Review

MOLECULAR METABOLISM

The metabolic sensor AMPK: Twelve enzymes in one

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ABSTRACT

Background: AMP-activated protein kinase (AMPK) is an evolutionarily conserved regulator of energy metabolism. AMPK is sensitive to acute perturbations to cellular energy status and leverages fundamental bioenergetic pathways to maintain cellular homeostasis. AMPK is a heterotrimer comprised of αβγ-subunits that in humans are encoded by seven individual genes (isoforms α1, α2, β1, β2, γ1, γ2 and γ3), permitting formation of at least 12 different complexes with personalised biochemical fingerprints and tissue expression patterns. While the canonical activation mechanisms of AMPK are well-defined, delineation of subtle, as well as substantial, differences in the regulation of heterogenous AMPK complexes remain poorly defined.

Scope of review: Here, taking advantage of multidisciplinary findings, we dissect the many aspects of isoform-specific AMPK function and links to health and disease. These include, but are not limited to, allosteric activation by adenine nucleotides and small molecules, co-translational myristoylation and post-translational modifications (particularly phosphorylation), governance of subcellular localisation, and control of transcriptional networks. Finally, we delve into current debate over whether AMPK can form novel protein complexes (e.g., dimers lacking the α-subunit), altogether highlighting opportunities for future and impactful research.

Major conclusions: Baseline activity of α1-AMPK is higher than its α2 counterpart and is more sensitive to synergistic allosteric activation by metabolites and small molecules. α2 complexes however, show a greater response to energy stress (i.e., AMP production) and appear to be better substrates for LKB1 and mTORC1 upstream. These differences may explain to some extent why in certain cancers α1 is a tumour promoter and α2 a suppressor. β1-AMPK activity is toggled by a 'myristoyl-switch' mechanism that likely precedes a series of signalling events culminating in phosphorylation by ULK1 and sensitisation to small molecules or endogenous ligands like fatty acids. β2-AMPK, not entirely beholden to this myristoyl-switch, has a greater propensity to infiltrate the nucleus, which we suspect contributes to its oncogenicity in some cancers. Last, the unique N-terminal extensions of the γ2 and γ3 isoforms are major regulatory domains of AMPK. mTORC1 may directly phosphorylate this region in γ2, although whether this is inhibitory, especially in disease states, is unclear. Conversely, γ3 complexes might be preferentially regulated by mTORC1 in response to physical exercise.

Keywords AMPK; mTORC1; Signalling; Metabolism; Cancer; Exercise

1. INTRODUCTION

Central to the survival and growth of all living organisms is the synchronous regulation of cellular metabolic pathways coupled with fluctuating levels of systemic hormones and nutrients. The AMP-activated protein kinase, AMPK, is a nutrient-sensitive fundamental regulator of cellular growth and metabolism. Dubbed the metabolic "fuel gauge" [1], the major function of AMPK is to directly sense changes in energy and nutrient levels and respond by phosphorylating downstream targets involved in virtually every branch of cellular metabolism. The result is potentiation of catabolic, ATP-generating processes (e.g., fatty acid oxidation, mitochondrial biogenesis, glucose uptake) and repression of anabolic, ATP-consuming processes (e.g., protein and lipid synthesis), ultimately restoring cellular and whole-body metabolic homeostasis. Myriad bioenergetic perturbations switch on AMPK kinase activity, including physiological stimuli like physical exercise and caloric restriction, and noxious stimuli like cachexia and cardiac ischemia. Dysregulated cellular metabolism is a hallmark of numerous diseases, particularly obesity, type 2 diabetes and cancer, and AMPK's central role as a metabolic sensor has made this enzyme an incredibly attractive drug target for these, and other pathologies relatedly characterised by defective metabolism.

AMPK is a phylogenetically conserved, heterotrimeric serine/threonine protein kinase comprised of a catalytic α-subunit, and two regulatory β- and γ-subunits. In invertebrates, each subunit is encoded by a single gene [2], but in humans, the presence of seven different genes give rise to multiple isoforms of each subunit; *PRKAA1* and *PRKAA2* (encoding α1 & α2), *PRKAB1* and *PRKAB2* (encoding β1 & β2), and

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Abbreviations

α-KG	α-ketoglutarate
α-γ-SBS	α-γ-subunit binding sequence
β-SID	β-subunit interacting domain
53BP1	p53-binding protein 1
ACC	Acetyl-CoA carboxylase
ADaM	Allosteric drug and metabolite
AgRP	Agouti-related peptide
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AID	Autoinhibitory domain
AMPK	AMP-activated protein kinase
ARC	Arcuate nucleus
ARHGEF9	Cdc42 guanine nucleotide exchange factor 9
AS160	Akt substrate of 160 kDa
CaMKK2	Ca^{2+}/calmodulin-dependent protein kinase kinase 2
CBM	Carbohydrate-binding module
CBS	Cystathionine β synthase
Cdc	Cell division cycle protein
CDK2	Cyclin-dependent protein kinase 2
CHD1L	Chromodomain helicase DNA-binding protein 1-like
CLEAR	Coordinated lysosomal expression and regulation
CpdC	Compound C
DHAP	Dihydroxyacetone phosphate
dKO	Double knockout
DNA-PK	DNA-activated protein kinase
EDL	Extensor digitorum longus
eEF2	Eukaryotic translation elongation factor 2
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
EXO1	Exonuclease 1
FA-CoA	Fatty acyl-CoA
FLCN	Folliculin
FOXO3a	Forkhead box transcription factor 3a
FBP	Fructose-1,6 bisphosphate
GDH	Glutamate dehydrogenase
GLUT4	Glucose transporter 4
GSIS	Glucose-stimulated insulin secretion
HDX-MS	Hydrogen deuterium exchange mass spectrometry
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase
HSP90	Heat shock protein 90
HUNK	Hormonally up-regulated Neu-associated kinase
KD	Kinase domain
LKB1	Liver kinase B1
mTORC1	mammalian target of rapamycin complex 1
NES	Nuclear export signal
NLS	Nuclear localisation signal
NMT1	N-myristoyltransferase 1
NOX2	NADPH oxidase 2
NPC	Nuclear pore complex
NPY	Neuropeptide Y
NTE	N-terminal extension
NUAK2	NUAK family SNF1-like kinase 2
Nur77	Nuclear receptor 4A1
PARP1	Poly (ADP-ribose) polymerase 1
PCoA	Palmitoyl-CoA
PDB	Protein databank
PDHc	Pyruvate dehydrogenase complex catalytic subunit
PGC-1α	PPARγ-coactivator-1α
PHF2	PHD finger protein 2
PKA	Protein kinase A
POMC	Pro-opiomelanocortin
PPP6C	Protein phosphatase 6C
RIM	Regulatory subunit-interacting motif
ROS	Reactive oxygen species
RYR2	Ryanodine receptor 2
SNP	Single nucleotide polymorphism
STK11	Serine/threonine kinase 11
ST loop	Serine/threonine-rich loop
STU	Staurosporine
TBC1D1	TBC1 domain family member 1
TFEB	Transcription factor EB
TIF-1A	Transcription initiation factor 1A
TRPV2	Transient receptor potential cation channel subfamily V
UHRF1	Ubiquitin-like with PHD and RING finger domains 1
ULK1	Unc-51-like kinase 1
v-ATPase	Vacuolar H^+-ATPase
$\dot{V}O_{2peak}$	Peak oxygen consumption

PRKAG1, *PRKAG2* and *PRKAG3* (encoding γ1, γ2 & γ3), altogether permitting assembly of 12 distinct complexes without even accounting for splice variants. This evolutionary phenomenon ensures that isoforms with intrinsically unique biochemical properties are expressed in, and accommodate the bioenergetic needs of, metabolically heterogenous cells and tissues. Examples of known or predicted heterotrimeric combinations of AMPK in different human tissues are presented in Figure 1A.

Since the last devoted AMPK isoform review by Ross and colleagues in 2016 [3], a substantial amount of literature has described advances and new concepts in isoform-specific AMPK regulation and the physiological implications. For instance, there has been a push to re-evaluate the role of AMPK isoforms in cancer [4]. Traditionally viewed as a tumour suppressor, AMPK is now often contextually labelled a tumour promoter depending on the expression and behaviour of different isoforms in certain malignancies. The number of reported AMPK isoform-specific post-translational modifications has also grown considerably since 2016 [5]. Both up and downstream of AMPK is the mammalian (or mechanistic) target of rapamycin (mTOR), another serine/threonine kinase that assembles into at least two functionally and structurally dissimilar complexes (mTORC1/2). mTORC1, which is highly oncogenic and drives cellular growth and proliferation in response to hormonal and nutrient cues [6], is negatively regulated by AMPK and itself counterbalances AMPK activity by direct and indirect phosphorylation [7–11]. This constitutes a fundamental feedback loop integral to cellular homeostasis, as the vast majority of processes mTORC1 is responsible for are antagonistic to AMPK. However, the relationship AMPK shares with mTORC1 varies depending on a given subunit isoform (e.g. [12]), which becomes especially pertinent in a variety of disease states where both kinases can be simultaneously active, an area we delve into herein.

This review provides comprehensive analysis on the sophisticated nature of isoform-specific AMPK function. For a more detailed outline of the numerous cellular processes regulated by AMPK in general, including broader implications for health and disease, we refer the reader to several excellent reviews written by leading experts in this field [13–17]. Here, to dissect isoform-specific AMPK function, we draw upon knowledge gained from multidisciplinary approaches ranging from basic, structural and biophysical studies to clinical trials and large-scale genome-wide association studies. We also discuss

