disease. Type 2 diabetes is an independent risk factor for CVD and accounts for most deaths in people with type 2 diabetes. The majority of heart attacks and strokes are caused by thrombus formation superimposed on disrupted atherosclerotic plaques[241]. This process is accelerated with type 2 diabetes and is linked to both elevations in low-density lipoprotein cholesterol (LDL-C) and chronic low-grade inflammation, both of which are regulated by AMPK. Given that the activation of AMPK catalyses the inhibitory phosphorylation on HMGR (Ser872), it could be anticipated that the corresponding suppression of cholesterol synthesis would promote the activation of SREBP1C, resulting in increased LDL receptor expression in the liver and subsequent reductions in LDL-C, similar to the effects observed with statin therapy[76]. Supporting this hypothesis, recent studies using a mouse model in which the AMPK phosphorylation site on HMGR is mutated to Ala have demonstrated that this phosphorylation event inhibits cholesterol synthesis and is important for suppressing serum and liver cholesterol levels[73]. Interestingly, these mice also develop fatty liver disease and insulin resistance, effects that are attributed to increases in SREBP1C and enhanced expression of lipogenic enzymes such as ACC and FASN. Although initial studies in mouse models using a variety of AMPK activators reported equivocal results in relation to plasma LDL-C or atherosclerosis progression[242–248], recent studies in a mouse model that has cholesterol profiles more closely resembling those seen in humans and in non-human primate models found that PF-06409577 lowered liver cholesterol, leading to the activation of SREBP1C and reductions in LDL-C[25]. Consistent with an inhibitory role for AMPK on cholesterol synthesis,

Atherosclerosis
The formation of solid plaques within arteries that block normal blood flow.

metformin has a modest effect on lowering LDL-C in patients with type 2 diabetes[249]. Future studies investigating whether the effects of AMPK activators are mediated through the phosphorylation of HMGR and whether this may be effective in reducing atherosclerosis in preclinical models and possibly in humans are now required. Supporting the therapeutic potential of targeting this pathway, the prodrug bempedoic acid (ETC-1002) is converted to ETC-1002-CoA in the liver, which allosterically activates AMPK and inhibits ACLY, resulting in lower LDL-C and atherosclerosis in mice[248]. The mechanisms by which ETC-1002-CoA directly activates AMPK are currently undefined but appear to involve direct interactions with the β1 isoform[248]. Phase III trials with ETC-1002 for CVD are currently underway, with early studies indicating reductions in LDL-C and inflammation in patients taking statins[250].

Reducing lipid-laden and inflamed macrophages within atherosclerotic plaques may also be an important strategy for reducing cardiovascular events[241]. In addition to lowering LDL-C, AMPK activation in response to salicylate and A769662 enhances reverse cholesterol efflux from macrophages owing to upregulation of the transport proteins ATP-binding cassette subfamily A member 1 (ABCA1) and ABCG1 (REFS[251,252]), or by increasing scavenger receptor class B type 1 (SRB1)-mediated hepatic delivery[247]. AMPK suppression of macrophage inflammation within plaques[229,231,253] and adipose tissue[220] may also be important for suppressing atherosclerosis development. In addition to regulating macrophage polarization, increased proliferation of monocytes to macrophages and reductions in autophagy are important for atherosclerosis[254], and in this regard, AMPK inhibition of cellular proliferative pathways such as mTOR and p53 as well as the induction of autophagy would be expected to reduce atherosclerosis progression[255]; however, this has not been observed in all studies[256]. Similarly, the induction of autophagy may reduce atherosclerosis and improve plaque stability, further supporting the possible beneficial role of activating macrophage AMPK[257]. However, recent clinical trials with salsalate in combination with statin therapy for 30 weeks reported no reductions in atherosclerosis compared with statin therapy alone[258]; however, there were improvements in glycaemia, supporting the potential role for this therapy in people at high risk of developing type 2 diabetes[259]. Further studies in mice with AMPK-activating and AMPK-inactivating mutations are now needed to determine whether targeting this pathway may be effective in reducing atherosclerosis and blood glucose.

Besides elevations in LDL-C and inflammation, an important risk factor for CVD is hypertension. Numerous studies have indicated that AICAR lowers blood pressure in both rodents[260] and humans[237], findings that have more recently also been observed using PF-06409577 (REF.[27]) and O304 (REF.[28]). While the exact mechanisms mediating these hypotensive effects are not entirely clear, activation of AMPK using pharmacological stimuli (for example, AICAR[28,260] or O304 (REF.[28])) consistently promotes vasodilation. AMPK may facilitate vasodilation by increasing endothelial nitric oxide through phosphorylation of endothelial nitric oxide synthase (eNOS) (Ser633

and Ser1177)[261] and angiotensin-converting enzyme 2 (Ser680)[262], and by promoting an increase in calcium within vascular smooth muscle cells[263]. Importantly, these anti-hypertensive effects have also been observed in a recent phase IIa study in people with type 2 diabetes using O304, suggesting that this may be an important mechanism by which AMPK activation could reduce cardiovascular events.

Much of the mortality associated with CVD is the result of heart failure. Cardiac AMPK is increased in most cases of heart failure, consistent with lower levels of ATP and reductions in oxidative metabolism (reviewed in REF.[264]), suggesting that the activation of AMPK in this context is a consequence and not a cause of heart failure. Loss of cardiac AMPK reduces systolic and diastolic function, an effect that results in ventricular shortening in the absence of changes in fatty acid or glucose metabolism or cardiac hypertrophy[95,265]. However, hypertrophy is also observed with chronic genetic and pharmacological activation of AMPK, effects that, in contrast to Wolff–Parkinson–White syndrome, have been associated with increased stroke volume[26,195,266] and subsequent reductions in heart rate[267], suggesting that activation of cardiac AMPK may be potentially beneficial in people with heart failure. However, the pathways controlling cardiac hypertrophy are complex, and under some conditions, the activation of AMPK has been shown to be protective against cardiac hypertrophy[268]. Activating cardiac AMPK may also help protect against cardiac reperfusion injury by enhancing glucose uptake[269,270] and suppressing ER stress[271]. Future studies investigating the effects of direct pharmacological AMPK activators in the context of heart failure and cardiac reperfusion injury are required.

Summary. As detailed above, the pharmacological activation of AMPK exerts positive effects on many aspects of cardiometabolic disease including hyperglycaemia, hyperlipidaemia, NAFLD, insulin resistance, hypertension and chronic low-grade inflammation. These effects are likely mediated through the simultaneous modulation of multiple molecular targets in several different tissues including adipose tissue, liver, immune cells such as macrophages, skeletal and cardiac muscle and the kidney (discussed below). In aggregate, these diverse disease-modifying activities might be expected to exert substantial positive effects on cardiometabolic risk in a more effective manner than existing standards of care such as metformin, GLP1 agonists and SGLT2 inhibitors. Clinical studies investigating these actions are currently underway with some AMPK-activating therapies.

Cancer

Mutations in tumour suppressors and oncogenes such as phosphatase and tensin homologue (PTEN), LKB1, mTOR, p53, RAS and MYC are often associated with reductions in AMPK activity owing to alterations in transcription[272], phosphorylation[32,43,273,274] or ubiquination[275,276]. Similarly, increases in AMPK expression and activity have been linked to improved survival in multiple tumour types in humans[277], suggesting a potentially important role for AMPK in cancer.

Wolff–Parkinson–White syndrome
An electrical conductance abnormality that leads to increased heart rate (tachycardia); mutations in AMP-activated protein kinase γ2 are often associated with this syndrome.