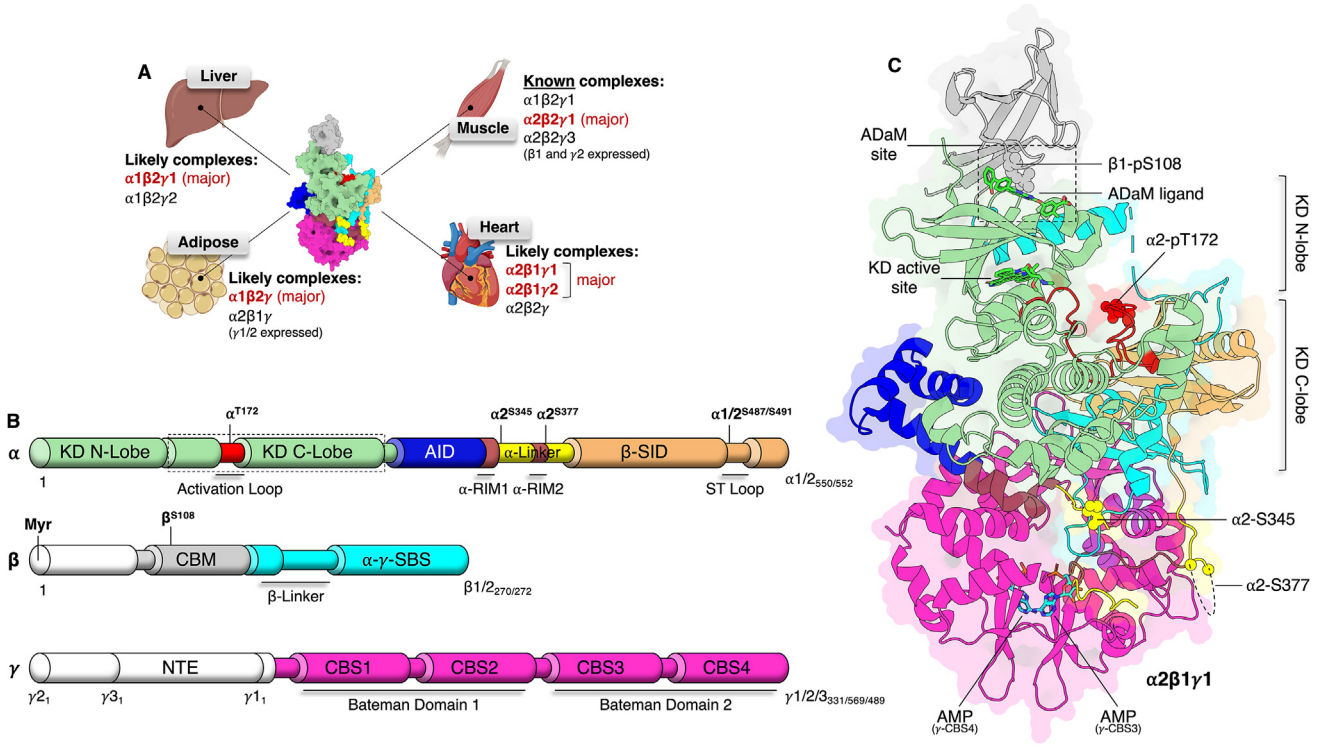

Figure 1: Structure of the active AMPK heterotrimer. A) Known and likely heterotrimeric combinations of AMPK in human tissues based on individual studies reporting isoform expression and/or complex assembly in liver [106,107], heart [106,219], adipose tissue [263,330,331] and skeletal muscle [282,285]. **B**) Schematic representation of the organisation of each AMPK subunit. Major structural features are presented as a linear tube with relevant phosphorylation sites indicated; Myr denotes myristoylation of the β-subunit. **C**) Cartoon representation of the crystal structure of an active AMPK α2β1γ1 heterotrimer in complex with staurosporine in the active site (situated between the N-lobe and C-lobe), AMP bound to γ-CBS3 and γ-CBS4, and the small molecule SC4 in the ADaM site formed between the β1-CBM and α2-kinase domain (KD) (PDB: 6B1U). The structure is colour coded according to the schematic in **B**.

several unresolved, and at times controversial topics, with the aim to highlight gaps in our understanding that ideally will be filled by collaborative efforts in the years to come.

2. AMPK SUBUNIT ISOFORM STRUCTURE AND REGULATION

Domain structures of all three AMPK subunits are shown in Figure 1B alongside a crystal structure of an active α2β1γ1 human complex (Figure 1C) [18]. Amino acid numbering and the level of sequence conservation of all subunit isoforms and their major domains are presented in Table 1, with an accompanying summary of each function that we discuss in detail in the following section.

2.1. α-subunit

α1 is ubiquitously expressed, with high levels in adipose and breast tissue, lung, liver and brain [19]. α2 has greater tissue specificity, being abundantly expressed in skeletal and cardiac muscle, and lowly expressed in the kidney and brain [19]. The AMPK kinase domain lies within the N-terminal region of the α-subunit and is the most well-conserved domain between the two α isoforms (89%; Table 1). The kinase domain adopts an archetypal bilobal fold comprised of a smaller N-lobe (five β-strands and two α-helices; αB & αC) and a larger, mostly helical (αD-αI) C-lobe with the catalytic cleft sandwiched between the two (Figure 1C). Indicative of many eukaryotic protein kinases, the kinase domain C-lobe of α1 and α2 contains a completely conserved and flexible activation loop stabilised by phosphorylation of a specific threonine residue to facilitate substrate binding. This primary phosphorylation site is T174 in α1 and T172 in α2, although generally T172 nomenclature is used. The two principal α-T172 kinases are liver kinase B1 (LKB1) and the Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2) [20–25], with phosphorylation enhancing AMPK activity by 50- to 100-fold [22,26]. Active AMPK displays all the hallmarks of a catalytically competent protein kinase, including a salt bridge formed between α-K47 in the β3-strand and α-E66 in the αC-helix (α1 numbering) [18], along with assembly of two hydrophobic regulatory and catalytic "spines" that traverse both lobes of the kinase domain connecting its critical elements [27].

C-terminal to the kinase domain is a tri-helical bundle termed the autoinhibitory domain (AID). The α-AID makes direct contacts with the hinge region (between N- and C-lobes) at the back of the kinase domain in AMPK's inactive state but rotates away during the activation cycle [28]. Immediately adjacent to the α-AID is the α-linker, which contains two γ-subunit-interacting α-RIM (Regulatory Subunit-Interacting Motif) modules integral to nucleotide-sensing [29]. The C-terminus of the α-subunit is completed by a β-subunit interacting domain (β-SID), with the tail containing a nuclear export signal (NES) that is functional in both α1 and α2 [30]. Although α2, and possibly α1, contain putative nuclear localisation signals (NLSs) in the kinase domain C-lobe (α1: 226–228; α2: 224–227) [31], the NLS is not always required for AMPK nuclear translocation [30]. The most noticeable difference between the two isoforms is a divergent and supposedly unstructured C-terminal (~60 amino acids; Table 1) serine/threonine-rich 'ST loop'. The ST loop is decorated by a range of phospho-sites, for which the most well-characterised is α1-S487 that inhibits α1-T174 phosphorylation by LKB1, and the equivalent α2-

Table 1 — Human AMPK subunit isoform sequence conservation of major structural elements.

Major domains	Amino acids	Isoform Conservation	Domain Features
Human α-subunit: α1 (550 residues), α2 (552 residues); 77% sequence conservation			
Kinase Domain	α1: 1—289 α2: 1—287	89%	• Adopts a classical bilobal fold indicative of eukaryotic protein kinases. • Harbours the activation loop.
Activation loop	α1: 159—185 α2: 157—183	100%	• Contains a phosphorylatable threonine (α1-T174, α2-T172) targeted by upstream kinases.
AID	α1: 290—333 α2: 288—331	75%	• Tri-helical bundle that binds the kinase domain and inhibits AMPK. • Rotates away from the kinase domain upon AMP binding to the γ-subunit.
α-linker	α1: 334—395 α2: 332—399	55%	• Harbours the RIM domains implicated in nucleotide-sensing. • Region of mTORC1 phosphorylation.
α-RIM1	α1: 334—344 α2: 332—342	73%	• Associates with nucleotide-free γ-CBS2.
α-RIM2	α1: 360—370 α2: 364—374	64%	• Associates with AMP bound to γ-CBS3.
β-SID	α1: 396—550 α2: 400—552	63%	• Forms a complex scaffold with the β-subunit. • Extreme C-terminal portion contains a nuclear export signal.
ST loop	α1: 471—530 α2: 475—532	36%	• Unstructured region modified heavily by phosphorylation.
Human β-subunit: β1 (270 residues), β2 (272 residues); 71% sequence conservation			
N-terminus	β1: 1—67 β2: 1—66	42%	• Predicted to be mostly unstructured. • Co-translationally myristoylated at glycine 2. • Required for AMPK complex stability.
CBM	β1: 68—158 β2: 67—158	77%	• Positioned on top of the kinase domain in active AMPK. • Forms the 'ADaM' site required for allosteric AMPK activation. • Contains serine 108 *cis*-autophosphorylation site or substrate of an upstream kinase (e.g., β1-S108).
β-linker	β1: 159—185 β2: 159—187	67%	• Provides a helix that interacts with the kinase domain αC-helix that protects the activation loop against phosphatases. • C-terminal β-loop phosphorylated on β-S182/184.
α-γ-SBS	β1: 186—270 β2: 188—272	88%	• Three-stranded anti-parallel β-sheet that forms part of the AMPK core. • Interacts with a β-sheet from the γ-subunit scaffold region.
Human γ-subunit: γ1 (331 residues), γ2 (569 residues), γ3 (489 residues); γ1 vs. γ2 = 71%, γ1 vs. γ3 = 59%, γ2 vs. γ3 = 49% sequence conservation			
NTE	γ1: 1—27 γ2: 1—259 γ3: 1—182	γ1 vs. γ2: 22% γ1 vs. γ3: 16% γ2 vs. γ3: 19%	• Completely unrelated among all three isoforms. • Predicted to be mostly unstructured. • Region of post-translational modifications, especially for γ2 by proline-directed kinases (likely mTORC1).
Pre-CBS1	γ1: 28—42 γ2: 260—274 γ3: 183—197	γ1 vs. γ2: 60% γ1 vs. γ3: 47% γ2 vs. γ3: 60%	• Required for complex formation. • Forms a kinked helix in crystal structures of γ1. • Predicted to be helical in γ2 and γ3.
Scaffold	γ1: 45—52 γ2: 277—284 γ3: 200—207	γ1 vs. γ2: 100% γ1 vs. γ3: 88% γ2 vs. γ3: 88%	• First β-sheet of γ-CBS1. • Interacts with the α-γ-SBS to stabilise the AMPK complex. • Possibly involved in AMPK βγ dimer formation.
γ-CBS1	γ1: 43—103 γ2: 275—335 γ3: 198—258	γ1 vs. γ2: 92% γ1 vs. γ3: 84% γ2 vs. γ3: 84%	• Low-affinity site of nucleotide exchange. • Combines with γ-CBS2 to form Bateman domain 1.
γ-CBS2	γ1: 125—185 γ2: 357—417 γ3: 280—340	γ1 vs. γ2: 80% γ1 vs. γ3: 67% γ2 vs. γ3: 70%	• Incapable of nucleotide binding due to a ribose-interacting aspartate found in other CBS domains replaced with an arginine. • Combines with γ-CBS1 to form Bateman domain 1.
γ-CBS3	γ1: 198—260 γ2: 430—492 γ3: 353—415	γ1 vs. γ2: 75% γ1 vs. γ3: 60% γ2 vs. γ3: 59%	• Principle site of nucleotide exchange. • Forms an interaction network with α-RIM2 that leads to AMPK allosteric activation and protection of α-T172 against dephosphorylation. • Combines with γ-CBS4 to form Bateman domain 2.
γ-CBS4	γ1: 272—329 γ2: 504—561 γ3: 427—484	γ1 vs. γ2: 66% γ1 vs. γ3: 53% γ2 vs. γ3: 53%	• Permanently houses AMP. • Stabilises γ-CBS3 upon AMP binding. • Combines with γ-CBS3 to form Bateman domain 2.

S491, both an autophosphorylation site and substrate of inhibitory upstream kinases [9,32–34].

2.2. β-subunit

β1 is highly expressed in the kidney, cardiac tissue and lung, whereas β2 is abundant in skeletal muscle, liver, adipose tissue and cardiac muscle. The N-terminal, unstructured regions of the β isoforms display the highest level of sequence divergence (42% conservation; Table 1). Both isoforms are co-translationally myristoylated (attachment of C14 myristic acid) at glycine 2 after removal of the initiator methionine [35,36], which enables membrane association of AMPK with intracellular organelles such as mitochondria and lysosomes [37,38]. Following this region is a highly dynamic carbohydrate-binding module (CBM). Despite conservation of residues required for glycogen binding by the CBM, the β2-CBM binds linear and single α1,6-branched carbohydrates with greater affinity than β1-CBM [39,40], in part due to an additional threonine residue in β2 (T101) that increases flexibility of a loop accommodating the α1,6-branch [41]. Mice with genetically disrupted glycogen-binding of both β isoforms (β1-W100A, β2-W98A) have increased fat deposition in liver (β1-W100A) and muscle (β2-W98A), yet only β2 mutant mice have greater overall adiposity, impaired glucose handling and reduced maximal running capacity [42]. Poorer exercise performance is a consequence of the β2-W98A mutant exacerbating glycogen utilisation in muscle [43]. Loss of glycogen-binding capacity destabilises the AMPK heterotrimer in general, without retarding intrinsic kinase activity and downstream signalling [42,43].

In crystal structures of active AMPK, the CBM of both β1 and β2 sits atop the kinase domain N-lobe to form a hydrophobic cavity termed the allosteric drug and metabolite (ADaM) site (Figure 1C) [18,44–46]. This pocket is stabilised by phosphorylation of the CBM residue S108, particularly for the β1 isoform [35,44,47,48], although phospho-β2-S108 could have a similar role [18,49], leading to the prediction there existed an endogenous, AMPK-regulating metabolite(s) capable of engaging the ADaM site [46]. Strong candidates for this metabolite, at least for β1, are long-chain fatty acyl-CoA (FA-CoA) esters [50]. β1-S108 is a *cis*-autophosphorylation site and substrate of at least one upstream kinase, the autophagy initiator unc-51-like kinase 1 (ULK1) [47,48]. Flanking the CBM is the β-linker that in response to ADaM site ligand binding of both β isoforms forms an α-helix at its N-terminus that packs against and stabilises the αC-helix of the α-subunit kinase domain [18,44]. Termed the αC-interacting helix, it is required for ADaM ligand-mediated allosteric activation and protection of the activation loop against phosphatases [44,51]. In the inactive (β2-containing) AMPK structure, the CBM and αC-interacting helix completely dissociate from the kinase domain, exacerbating phosphatase pressure toward phosphorylated α-T172 and presumably β-S108 [51]. At the extreme C-terminus of the β-subunit is a well-conserved α-γ-subunit binding sequence (α-γ-SBS) connected to the β-linker by a flexible β-loop. The β-loop contains a serine residue stoichiometrically phosphorylated on β1 (S182) and sub-stoichiometrically on β2 (S184) in mammalian cells and tissues [35,52,53]. The α-γ-SBS forms part of the core region of AMPK and interacts with the activation loop in both its phosphorylated and unphosphorylated form [54,55]. The α-γ-SBS forms a three-stranded antiparallel β-sheet, whereby the conserved residues T263 and Y267 (β1 numbering) of the third β-strand form critical interactions with a β-strand provided by the γ-subunit [56].

2.3. γ-subunit

γ1 and γ2 are generally ubiquitously expressed; γ1 is prevalent in skeletal and cardiac muscle and adipose tissue, whereas γ2 is a major isoform in cardiac muscle, but also displays reasonable expression in the small intestine, adipose tissue, brain and liver [19]. By contrast, γ3 expression is extremely tissue-specific, being almost exclusively confined to skeletal muscle [19,57,58]. γ2 and γ3 isoforms are identifiable by completely unrelated 259 and 182 amino acid N-terminal extensions (NTEs), respectively (Figure 1B), whose functions are poorly defined, and at least for γ2, is a 'hotspot' of post-translational control, particularly by phosphorylation [5]. Understanding of γ2 and γ3 is significantly hampered by all structural information published to date existing solely for γ1. These NTEs have previously been defined as 241 and 168 amino acids for γ2 and γ3 [5]. We update this to include additional C-terminal residues that are similarly predicted to be unstructured and show virtually zero conservation among the three γ isoforms (Table 1; Supplementary Figure 1). The new definition of the γ2- and γ3-NTEs brings their end point to the start of the pre-cystathionine β-synthase 1 (CBS1) sequence, a region previously shown to be critical for AMPK complex formation [59] that forms a kinked helix in crystal structures of γ1, and is predicted to form similar structures in γ2 and γ3 (Supplementary Figure 1).

Each γ-subunit contains four tandem CBS repeats that assemble into two Bateman domains, creating four potential nucleotide-binding sites that endow AMPK with its energy-sensing capabilities. In crystal structures of γ1, CBS site 2 (CBS2) is permanently vacated due to an arginine (γ1-R171, corresponding to γ2-R403 and γ3-R326) replacing a ribose-binding aspartate present in the other CBS motifs [45,54,60]. CBS4 appears to always accommodate AMP, although it is required to stabilise CBS3 upon AMP binding [60,61]. The two remaining sites are exchangeable for ATP, ADP and AMP, yet CBS1 retains greater affinity for ATP even under conditions resembling energy stress (e.g., 3.8 mM ATP, 300 μM AMP), resulting in its classification as a low-affinity site, leaving CBS3 as arguably the lone, physiologically relevant region of nucleotide exchange [62]. Of note, γ1-CBS1 contains a short, N-terminal β-strand that interacts with α-γ-SBS in crystal structures to stabilise the AMPK complex. This sequence is conserved in γ2 and γ3 (Table 1), suggesting it performs the same scaffolding role.

3. INSIGHT INTO THE REGULATION OF AMPK ISOFORM ACTIVITY

AMPK is generally activated in mammalian cells by activation loop α-T172 phosphorylation by LKB1 and CaMKK2 following energy stress or intracellular Ca^{2+} release, respectively. Nucleotide sensing is the most comprehensively studied canonical activation mechanism of AMPK, whereas non-canonical activation mechanisms occur independent of overt changes to cellular energy balance, often harnessing the lysosome as the activation platform. The discovery of the ADaM site unveiled another cellular function of AMPK, fatty acid sensing, and drug discovery efforts aimed at developing ADaM site ligands have greatly enhanced our understanding of AMPK isoform biology. We address these forms of AMPK activation below, drawing on structural information where available, whilst acknowledging some isoform-specific differences in regulation.

3.1. Canonical nucleotide sensing by AMPK

The conventional, 'tripartite' mode of AMPK activation by AMP binding at γ-CBS3 involves 1) promotion of α-T172 phosphorylation by LKB1, 2) further allosteric activation by 1.5–10-fold depending on the γ isoform, and 3) protection of phosphorylated α-T172 from phosphatases [22,36,54,63,64]. ADP can also promote α-T172 phosphorylation and buffer phosphatases, although AMP is argued to be the

principle physiological activator with only secondary contributions from ADP [54,63]. Activation of AMPK by AMP is governed by the coordinate actions of each α-RIM module in the α-linker. α-RIM1 forms an interaction surface on the nucleotide-free γ-CBS2, and α-RIM2 directly associates with AMP stationed at γ-CBS3. Combined, these events sequester the α-AID away from the kinase domain to relieve inhibition [29]. The α-RIM2 residue α-E364 (α1 numbering) forms a hydrogen bond with γ1-R70 that binds the AMP phosphate moiety in CBS3, while the adjacent α-R365 forms a well-conserved 'R365 pocket' (Figure 2A) with a portion of the β-subunit α-γ-SBS (β1: T219-P225; β2: T221-P227). Mutation of either of these two α-subunit residues to alanine abolishes both AMP-mediated allosteric AMPK activation and protection of α-T172 from dephosphorylation [51,65]. As expected, ATP binding breaks the α-linker/γ-CBS3 connection, releasing the α-AID to reassociate with the kinase domain [29], as well as collapsing the regulatory spine that results in an outward rotation of the αC-helix (relative to the AMP-bound conformation), breaking several key, catalytic interactions (Figure 2B—D). ATP also causes a 6 Å shift of the R365 pocket, destabilising an interaction between the β-linker and activation loop and exposing α-T172 to phosphatases [51].

Hydrogen deuterium exchange mass spectrometry (HDX-MS) measures the exchange of hydrogens within the amino acid backbone amide against a deuterium-containing solvent [66], and has been utilised to detect AMP-induced conformational changes to AMPK [62]. In non-phosphorylated α1β2γ1, in addition to expected protection against hydrogen exchange at γ1-CBS sites, AMP provided modest protection to the activation loop [62], which improved once phosphorylated on α-T172 [51]. From these data it is challenging to discern exactly where in the activation cycle AMP facilitates LKB1 phosphorylation of α-T172, with one possibility being AMP provides just enough exposure of the activation loop for phosphorylation at physiological ATP before it is subsequently protected from surrounding phosphatases [62]. Regardless, given that ATP exists in cells at markedly higher concentrations than AMP, even during energy shortfalls, exemplifies just how fine-tuned this surveillance system is to recognise even the slightest perturbation to energy balance [67].

Several pro-drugs that are converted into AMP analogues have been described in the literature. The most well-known is 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), which is transported into cells and converted by adenosine kinase to ZMP that mimics all three actions of AMP [68]. AICAR is not without several off-target effects however, otherwise precluding its utility as a tool compound to study AMPK [69,70]. In terms of isoform specificity, the cell-permeable drug C13 that in cells is cleaved to release the adenosine analogue C2, is a potent allosteric activator of α1-containing AMPK [71]. C2 binds to novel regions on the γ-subunit, occupying the interface between CBS1, CBS3 and CBS4 sites [72]. The preference for α1-AMPK can be attributed to stronger engagement of the α1-RIM2 upon C2 binding [72]. However, C13 activates both α complexes in mammalian cells since its cleavage results in the release of formaldehyde, an inhibitor of mitochondrial function that elevates cellular AMP [73]. Besides AMP and ADP, other regulatory endogenous metabolites (e.g., NADH, NADPH, inositol [54,62,74]) or natural products (e.g., cordycepin [75]) thought to bind the γ-subunit exist, but whether they modulate AMPK activity in an isoform-specific manner requires further characterisation.