When mice lacking AMPK α1 (REF.[278]) and β1 (REF.[279]) subunits are crossed with MYC-overexpressing mice or p53-null mice, respectively, there is an increase in the appearance of T cell lymphomas and reduced survival. A defining feature of the tumours lacking AMPK was reductions in ACC phosphorylation and an increased rate of de novo lipogenesis[278,279]. In both cells and mice in which ACC is insensitive to AMPK phosphorylation, lipogenesis, cell proliferation and tumorigenesis are also increased[217]. Similarly, the inhibition of de novo lipogenesis in response to indirect (metformin, phenformin or canagliflozin) and direct (salicylate, MT-6378 or 991) AMPK activators correlates strongly with decreased proliferation and colony formation in many tumour types[21,280–282], effects that are enhanced when direct and indirect activators are used in combination[280] and could involve the suppression of mitosis[283]. However, it should be noted that some of these effects on lipogenesis may be related to alterations in mitochondrial function that do not require AMPK[21,162,282,284]. Further supporting the concept that inhibiting lipogenesis may be important for limiting cancer cell proliferation, small-molecule inhibitors of ACC, which mimic the effects of AMPK phosphorylation of ACC to prevent dimerization, suppress the growth of both non-small-cell lung carcinoma[216] and hepatocellular carcinoma[217] in mice. In addition to directly inhibiting tumour lipogenesis, AMPK activation would also be expected to exert multiple beneficial systemic effects to inhibit tumour growth. This includes the phosphorylation and degradation of programmed cell death ligand 1 (PD-L1), which enhances immune destruction of tumours[285], along with lowering of blood glucose and insulin, which would be expected to restore AMPK activity, stabilizing the epigenetic modifying enzyme TET2 (REF.[286]), and enhance the effectiveness of PI3K inhibitors[287]. Collectively, these tumour-directed and systemic roles for AMPK have laid the foundation for the initiation of many clinical trials examining the effects of metformin, phenformin or salicylates in preventing many cancers or for enhancing the effects of radiation and chemotherapeutics. Future studies examining whether similar beneficial effects are also observed in preclinical models in vivo with the new generation of direct AMPK activators are warranted.

AMPK may also exert anti-neoplastic activities though both direct and indirect regulation of many other important pathways vital for regulating cell growth and proliferation. AMPK inhibits mTOR activity through direct phosphorylation of TSC and Raptor[114,273], which in turn suppresses protein synthesis and translation through inhibition of 4EBP1 and S6 kinase, respectively. Cell growth is further limited through AMPK inhibition of both the Hedgehog and Hippo pathways, effects that are mediated through phosphorylation of GLI1 and YAP[288,289]. AMPK might also indirectly inhibit the Hippo pathway through phosphorylation of HMGR, which would be expected to reduce cholesterol synthesis, protein prenylation and RHO GTPase activity, which is essential for activating YAP. Similarly, in some tumour types, AMPK may also inhibit RAS activity through both direct phosphorylation and inhibition of BRAF (Ser729)[290] and indirectly through inhibition of HMGR

and subsequent protein prenylation[73,76], which is required for activation of this pathway and subsequent increases in MAPK–ERK signalling. AMPK also promotes cell cycle arrest through activation of tumour suppressors such as p53 (REFS[291,292]), retinoblastoma protein[293] and p27 (REF.[294]), effects that may be mediated through direct phosphorylation or indirectly through the phosphorylation of proteins critical for controlling acetylation such as SIRT1 (Thr344)[77]. Further fine tuning of cell cycle progression may involve phosphorylation of key components of the mitotic machinery such as protein phosphatase 1 regulatory subunit 12 C, p21-activated protein kinase, SNX17 (Ser437) and CDC42EP1 (Ser192)[295]. Consistent with a role in inhibiting cell division, AMPK also phosphorylates and inhibits proteins critical for cell invasion, migration and/or motility and adhesion such as neuroepithelial cell-transforming 1 (NET1) (Ser46), cingulin (Ser137) and CLIP170 (REF.[296]) while at the same time stabilizing existing cell junctions to maintain cell polarity through phosphorylation of Gα-interacting vesicle-associated protein (Ser245)[297]. AMPK activation therefore modulates numerous distinct pathways, which collectively limits cell proliferation and growth, suggesting that therapies targeting AMPK may be effective for preventing disease or slowing disease progression. However, activation of these pathways is unlikely to induce apoptosis or cell death, indicating that AMPK activation may not be sufficient for treating established disease.