3.2. Non-canonical AMPK activation at the lysosome

The lysosome is a major intracellular hub where AMPK is activated [12,76,77], and in response to energy stress that would disrupt cellular ATP levels, lysosomal LKB1 is the predominant α-T172 kinase [76]. Alternatively, non-canonical activation of AMPK by LKB1 at the lysosome, independent of changes in adenine nucleotide levels, has been examined largely in the context of glucose starvation. The current model asserts that in the glucose-replete state, the glycolytic enzyme aldolase occupied by its substrate fructose-1,6 bisphosphate (FBP) associates with and activates the lysosomal vacuolar H^+-ATPase (v-ATPase) [38,78], which acidifies the lysosome. Upon glucose withdrawal and depletion of FBP, aldolase liberated from its substrate inhibits the endoplasmic reticulum Ca^{2+}-release channel TRPV at physical contact points with the lysosome [79]. This triggers a reduced Ca^{2+} concentration in this compartment, leading to interaction of TRPV with aldolase and v-ATPase, in turn altering v-ATPase's conformation and ultimately causing formation of an AXIN/LKB1 complex that translocates to the lysosome and activates AMPK [38,77,79]. Notably, low doses (<0.5 mM) of the anti-diabetic drug metformin, ordinarily an inhibitor of mitochondrial complex I that increases the cellular AMP:ATP ratio, but not at these concentrations, similarly invokes the lysosomal AMPK activation pathway by inhibiting v-ATPase [80]. AXIN is thought to bring LKB1 to the lysosome by docking onto v-ATPase and a pentameric complex termed 'Ragulator' [77], itself a scaffold for Rag-GTPases that recruit mTORC1 to the lysosome in response to nutrient sufficiency [81]. There is some debate over the accuracy of this AXIN/LKB1-mediated model [82,83], arguably attributable to expression of different AXIN isoforms in various cells and tissues, or the duration of glucose starvation [12]. Nevertheless, these studies highlight that bidirectional nutrient-sensing culminates in AMPK and mTORC1 sharing identical lysosomal activation platforms. Interestingly, in response to DNA-damaging agents like etoposide, DNA-dependent protein kinase (DNA-PK) was found to directly phosphorylate the γ1-subunit isoform residues S192 (N424 in γ2; T347 in γ3) and T284 (I516 in γ2; S339 in γ3), eliciting lysosomal enrichment of AMPK and association with AXIN/LKB1 in cancer cells [84], suggesting this mechanism might be more commonplace than is currently appreciated.

Whilst there is some degree of α isoform specificity of AMPK activation by LKB1 at the lysosome [12] that we address in Section 5.2, the extent to which it is modulated by this non-canonical, AXIN-dependent pathway is unknown.

3.3. Allosteric AMPK activation by ADaM site ligands

Since the discovery of the first-generation synthetic compound A-769662 [85], there has been incremental progress in the development of ADaM site allosteric AMPK activators. The ADaM ligand pocket samples a variety of conformational states that differ in size and shape [86]. Ligand-free ADaM sites have been observed in structures of rat α1β1γ1 and human α1β2γ1 [28,45], whereby the size of the pockets were calculated as 195.0 and 396.1 $Å^3$, respectively [86]. Similarly, the size of ligand-occupied ADaM pockets range from 374.5 to 547.1 $Å^3$ [86], illustrating its adaptability to accommodate a range of chemically distinct molecules.

ADaM ligands can now be categorised based on their AMPK isoform specificity, in particular the β-subunit isoforms. **β1-selective activators** include the synthetic compounds A-769662, MT47-100, PXL770 and PF-06409577 [47,87—89], natural plant products salicylate and lusianthridin [90,91], and nutrient metabolites, namely long-chain FA-CoAs with acyl chain lengths > C12 such as palmitoyl-CoA [50]. Selectivity here is further defined by an absolute requirement for β1-S108 phosphorylation. Unlike ADaM site drugs [44,51,92], FA-CoAs are unable to prevent phosphatase-induced dephosphorylation of purified β1-AMPK [50]. Molecular dynamics simulations indicate that efficacy of a given ADaM site drug is defined by its ability to serve as a

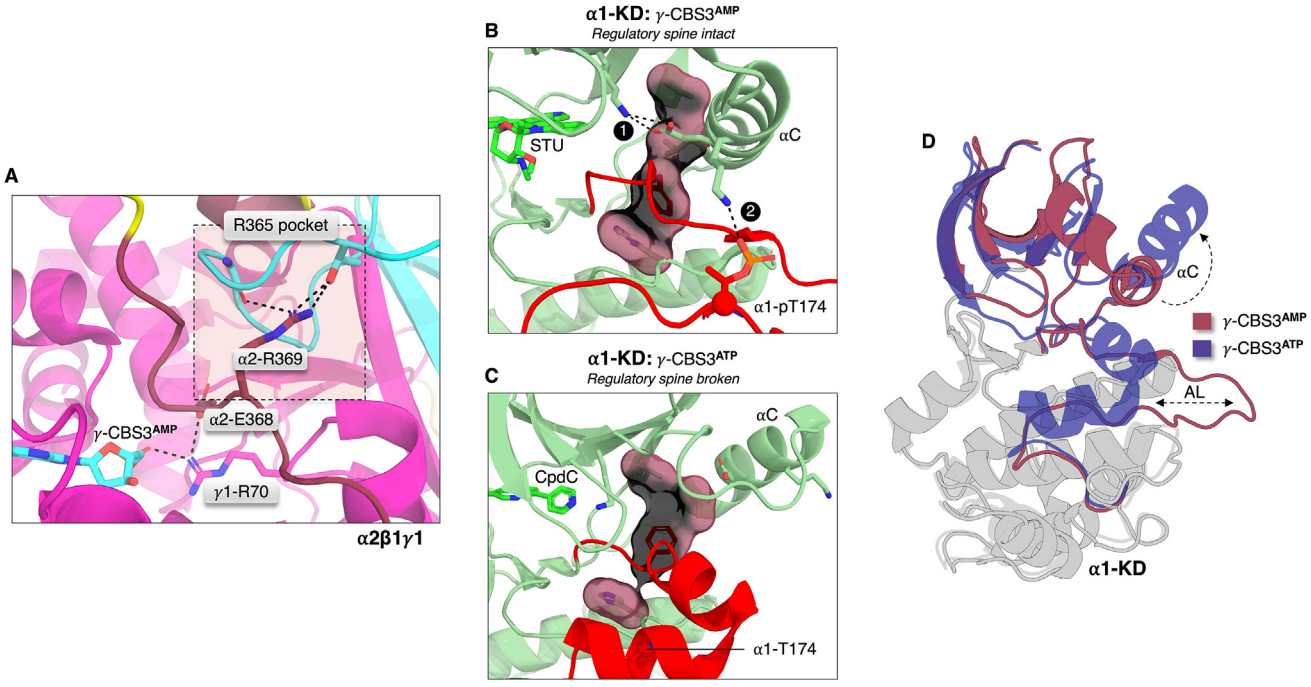

Figure 2: Structural comparisons of AMP- versus ATP-bound AMPK. A) Nucleotide sensing is transmitted to the kinase domain (KD) via establishment of an R365 pocket. A close-up cartoon view of the pocket is shown in the crystal structure of the active AMPK α2β1γ1 heterotrimer (PDB: 6B1U) with the γ-subunit displayed as magenta, β-subunit as cyan, α-linker as yellow and α-RIM2 as maroon. This pocket is formed by the α-RIM2 residues α-E364 and α-R365 (α1 numbering, equivalent α2 residues shown) that bind γ-CBS3 and the β-subunit C-terminus, respectively. The R365 pocket is required for allosteric AMPK activation by AMP and protection of phosphorylated α-T172 from phosphatases. **B**) AMP binding to γ-CBS3 induces an active configuration of the KD including, but not limited to, ❶ formation of a salt bridge in the N-lobe between α1-K47 (third β-strand) and the α1-E66 (αC-helix), and ❷ an interaction between α1-K62 (αC-helix) and the phosphorylated α1-T174 residue (PDB: 4RER; STU: staurosporine). AMP also promotes the assembly of a hydrophobic regulatory spine anchored to the C-lobe that connects critical elements within the core of the KD. Amino acids comprising the regulatory spine are shown as sticks and its assembly is depicted by surface representation. **C**) ATP binding to γ-CBS3 disrupts these interactions, causing the regulatory spine to collapse (PDB: 7JHG; CpdC: compound C). **D**). Superimposition of the two KDs from **B** and **C** with AMP or ATP bound to γ-CBS3 demonstrates that movement of the αC-helix constitutes a fundamental mechanism controlling the AMPK activation cycle. ATP binding causes an outward rotation of the αC-helix away from the KD core, completely disabling the catalytic machinery described above.

'molecular glue' connecting the CBM to the N-lobe of the kinase domain [49,93,94]; notably, A-769662 fails to achieve this for β2 complexes despite it still being able to stabilise the β2-CBM [95].

Intermediate activators 991 and SC4 show some semblance of isoform selectivity. 991 is more potent toward β1 than β2 owing to partial dependency on phospho-β1-S108 [44], while SC4 activates both β isoforms provided β2 is complexed with α2 [18]. SC4-induced activation of β2-AMPK is mediated by a non-conserved β2-D111 residue (N111 in β1) in the CBM [18]. The final category is the **pan-activators** MK-8722, PF-739, I-3-40 and MSG011 capable of switching on all 12 AMPK combinations [96–99]. Despite promising pre-clinical findings of improved glucose homeostasis in animal models, progress of systemically administered pan-activators to clinical use has been hindered by off-target effects such as cardiac hypertrophy [98]. This may involve activation of heart γ2-AMPK, as activating γ2 mutations are linked to Wolff-Parkinson-White syndrome, a cardiac condition defined by abnormal glycogen accumulation and left ventricular hypertrophy [100–102]. Strategies in this exciting area are now turning to tissue-specific delivery systems, or medicinal chemistry approaches that leverage unique AMPK isoform tissue distribution profiles to activate AMPK only in desirable tissues, namely β2-containing complexes expressed majoritively in human skeletal muscle, liver and adipose tissue, while constituting only a minor component of heart AMPK (Figure 1A).

The finding that long-chain FA-CoAs are ADaM site metabolites expanded the repertoire of AMPK's nutrient-sensing capabilities [50]. Relevance in a physiological context was illustrated by whole-body β1-S108A knock-in mice that have elevated liver triglycerides in response to high-fat availability, materialising from impaired mitochondrial function and turnover [103]. Naturally this assumes that detection of FA-CoAs by β1-AMPK is coupled to their oxidation via acetyl-CoA carboxylase (ACC) phosphorylation, an inhibitor of fatty acid catabolism that exists as two isoforms (cytosolic ACC1, mitochondrial ACC2). FA-CoAs themselves inhibit ACC [104], and phosphorylation by AMPK lowers the concentration at which palmitoyl-CoA effectively inhibits ACC [105]. Despite human liver expressing vastly more β2 than β1 [106,107], β1-specific activators still elicit indices of AMPK activation in human hepatocytes [89,108,109], suggesting the AMPK FA-CoA-sensing apparatus in human liver is intact. Future work should aim to identify other tissue-specific physiological systems harnessing β1-dependent FA-CoA-sensing, such as the brain that governs feeding behaviour, in which global loss of β1 diminishes appetite and prevents high-fat diet-induced obesity [110].

4. α1- AND α2-AMPK ARE NOT THE SAME ENZYME

4.1. α-T172 phosphorylation and substrate selectivity

Higher basal activity for α1- versus α2-containing AMPK has been reported for recombinant human complexes expressed in bacteria and mammalian cells, as well as endogenous material isolated from rat liver and human skeletal muscle [12,99,111–114]. Part of the impediment toward α2-AMPK activity is lower α2-T172

phosphorylation shown in mammalian cells [12,53] and rat liver [26], possibly a result of greater susceptibility to phosphatases [113,115]. However, monomeric α1- and α2-kinase domains with virtually identical α-T172 phosphorylation stoichiometry still retain disparate levels of activity by over 3-fold [113].

Efficiency of protein kinase activity is governed by incredibly precise positioning of the phospho-donor ATP relative to the phospho-acceptor peptide substrate [27], and even small perturbations to the distance between reactants can affect catalysis [116]. Using 64 validated AMPK phosphorylation sites, Hardie et al. [117] defined the major residues forming the AMPK substrate consensus motif as serine as the preferred phospho-acceptor, a bulky hydrophobic residue at P−5 (L, M, I) and P+4 (typically L), and a basic residue at P−3. Basic residues at P−6, P−4 and P−2 are also positive determinants in some substrates [117]. Modelling of the α1-kinase domain with rodent ACC1 revealed two acidic patches in the kinase domain accommodating basic residues of the substrate [118]. Patch one (α1-E102/D105/D145; α2-E100/D103/D145) is proposed to interact with the P−3 or P−4 basic residue, and patch two (α1-D217/D218/D219; α2-D215/D216/E217) may be necessary for substrates containing the P−6 basic amino acid (e.g., ACC1) [118]. An amphipathic substrate-docking helix N-terminal to the P−5 position in ACC1 [118], and P−16 to P−1 in another AMPK substrate, HMG-CoA reductase (HMGCR) [119], is predicted to fit into a hydrophobic groove in the kinase domain C-lobe between the αD helix and αG/H-helix loop. Two small segments in the AMPK kinase domain contain stretches of sequence divergence between α isoforms: one in a loop connecting the αD and αE helices (α1: 110−118; α2: 108−116) and another from the C-terminal portion of the αG helix extending through to the majority of the αH helix (α1: 229−248; α2: 227−246). That these divergent α isoform sequences lie in proximity to putative substrate-binding sites raises the likelihood of minor structural discordance eliciting meaningful changes in substrate affinities and AMPK activity; hence, the two isoforms probably have some preferred signalling targets.

4.2. Nucleotide sensitivity of α1 and α2

The concentration of AMP that elicits half−maximal activity (EC_{50}) of CaMKK2-treated human AMPK purified from bacteria is higher for α1- than α2-containing heterotrimers [113,120]. The presence of the β2 isoform in either α complex has also been shown to augment AMP responsiveness by either lowering its EC_{50}, or inducing a larger fold-change in activity compared to β1-containing counterparts [50,113]. The result, albeit from *Escherichia coli*-expressed AMPK, is α2β2γ1 being the most readily allosterically activated complex by AMP, as well as having the greatest AMP-mediated protection against α-T172 dephosphorylation [113]. α2-AMPK isolated from rat liver and human skeletal muscle have a superior net stimulation by AMP than α1 complexes [111,114,115], although these effects have not always been reproduced, at least when rat AMPK was assessed [121,122]. One possible explanation for enhanced AMP stimulation of α2-AMPK are intrinsic differences in AMP-sensing by the α-RIM2 module. A chimera of the α-subunit in which the α2-RIM2 sequence was replaced with α1-RIM2, whilst keeping the remainder of α2 the same, rendered α2-AMPK less responsive to AMP by elevating its EC_{50} to a concentration mirroring wild-type α1 [71]. Analysis of AMPK complexes expressed in mammalian and insect cells generally show a greater fold response of α2 versus α1 to a single (100 μM) AMP dose *in vitro* (insect/COS7: α2β1γ1>α1β1γ1; COS7: α2β1γ2>α1β1γ2) [18,50,71,123], although in some instances activity is comparable (COS7) [72,124], or even higher with α1 (HEK293T: α1β2γ1>α2β2γ1; α1β2γ2>α2β2γ2) [99]. Aside from canonical phospho-sites (α-T172, β-S108), variability in cell culture conditions subjects AMPK to a multitude of post-translational modifications sensitive to the prevailing metabolic conditions [5]. We suspect this equates to a broader spectrum of AMP/ADP sensitivity than is currently appreciated.

Notwithstanding these disparities, robust biophysical (HDX-MS) data unambiguously demonstrate the greater structural impact of AMP binding on α2-AMPK versus α1. AMP was shown to have a superior stabilising effect on the αC-helix, α-AID and γ-CBS3 site in α2β2γ1 versus α1β1γ1, as well as exclusively protecting vital catalytic segments (e.g., phosphorylated activation loop) and the R365 pocket [95]. The latter reinforces the notion of greater AMP-induced engagement of α-RIM2 in the α2 isoform. One consideration is that superior AMP sensitivity of α2-AMPK compensates for its lower basal activity compared to α1.

5. UNIQUE PHOSPHORYLATION PROFILES OF α1 AND α2 AND IMPLICATIONS FOR CANCER

The gene encoding LKB1, *STK11* (for 'serine/threonine kinase 11'), was found in the late 1990s to be mutated in the Peutz−Jeghers cancer syndrome characterised by benign gastric hamartomatous polyps, earning LKB1 the title of tumour-suppressor [125]. As a target of LKB1, this provided the first indication that AMPK could similarly function as a tumour suppressor. Conceptually it made sense in light of AMPK's fundamental role in preventing cell proliferation, particularly at the level of mTORC1 inhibition, a *bona fide* driver of cancer. Genetic evidence supported this theory, with whole-body loss of α1 or β1 accelerating tumour burden in two different mouse models of lymphoma [126,127]. However, it has since become clear that AMPK's role in cancer is context specific. AMPK affords tumours a survival advantage when confronted with energetic disturbances caused by conditions like anchorage-independent growth and solid tumour formation, oxidative stress, glucose deprivation, and metastasis [128−131]. Conversely, loss of AMPK function in *energy-replete* cancerous cells enhances their proliferation [128]. One argument is that AMPK might prevent the onset of cancer, hence formation of the tumour itself [4], explaining some of the anti-cancer properties of pharmacological agents like metformin [132]. A fascinating emerging topic is the behaviour of AMPK subunit isoforms in different cancers. Analysis of the TCGA PanCancer Atlas via the cBioPortal database revealed a high frequency of *PRKAA2* gene missense mutations in human cancer, especially cutaneous melanoma, whereas *PRKAA1* is frequently amplified, particularly in lung cancer (Figure 3A) [133,134]. The reader is referred to Supplementary Table 1 for the major cancers these genes are altered in and relationships with other relevant genetic alterations. That α1 could function as a tumour promoter and α2 a suppressor is paradoxical and something we suspect is related, in part, to their differential phosphorylation profiles.

5.1. LKB1 versus CaMKK2

In many respects, the α2 isoform of AMPK is more prominently targeted by LKB1 than α1. Genetic deletion of LKB1 in skeletal and cardiac muscle completely abolishes α2 activity in response to a variety of AMPK-activating stimuli, whereas α1 is largely unaffected [135−138]. In skeletal muscle, this led to the hypothesis that α1-AMPK is preferentially activated by CaMKK2 during prolonged exercise associated with Ca^{2+} oscillations [139,140], but later contradicted by work showing that neither a highly specific CaMKK2 inhibitor (SGC-CAMKK2-1), nor genetic loss of CaMKK2, had any impact on contraction-induced α1- and α2-AMPK activity in muscle [141]. In fact, substantial increases in α1 complex activity isolated from AICAR-