There are several contexts where increasing or maintaining cellular AMPK activity may be undesirable. For example, reductions in LKB1 and/or AMPK increase tumour sensitivity to mitochondrial complex I inhibitors such as phenformin[298]. Similarly, in KRAS and p53 lung tumours, a deficiency in AMPK reduces tumour load[299]. The treatment of tumour cells with a direct AMPK activator (A769662) has also been shown to promote proliferation under hypoxic conditions[284]. The mechanisms by which AMPK promotes survival are likely multifaceted and tumour-dependent. For example, under hypoxic conditions or in the presence of complex I inhibitors (that is, phenformin and/or metformin), AMPK activation may enhance mitochondrial biogenesis and/or spare respiratory capacity[284,300] and glucose uptake[134], together promoting cell survival. AMPK may also promote cell growth, metastasis and anchorage-dependent growth through AMPK phosphorylation of GFAT, which enhances angiogenesis[142], and ACC phosphorylation, which enhances fatty acid oxidation[301]. Lastly, as the activation of autophagy is an important mechanism by which cells avoid apoptosis, activation of this pathway by AMPK may lead to resistance to therapeutics such as RAS inhibitors[302]. Therefore, while activating AMPK may exert many positive effects with respect to cancer prevention, it will be important that therapeutics targeting AMPK are carefully considered and evaluated in the context of tumour type, microenvironment and treatment regimens such as radiation, chemotherapeutics and immunotherapy.

Emerging indications for AMPK

Neuromuscular disorders. AMPK exerts many effects on muscle function, which may be beneficial for the treatment of neuromuscular disorders[303]. Muscles from

mice lacking AMPK rapidly fatigue, an effect that is associated with an increased number of glycolytic fibres, centrally located nuclei and split and necrotic myofibres[123,304]. By contrast, the chronic activation of AMPK promotes the development of slow oxidative fibres[240,305], which are important for improving muscle function in a mouse model of Duchenne muscular dystrophy and may involve the upregulation of utrophin A[306]. AMPK activation also increases expression of the dystrophin-associated protein complex (DAPC), MYOD and myogenin[307] while also enhancing autophagic flux[150], all of which are impaired in many neuromuscular disorders[303]. AMPK is also important for maintaining capillary density and vascular perfusion effects associated with the phosphorylation (Ser1446) of neuronal NOSμ (nNOSμ)[308]. Interestingly, several years before this finding, AMPK had been shown to phosphorylate nNOS in muscle in response to exercise[309]. Furthermore, mice and humans treated with pharmacological AMPK activators have increased blood perfusion into muscle, which may also help improve muscle function[237,310,311]. Future studies examining the effects of muscle-specific AMPK activators in mouse models of myopathic disease such as Duchenne muscular dystrophy will be important.

Kidney disease. Chronic hypertension, obesity and type 2 diabetes accelerate the progression of chronic kidney disease and autosomal dominant polycystic kidney disease towards end-stage renal disease. Metformin-induced activation of AMPK improves renal function, an effect that has recently been linked to reductions in fibrosis and the phosphorylation of ACC[312]. Recent studies have also examined the effects of a direct pharmacological AMPK β1 activator in a mouse model of diabetes and hypertension and found marked reductions in proteinuria, improvements in kidney histology and reductions in S6 phosphorylation, suggesting that activation of AMPK may reduce the development of diabetic nephropathy by suppressing mTOR[27].

Chronic pain. A number of studies have revealed that AMPK activation might counteract pathways that promote neuronal activity involved in signalling pathological pain[313]. In preclinical models, the activation of AMPK can reduce the excitability of nociceptors, effects that are blocked in mouse models lacking AMPK α2 (REF.[314]). In the setting of chronic pain, AMPK activation inhibits the growth and plasticity of the neuronal circuits that detect pain[313] while also reducing interleukin-1β and increasing astrocyte glutamate clearance[315]. Mechanistically, these effects are attenuated in mice lacking AMPK α1 and may involve the inhibition of mTOR or MAPK. And although the precise role of AMPK in nociception is currently unclear, it provides a potentially attractive target for treatment of chronic pain.