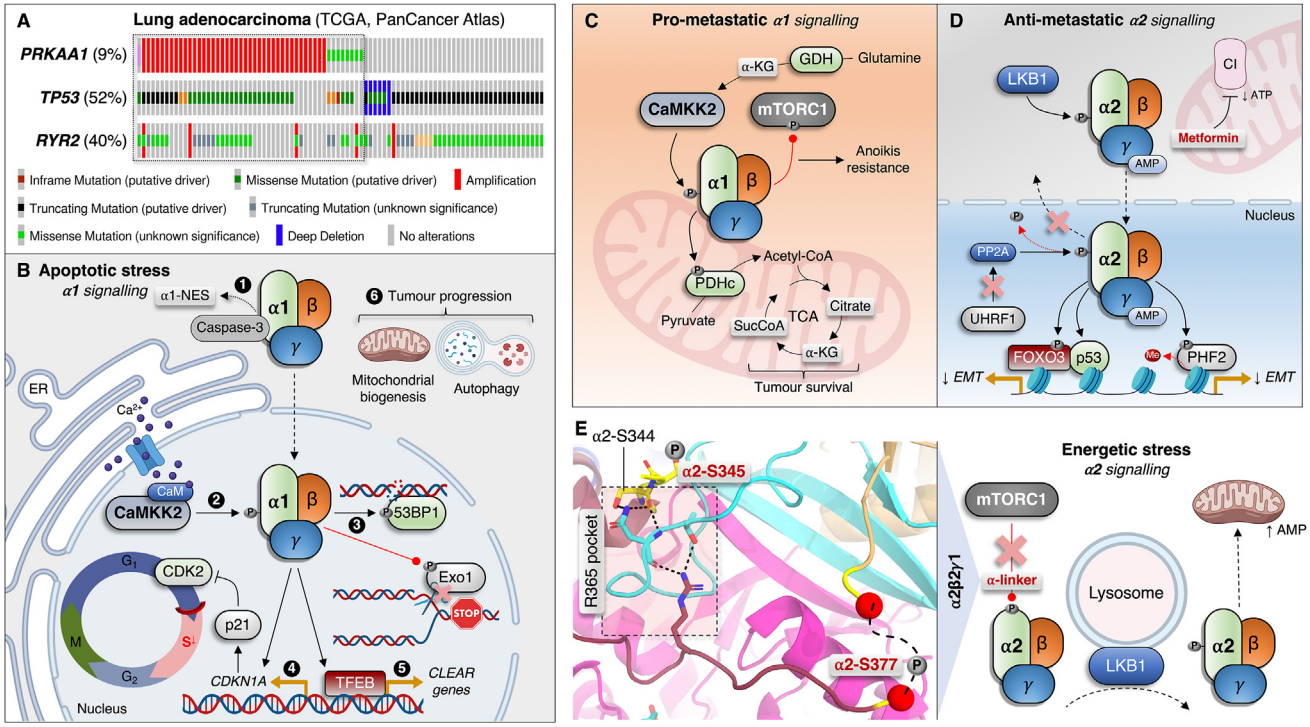

Figure 3: α1- and α2-AMPK regulation and implications for cancer. A) Analysis of the TCGA PanCancer Atlas lung adenocarcinoma dataset reveals a significant co-occurrence of amplification of the *PRKAA1* gene (encoding for the α1 isoform) with putative *TP53* (encoding for the p53 tumour suppressor) driver mutations and mutations of unknown significance to *RYR2*, which encodes for a Ca^{2+}-release channel. **B**) We present a hypothetical schema by which α1-AMPK could confer tumour promoter effects to compensate for mutations to p53, specifically in the context of apoptotic signalling. ❶ Apoptotic stress triggers the nuclear entrapment of α1-AMPK by caspase-3 cleavage of the α1-NES. ❷ Nuclear α1-AMPK is then activated by CaMKK2, itself stimulated by intra-nuclear Ca^{2+} flux for which the source could be RYR2 and/or TRPV2 Ca^{2+}-release channels embedded in the endoplasmic reticulum (ER) [150,332]. ❸ Activated α1-AMPK phosphorylates nuclear substrates implicated in cell survival (EXO1) and DNA-damage repair (53BP1), ❹ induces gene transcription that causes cell cycle arrest (e.g., p21-mediated blockade of CDK2), and ❺ upregulates TFEB-dependent 'CLEAR' network genes. ❻ The latter results in mitochondrial biogenesis and autophagy to ensure ongoing cell survival and progression in the nutrient-poor tumour microenvironment. Although steps one, two and four have been shown to be α1-AMPK-dependent, the α isoform dependency of nuclear substrate phosphorylation and TFEB activation (steps three and five) requires confirmation. **C-D**) Postulated pro- and anti-metastatic functions of each α isoform provides insight into differential regulation by CaMKK2 and LKB1. **C**) α1-AMPK phospho-activates the catalytic subunit of PDH to enhance TCA cycle activity and support the energy requirements of metastatic cells. α-KG, a product of glutaminolysis and intermediate of the TCA cycle, activates AMPK through CaMKK2, causing resistance to cell death (anoikis) during metastatic detachment from the extracellular matrix. **D**) Nuclear α2-AMPK may oppose metastasis by preventing the epithelial to mesenchymal transition (EMT) via direct (FOXO3/PHF2 phosphorylation) or indirect (p53 activation) modulation of the epigenome, which would require inhibition of the nuclear oncoprotein UHRF1. It appears that suppression of the EMT by agents like metformin are LKB1-dependent, thereby contributing to α2 activation selectivity. **E**) mTORC1 regulates LKB1 phosphorylation of α2-AMPK. Left panel: a close-up cartoon view of the R365 pocket is shown in the crystal structure of the active AMPK α2β2γ1 heterotrimer (PDB: 6B2E) colour coded according to Figure 1B. The two mTORC1 substrates α2-S345 and α2-S377 are located in the α-linker region of AMPK. Whilst α2-S377 is not resolved, α2-S345 can be seen bordering the nucleotide-sensing R365 pocket. Right panel: energy stress-induced inhibition of mTORC1 relieves α2-S345 phosphorylation of AMPK, causing lysosomal targeting where it is activated by LKB1. α2-AMPK is then further activated by AMP upon relocating to bioenergetic hubs like mitochondria. Where phosphorylation (Ⓟ) events are depicted in **B-E**, red lines ending with a circle denote inhibition of the target and black arrows activation.

or contraction-stimulated skeletal muscle can occur independent of α1-T174 phosphorylation [135], possibly driven by an alternate posttranslational modification, suggesting the intrinsically elevated basal activity of α1-AMPK may make it functionally less reliant on an α-T172 kinase.

Nevertheless, studies investigating AMPK function in cancer have demonstrated that in response to genotoxic stress, α1- but not α2-AMPK complexes translocate to the nucleus where they are activated by intranuclear Ca^{2+}-stimulated CaMKK2 [142]. α1-AMPK was shown to arrest the cell cycle in the G_1-phase, preventing entry into the DNA-replicating S-phase where cells are especially vulnerable to double-strand breaks [142,143]. The mechanism underpinning nuclear α1 specificity involves an initial activation of the apoptotic cascade by DNA-damaging agents, in which caspase-3 proteolytically cleaves α1-D520, resulting in removal of the C-terminal NES and sequestration of α1-AMPK in the nucleus where it orchestrates cell survival, presumably via induction of transcriptional programs (Figure 3B) [144]. Alternate stressors encountered by tumours, such as mitochondrial damage, hypoxia and serum deprivation that all induce caspase-3 mobilisation [145—148], would theoretically trigger α1-AMPK nuclear localisation; for example, mitochondrial damage-induced apoptosis is exacerbated by loss of α1 [37]. Additional studies have linked genotoxic stress to CaMMK2 activation of AMPK, inducing AMPK phosphorylation of the exonuclease EXO1 to prevent abnormal processing of stalled DNA replication forks [149,150], and p53-binding protein 1 (53BP1) to promote DNA double-strand break repair (Figure 3B) [151]. Finally, mitochondrial matrix-localised α1-AMPK phospho-activates the catalytic subunit of the pyruvate dehydrogenase complex (PDHc) in metastatic tumours to switch energy extraction from glycolysis to the tricarboxylic (TCA) cycle [129]. The TCA cycle metabolite and product of glutaminolysis, α-ketoglutarate (α-KG), activates CaMKK2 during anoikis, a form of programmed cell

death initiated upon detachment from the extracellular matrix [152]. Here, α-KG-mediated CaMMK2 activation of AMPK facilitated anoikis resistance and enhanced the metastatic potential of *STK11*-null lung cancer cells [152]. While it stands to reason α1 is the main target of CaMKK2 in response to genotoxic stress/anoikis, not all studies confirmed this.

By contrast, targeting mitochondrial metabolism with agents such as metformin to activate α2-AMPK may confer some therapeutic benefit in the solid tumour. Loss of α2 in melanoma exacerbates brain metastasis [153], which has been shown to be prevented by metformin in an AMPK/p53-dependent manner [154]. This signalling axis with p53, as well as AMPK phosphorylation of FOXO3a [155], diminishes expression of proteins implicated in the metastatic epithelial to mesenchymal transition (EMT). α2-AMPK was recently reported to prevent lung cancer metastasis by phosphorylating the histone demethylase PHF2 (PHD finger protein 2), relieving H3 methylation at lysine 9 and averting the EMT following metformin treatment [156]. The oncogenic nuclear protein UHRF1 (Ubiquitin-like with PHD and RING Finger domains 1) is an enhancer of DNA methylation [157] and negative regulator of AMPK by promoting α-T172 dephosphorylation and its nuclear export [158]. Whilst not delineating isoform specificity, additional studies have shown AMPK directly targets and supresses the activity of distinct oncogenic regulators of the epigenome [159,160], in each case being stimulated by metformin. Therefore, modulation of epigenetic marks, mainly DNA methylation, could underscore a general tumour suppressor effect of α2-AMPK. The anti-metastatic effects of metformin appear to require LKB1 [161,162], which may contribute to α2 selectivity. Hypothetical pro- versus anti-metastatic effects of α1 and α2, respectively, are juxtaposed in Figure 3C,D. We acknowledge these effects are generalised, as there are reported instances of α1 supressing, and α2 promoting, metastasis in breast cancer [163,164].

5.2. The role of mTORC1

In addition to LKB1, the α2 isoform is also significantly regulated by mTORC1. At least two functional mTORC1 sites located in the α-linker have been identified, α2-S345 and α2-S377. Mutation of either site to alanine is sufficient to delay mammalian cell proliferation during nutrient stress [8,12,165]. The location of these sites is noteworthy, since the α-linker is a 'hot-spot' for missense loss-of-function mutations in the *PRKAA2* gene in melanoma [153,166]. mTORC1 inhibition and α2-S345 dephosphorylation activates α2 complexes via a lysosomal targeting mechanism that colocalises AMPK with LKB1 [8,12]. Even though mTORC1 efficiently phosphorylates the equivalent α1-S347 residue [8], this does not impart an obvious regulatory effect [12]. α2-S345 is located immediately after α2-RIM1 in a region of the α-linker that borders the R365 pocket (Figure 3E) [18]. This predicts engagement of α2-S345 with the nucleotide-sensing machinery, especially since the α-carboxyl group of the preceding serine (S344) makes a series of interactions with β2 residues (N222, C225) lining the R365 pocket (Figure 3E; α2β2γ1 structure [18]). However, mTORC1 inhibition alone, hence in the absence of any changes to the adenylate energy charge (AMP:ATP and ADP:ATP ratios), is sufficient to enhance α2-AMPK lysosomal targeting and activation [8]. We should point out that under these conditions there is sometimes a lag between rates of α2-S345 dephosphorylation and α2-T172 phosphorylation [12]. Therefore, could mTORC1 inhibition be an elusive mechanism of AMP-mediated promotion of α-T172 phosphorylation by LKB1, at least for α2 complexes? This would assume α2-S345 dephosphorylation promotes nucleotide exchange on γ-CBS3 prior to lysosomal delivery of α2-AMPK to LKB1. However, in our hands, phosphorylation of α2-S345 had no bearing on allosteric AMP activation of AMPK [8]. Thus, the significance of α2-S345 being structurally modelled adjacent to the nucleotide-sensing machinery remains unknown. Another consideration is that mTORC1 inhibition, lysosomal targeting and α2-T172 phosphorylation precedes AMP occupancy of the γ-subunit once α2-complexes are redistributed to sites of ATP turnover like mitochondria (Figure 3E). This theory is supported by the kinetics of AMPK activation following energy stress, in which it is activated at lysosomes first, then delivered to mitochondria and later the cytosol [76].

The function of the second site, α2-S377, has not been as thoroughly examined. Following on from the α2-RIM2 module, α2-S377 lies in the vicinity of residues that coordinate AMP (γ1-R70-interacting α2-E368) and α2-R369 in the R365 pocket [18]. Because α2-S377 is not resolved in any α2-containing crystal structures, it is unclear how it might dictate AMPK function. α2-S377N is one of the missense mutations identified in melanoma [166], however reintroduction of α2-S377N into α1/2-double knockout (dKO) HEK293 cells did not impair α2-AMPK activity induced by phenformin [166], a more potent mitochondrial complex I inhibitor than metformin.

The oncogenicity of α1 is obviously multifaceted, with one possible scenario depicting proliferative mTORC1 activity commensurate with α1-mitigated cell survival. An analogous situation occurs in Birt-Hogg-Dubé syndrome driven by germline mutations in the *FLCN* gene. Loss of *FLCN* relieves inhibitory mTORC1 phosphorylation of the transcription factor TFEB without interfering with phosphorylation of its other substrates (e.g., S6K1, 4E-BP1) [167]. The result is a constitutively active nuclear TFEB that in addition to transcribing autophagy and lysosomal genes, upregulates the expression of lysosomal mTORC1 scaffolds RagC and RagD, in turn hyperactivating mTORC1 to create a lethal balance between anabolic and catabolic pathways to support proliferative demands [167,168]. Stable expression of α1 in α1/2-dKO lung adenocarcinoma cells enhances cell proliferation during glucose-deprived but not glucose-replete conditions, in a manner requiring AMPK-dependent TFEB/TFE3 activation and transcription of lysosomal genes [169], which may involve AMPK inhibition of FLCN [170]. TFEB/TFE3 transcription factors are known to amplify p53-dependent transcriptional programs in response to DNA damage [171]. Because *PRKAA1* is amplified alongside driver mutations in *TP53* (encoding for p53) in lung cancer (Figure 3A; Supplementary Table 1) [133,134], suggests an oncogenic α1-AMPK and TFEB/TFE3 axis might compensate for loss of p53 function. There is clearly a long way to go to understanding the function of different α isoforms in cancer, and unlike activator specificity, no α isoform-specific AMPK inhibitors are available.

6. THE SYNERGY QUANDARY

An unresolved question in the field is whether α-T172 phosphorylation is rate-limiting for AMPK activity in a physiological context. Initial work demonstrated the β1-selective ADaM site activator A-769662 could significantly activate bacterially-expressed AMPK lacking α-T172 phosphorylation provided β1-S108 was phosphorylated, achieving a level of enzyme activity comparable to that of α-T172-phosphorylated AMPK [47]. Furthermore, A-769662 dependence on β1-S108 phosphorylation was bypassed in the presence of AMP, since purified AMPK lacking phosphorylation at both β1-S108 and α1-T174 sites could be activated by dual incubation with A-769662 and AMP [47]. This "synergistic" activation has since been reported for AMP in combination with ADaM site drugs 991 and SC4, as well as the AMP mimetic C2 with A-769662 [18,55,72]. In each case, synergistic activation was either amplified with, or limited to, α1-containing complexes, in a twist to the normally weaker AMP sensitivity of α-T172-phosphorylated α1-AMPK compared to α2.

Despite a clear preference for α1 in a cell-free system, co-incubation of MEF cells exclusively expressing α2-T172A with A-769662 and phenformin still upregulated phospho-α-T172-independent AMPK signalling to ∼30% of that produced in similarly treated MEFs expressing wild-type α2 [48]. Since AMPK dephosphorylated on α-T172 represents by far the greatest pool of cellular AMPK, even under conditions of extreme cellular stress [47,55], we argue that dual liganded, dephosphorylated α1-containing AMPK is a major active form of the enzyme [18]. By contrast, 991 and 2-deoxyglucose (an inhibitor of glycolysis) treatment of LKB1-negative A549 cells, genetically deleted for CaMKK2 and therefore devoid of the two major upstream α-T172 kinases, induced negligible effects on AMPK signalling, bringing into question the physiological relevance of this regulatory mode [55]. Reasons for the inconsistencies are likely manifold, relating to differences in ADaM ligand potency, cell type α isoform distribution, and/or compartmentalised pools of AMP generated by different metabolic inhibitors.

Sensitivity to synergistic activation may relate to reduced dependence of α1-AMPK on an activation loop kinase. Basal β1-S108 phosphorylation stoichiometry of COS7-expressed α1β1γ1 is estimated to be only 6% [47], with comparable β2-S108 phospho-stoichiometry levels for β2 complexes expressed in HEK293T cells [53]. Despite this low stoichiometry, phosphorylated β1-S108 is more resistant to phosphatase treatment in α1-versus α2-AMPK [72]. From this we can also extrapolate that β1-ADaM ligands are another dominant form of α1-AMPK regulation in cells (note, we entertain a relevant, myristoylation-dependent signalling pathway in the subsequent section). Interestingly, CaMKK2 treatment renders α2β1-complexed AMPK markedly more responsive to A-769662 treatment in isolation than its α1 counterpart [72,113]. The overall heterogeneity by which ADaM ligands either synergise with AMP or act alone to stimulate AMPK opens up at least two possibilities. Firstly, more than one endogenous class of ADaM ligand exists, including a β2-specific metabolite that analogous to SC4, prefers α2-AMPK (e.g., generated in skeletal muscle). Secondly, there are additional β-S108 kinases other than AMPK and ULK1. Experimental profiling of the human kinome marked eight kinases not including ULK1 as having a higher probability of phosphorylating β1-S108 than AMPK [172]. For example, the top ranked β1-S108 kinase is hormonally up-regulated Neu-associated kinase or 'HUNK', known for its role in breast cancer metastasis [173,174]. The metastatic potential of breast cancer cells consuming large amounts of oleic acid is AMPK-dependent [175], which is literally food for thought when bearing in mind oleoyl-CoA is a β1-ADaM ligand for AMPK [50].

7. MYRISTOYLATION OF THE β-SUBUNIT

β-subunit myristoylation is a fundamental regulator of AMPK activity. Genetically preventing myristoylation (β-G2A) causes basal hyperactivation of AMPK yet attenuates responsiveness to AMP-elevating agents and ADaM site ligands [36,123,176,177]. Mechanistically, augmented myristoyl-free AMPK activity is the result of diminished colocalization with membrane-bound (also myristoylated) PPM1A/B phosphatases, in turn preserving α-T172 phosphorylation catalysed in the cytosol [176]. One exception was found in T cells lacking the N-myristoyltransferase enzyme NMT1, where AMPK has lower basal α-T172 phosphorylation due to defective lysosomal targeting [178]. In these cells, normally myristoylated phosphatases (i.e., PPM1A/B) would also presumably disengage from the lysosome, facilitating encounters with cytosolic AMPK. The "myristoyl-switch" hypothesis proposes stimulus-driven cycling of the myristoyl group on and off the kinase domain [36]. Unstimulated, the myristoyl group is postulated to occupy an intramolecular hydrophobic pocket (switch: OFF) formed by a segment of the αE helix just prior to the catalytic loop [179], with comparable orientations found in other myristoylated protein kinases (Figure 4A) [180,181]. AMP-induced conformational changes lead to ejection and exposure of the myristoyl group (switch: ON), permitting AMPK membrane targeting and colocalization with activatory kinases [36].

While myristoylation is generally required to stabilise the AMPK heterotrimer in mammalian cells, only β1- and not β2-containing complexes were shown to display basal, β-G2A-induced hyperactivity in MEFs isolated from β1-G2A or β2-G2A knock-in mice [176]. Nevertheless, myristoylation-dependent organelle localisation and phosphorylation of AMPK has been reported for both β complexes [37,38,48,182]. Moreover, in several tissues the mitochondrial abundance of β2 is either higher than β1 (liver, kidney), or there is no mitochondrial association of β1 at all (skeletal muscle, heart) [183]. β1 myristoylation controls mitochondrial targeting during mitochondrial autophagy (mitophagy) [37], indicating these complexes have greater motility insofar as being prompted to move from a cytosolic compartment to a membrane-bound organelle; thus, β1 appears to be the predominant β isoform regulated by the myristoyl-switch. It is also worth highlighting that AMPK is a lysosomal resident even in the basal, nutrient-replate state [12,77], in which case glucose starvation-induced LKB1 translocation to the lysosome probably activates a pool of AMPK already stationed at this organelle [38]. Whether β2 makes up the bulk of these "stickier" membrane-bound complexes is open for interrogation.

The apparent specificity of the myristoyl-switch is most likely intrinsically mediated by poorly conserved elements in the β-subunit N-terminus interacting with the α-subunit. The only structural information relates to a short α-helix in β2 (F59—S69) found in an open groove surrounded by the α2-AID [18], which shifted the first two α2-AID helices by 11 Å (relative to α2β1γ1) toward the γ1-CBS2 domain. An AMPK construct expressed in bacteria with the first 68 β2 residues missing was efficiently activated by SC4 and AMP but failed to express in COS7 cells [18]. This is in stark contrast to the hyperactivation of α1β1γ1 in COS7 cells expressed with a near-identical β1 deletion (removal of the first 63 amino acids) [123], demonstrating that for β2, myristoylation in combination with other elements in its N-terminus, might control the formation of the entire AMPK heterotrimer in mammalian cells.

7.1. The ULK1 ligand-switch

ULK1 is classically thought to be activated by AMPK and inhibited by mTORC1 via direct phosphorylation at distinct sites [184,185], but it is now evident these three kinases engage in more elaborate crosstalk with one another, earning them the moniker of 'kinase triad' [186]. First, the AMPK myristoyl-switch triggers ULK1 phosphorylation of β1-S108 (meaning the modification occurs at a membranous site) in mammalian cells, sensitising AMPK to ADaM ligands [48]. In addition to β1-S108, ULK1 targets up to five sites on the α-subunit whose individual functions are unclear yet result in suppression of α-T172 phosphorylation [187]. Indeed, dephosphorylation kinetics of β1-S108 are slower than α-T172 in mammalian cells following removal of an AMPK-activating stimulus [47], which would preserve ADaM ligand sensitivity once α-T172 has been stripped of its phosphate. Based on recent measurements of AMPK subcellular distribution in response to cellular stress, we provide an updated model of the ULK1 ligand-switch [48], in which an initial membrane-partitioning stimulus (AMP) causes lysosomal α-T172 phosphorylation by LKB1 [76]. AMPK is then rapidly