Ageing. It has been known for many years that AMPK activity is diminished with ageing in tissues of humans and rodents[316–318]; however, the mechanisms by which this occurred were unclear. Recent studies have indicated that DNA-dependent protein kinase (DNA-PK), whose activity is increased in response to DNA double-strand breaks that are increased with ageing, inhibits AMPK activity by repressing Thr172 phosphorylation through upregulation of a protein chaperone that inhibits LKB1 activity[319]. Many therapies that activate AMPK such as endurance exercise, caloric restriction, resveratrol and metformin also improve longevity and health span[98,320]. The mechanisms by which AMPK elicits these effects are likely complex; however, one important and obvious link involves the induction of autophagy, which is reduced in numerous organs with ageing and has been identified as a critical factor that may reduce longevity[321–323]. Of particular importance may be AMPK regulation of mitochondrial fusion and autophagy or mitophagy[97,98,324], as ageing is often associated with the accumulation of giant, defective mitochondria that have a decreased respiratory capacity and are unable to take part in fusion, which is required to replenish contents and initiate mitophagy[325]. The inability to remove these large mitochondria perpetuates further growth and an increased population of damaged mitochondria that contribute poorly to ATP production, which may further exacerbate ageing-related declines[326]. Consistent with this idea, mice lacking skeletal muscle AMPK have defects in mitophagy that lead to large dysfunctional mitochondria and sarcopenia[150]. Interestingly, skeletal muscle AMPK is also important for controlling the ageing of skin, effects that are mediated through transcriptional control of interleukin-15, which is an important cytokine regulating mitochondrial biogenesis[327]. Activation of AMPK may also be beneficial for ageing owing to its suppressive effects on multiple inflammatory pathways[227,228], which may involve the control of mitochondrial content[220]. Indeed, metformin increases AMPK and mitochondrial function in multiple tissues, effects that are associated with reductions in oxidative damage, chronic low-grade inflammation and improved health span and lifespan in mice[320]. The AMPK activator quercetin when used in combination with dasatinib has also been shown to exert anti-ageing effects by delaying senescent cell accumulation or reducing senescent cell burden in numerous tissues of rodents[328]. Whether these beneficial effects are replicated in humans and/or are observed with the new generation of direct AMPK activators that do not inhibit mitochondrial function remains to be determined.

Challenges and outlook

There is now overwhelming evidence supporting the hypothesis that AMPK activation is beneficial for both the prevention and treatment of a wide variety of chronic diseases. The identification of small-molecule activators, coupled with detailed mechanistic and structural information[9] regarding the nature of their binding and mode of action, as well as unique tissue-specific genetic tools, now provides the basis for trials that will directly address the efficacy of AMPK activation across distinct disease conditions. Indeed, studies have already demonstrated that in cardiometabolic disease AMPK activation may have substantial beneficial effects in lowering plasma glucose and lipids, NAFLD and blood pressure in preclinical models, findings that have been confirmed in recent phase II clinical trials with direct

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Nociceptors
Neurons that respond to damaging stimuli and transmit a response to the brain that is perceived as pain.

REVIEWS

AMPK activators such as O304. Future studies examining whether these effects are additive or perhaps synergistic with metformin, SGLT2 inhibitors or GLP1 receptor agonists will be important.

However, like all therapies for chronic diseases, there remain important safety issues that need to be carefully considered and examined, such as the potential for AMPK activation to promote cardiac hypertrophy or the survival of cancer cells under hypoxic conditions. To avoid these potential liabilities, the development of AMPK activators that can be targeted to specific tissues (for example, liver, muscle and macrophages) by taking advantage of isoform-specific selectivity may be beneficial. For example, the availability of AMPK γ3-selective activators could restrict AMPK activation to skeletal muscle, reducing the risk of adverse effects of AMPK activation in other tissues and cell types. Another important consideration would be the optimal pharmacokinetics and pharmacodynamics of a

small molecule designed to activate AMPK. For example, both acute endurance exercise and metformin exert positive health benefits associated with transient activation of AMPK[329]. Therefore, although speculative, it is possible to envision that, unlike typical drug design, where prolonged target engagement is often desired, the optimal strategy to maximize benefits and minimize liabilities may be to develop a therapy that acutely switches on AMPK once or twice per day following a meal.

Despite the potential challenges, ultimately, the only way to determine the long-term safety and efficacy of AMPK activators will be to conduct more extensive clinical trials. Until these studies are completed, the field will be poised for the answer to the long-awaited question of whether AMPK will provide the target for a metabolic wonder drug.

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