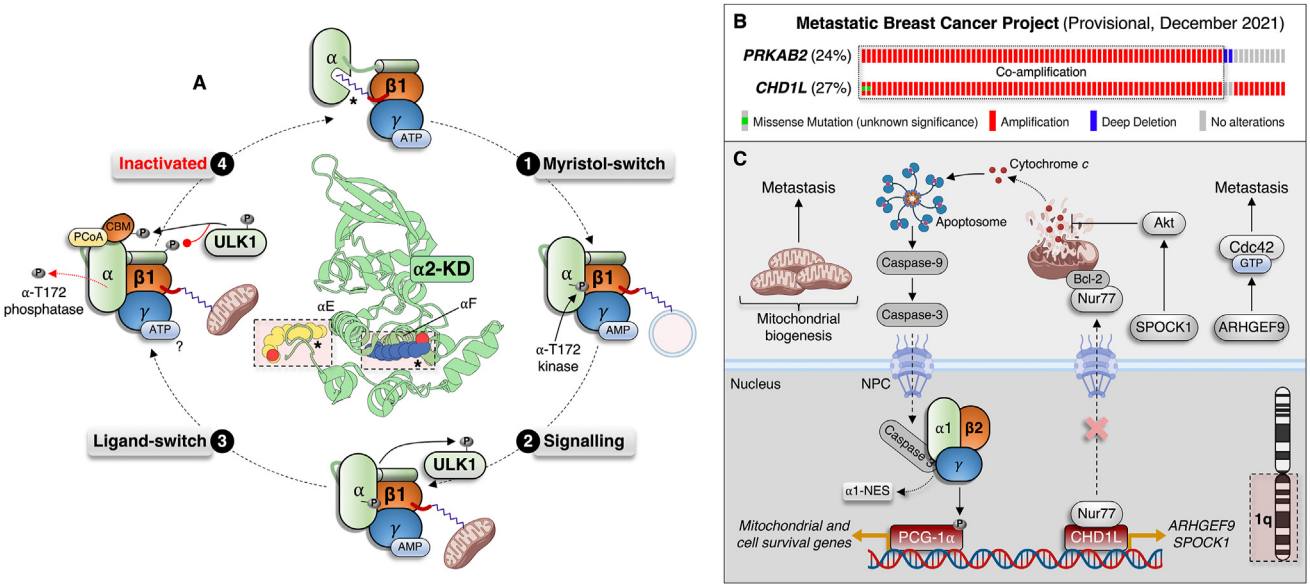

Figure 4: Divergent effects of β-subunit myristoylation on AMPK function. A) The myristoyl- and ligand-switch are two intertwined processes involving the β1 isoform. The N-terminal myristoyl group is hypothesized to occupy a hydrophobic pocket in the kinase domain (KD) of AMPK. Pictured in the centre of the schematic is the cartoon view of the α2-KD crystal structure (PDB: 6B1U) with superimposed myristoyl groups (indicated by asterisks) from two other myristoylated protein kinases (after alignment of the KDs), c-Abl (blue; PDB: 1OPJ) and PKA (yellow; PDB: 1CMK), which may provide some clues as to where the myristoyl group lies in inactivated AMPK. ❶ AMP binding triggers the myristoyl-switch of β1-AMPK in which the myristoyl group is ejected from the KD to facilitate organelle (e.g., lysosome) association of AMPK and α-T172 phosphorylation. ❷ AMPK signalling commences, including phosphorylation of the autophagy initiator ULK1 at a membranous location such as mitochondria. ❸ Activated ULK1 eventually phosphorylates β1-S108 in a manner requiring β-subunit myristoylation, sensitising AMPK to ADaM ligands like palmitoyl-CoA (PCoA) that would be getting imported into mitochondria for oxidation and ATP production. ULK1 also phosphorylates the α-subunit of AMPK, inhibiting α-T172 phosphorylation and thereby switching ligand dependency from the γ-CBS3 site to ADaM site to prolong AMPK activity. Whether this involves nucleotide exchange and ATP occupancy of γ-CBS3 is unclear. ❹ The myristoyl group reassociates with the kinase domain and AMPK is ultimately deactivated once cellular homeostasis is restored. **B**) The gene encoding β2 (*PRKAB2*) is located on chromosome 1q21, a region frequently amplified in cancer. In metastatic breast cancer, *PRKAB2* is co-amplified with the tumour promoter gene *CHD1L*. **C**). These collaborative effects between co-amplified genes are presented in a hypothetical schema. First, CHD1L is a nuclear protein that supresses apoptosis (e.g., via SPOCK1-mediated Akt activation and Nur77 nuclear sequestration) and transcriptionally promotes metastasis through ARHGEF9, a GTPase activating protein for Cdc42. Second, β2-AMPK complexes are highly prone to nuclear translocation; thus, the prediction is that α1β2-containing AMPK is especially oncogenic. Apoptotic signalling, in this instance anoikis triggered upon metastatic detachment, would elicit caspase-3 cleavage of α1 (note, caspase-3 can enter the nucleus by passive diffusion across the nuclear pore complex (NPC)), sequestering AMPK in the nucleus. Nuclear AMPK complexes may reprogram metastatic cancer cell metabolism toward a more oxidative phenotype (e.g., PGC-1α-induced mitochondrial biogenesis), ensuring survival once cells enter the circulation. Where phosphorylation (Ⓟ) events are depicted in **A** and **C**, red lines ending with a circle denote inhibition of the target and black arrows activation.

redistributed to organelles like mitochondria where it phosphorylates ULK1 on S556 (mouse S555 numbering most often used), a key residue implicated in mitophagy [185,188,189]. Multi-site phosphorylation by ULK1 (including β1-S108) switches allosteric ligand dependency from AMP to the ADaM site where ligands like long-chain FA-CoAs, imported into mitochondria to be oxidised under metabolic stress, can prolong AMPK activity (Figure 4A). Either way, this entire β1-specific operation is governed by the myristoyl-switch and uncovers further opportunities to explore other relevant signalling pathways and biological outcomes.

One of interest concerns exactly how AMPK regulates mitochondrial dynamics and mitophagy in response to stress. The current paradigm delineates that the initial reaction to agents that cause mitochondrial dysfunction is AMPK-dependent (phosphorylation of mitochondrial fission factor [190]) fragmentation or 'fission' of mitochondria that generates two daughter organelles, whereby the depolarised/damaged one is segregated for mitophagic clearance [15]. ULK1-S555 phosphorylation is rapidly enhanced (within 2 min) in response to these conditions, constituting one of the most proximal steps in the mitophagic signalling cascade [191]. Paradoxically, despite strengthening binding of the two enzymes, AMPK phosphorylation of ULK1-S555 (and likely other sites; e.g., T660) is thought to *inhibit*, rather than activate, ULK1 kinase activity, in part by preventing dephosphorylation of the mTORC1 site on ULK1, S757 [192]. This explains findings of how AMPK puts the brakes on autophagy as an acute response to energy and nutrient stress [192—194]. Pharmacological suppression of mTORC1 and amino acid starvation, potent inducers of autophagy, trigger marked dephosphorylation of both mTORC1 *and* AMPK sites on ULK1, breaking the interaction between the latter two kinases [192,194,195]. In all likelihood this is simply a reflection of AMPK's most basic function in conserving ATP, as formation of autophagosomes that sequester unwanted cellular material like mitochondria, is an energy-consuming task [196]. Conversely, the phosphorylation-mediated interaction of ULK1 with AMPK is thought to protect ULK1 from caspase-mediated degradation with glucose starvation and mitochondrial dysfunction, eventually facilitating autophagy induction once the initial stress is cleared [192].

With prolonged exposure to mitochondrial stress (>2 h), AMPK signalling (including levels of phosphorylated α-T172 and ULK1-S555) eventually drops off, coinciding with mTORC1 inhibition and restoration of energy balance [191,197]. Despite this effect, the mitophagic disposal of damaged mitochondria, at a time where ATP levels would otherwise be stable, is not only dependent on myristoylation of the β1-subunit isoform, but also the kinase activity of AMPK [37], suggesting

β1-AMPK remains active throughout the entire process. Therefore, whilst the spatiotemporal dynamics of the ULK1 ligand-switch are still a mystery, conceptually it makes sense that it would occur under conditions of reduced ATP consumption (and less AMP generation) to sustain β1-AMPK activity and complete the mitophagic assignment (Figure 4A). Because mitochondrial fission increases fatty acid oxidation (which would help restore energy balance) [198], FA-CoAs might rapidly occupy the ADaM site following mitochondrial stress, with ULK1 ensuring it remains there (via β1-S108 phosphorylation) well after cellular ATP has been replenished. Whether fully dephosphorylated (on AMPK/mTORC1 sites) and maximally active ULK1 is required for this event, remains to be seen.

7.2. Implications of β-subunit myristoylation in disease

Few studies have assessed the therapeutic potential of targeting AMPK myristoylation. β1-G2A knock-in mice are protected from high-fat diet-induced obesity and hepatic steatosis because of hyper-phosphorylation of ACC1 in the liver (repressing lipid synthesis) and induction of genes involved in fatty acid oxidation in brown adipose tissue [176]. Mouse C2C12 myoblasts treated with leptin demonstrated increased nuclear localisation of AMPK nucleated by α2 and β2, but not α2 and β1, unless β1 carried the G2A mutation [31]. This illustrates that certain upstream cues converging on intracellular organelles where β2 abounds somehow prioritise delivery of these AMPK complexes to the nucleus. Findings of circadian and rhythmic nuclear infiltration of α1 commensurate with β2 and not β1 expression in rat liver, lends some support to that idea [199]. In addition, dephosphorylation of β2-S184 but not the analogous β1-S182 site, enhances nuclear AMPK activity [53].

Analysis of the TCGA PanCancer database demonstrates amplification of *PRKAB2* in a range of cancers (Supplementary Table 1), such as hepatocellular carcinoma and breast invasive carcinoma [133,134]. *PRKAB2* is located on chromosome 1q21, and gains of 1q occur at a high frequency in cancer [200—202]. Amplification of 1q21 often associates with a metastatic phenotype and/or disease recurrence [201,203,204], and *PRKAB2* co-amplifies with the oncogene *CHD1L*, which has documented anti-apoptotic and metastatic functions [205]. Provisional data from The Metastatic Breast Cancer Project (taken from 301 patients) demonstrates significant co-amplification of *PRKAB2* and *CHD1L* (Figure 4B; Supplementary Table 1) [133,134]; their hypothetical collaborative effects are depicted schematically in Figure 4C. Caspases increase the diffusion limit of nuclear pores [206], allowing caspase-3 to accumulate in the nucleus upon apoptotic stress where it could cleave the α1-NES [144]. Overall, α1β2-containing complexes may have the greatest oncogenic potency due to their propensity to localise to the nucleus and direct anti-apoptotic pathways (e.g., transcriptional induction of mitochondrial biogenesis). This may underpin a role for these AMPK complexes during metastasis and/or disease recurrence. That *PRKAA1* and *PRKAB2* are both amplified in lung adenocarcinoma, although without significant overlap (Supplementary Table 1), suggests elevated production of β2 may increase the steady-state levels of nuclear AMPK, arming cancerous cells with reinforced defences against genotoxic stress and nutrient starvation. It is interesting to observe that a small pool of nuclear AMPK is targeted for poly-ADP-ribosylation by the polymerase PARP1 in response to nutrient starvation, triggering AMPK nuclear *export* and autophagy initiation [207].

Comparatively little is known about the role of β1 in cancer, although one study demonstrated progressive loss of β1 expression with more aggressive stages of ovarian cancer [208]. Here, genetic silencing of *PRKAB1* exacerbated the oncogenicity of ovarian cancer cells, whereas overexpression had the opposite effect [208]. It is unlikely that the oncogenic impact of removing β1 was simply due to destabilisation of the AMPK heterotrimer, since benign and cancerous ovarian tissue express β2 [209]. Collectively, we propose that differences in the oncogenicity of β1 and β2 are spatiotemporal, orchestrated by disparate functional outcomes of N-terminal myristoylation and β-loop phosphorylation. Such differences also highlight the therapeutic potential of ADaM compounds like MT47-100, which apart from its β1 selectivity for AMPK activation, inhibits β2 complexes [87].

8. THE DICHOTOMY OF γ2 IN HEALTH AND AGING

Transcription of *PRKAG2* from different promoters produces several protein products differing in length from the canonical γ2-A (or 'long') sequence. These include γ2-B (or 'short') and γ2-C, which lack the first 241 and 44 amino acids, respectively [210], as well as a so-called γ2-3B whose first 32 amino acids are unique and is missing roughly half of the NTE [211]. γ2-3B is the most abundant variant in the human heart [211]. Basal activity of γ2-containing AMPK expressed in mammalian cells is elevated above other γ isoforms [18,99], which is due to the γ2-NTE protecting α-T172 from phosphatases and/or potentially promoting its phosphorylation by an upstream kinase [53,120,212]. This points to a direct interaction of the γ2-NTE with the kinase domain to enhance net α-T172 phosphorylation. AMP fulfills all three of its activatory obligations for γ2-AMPK [120]. Of note is that the EC_{50} values of AMP and ADP that protect against α-T172 dephosphorylation in γ2 complexes are comparable (130 μM and 180 μM, respectively), which is in contrast to γ1- and γ3-AMPK that have EC_{50} values one order of magnitude higher for ADP than AMP [120]. This effective concentration of ADP for γ2 is below the amount detectable in energy balance (400 μM) [62]. Thus, γ2-AMPK is intrinsically 'calibrated' to remain active even under conditions of homeostatic ATP turnover devoid of appreciable AMP production. Given that constitutive γ2-AMPK activity precipitates several disease states [100,213], emphasises the importance of counter-regulatory mechanisms to keep any untoward activity at bay.

The human γ2-NTE is home to well over 30 putative serine and threonine phospho-sites, many of which are conserved in mice [214]. The γ2-NTE also contains 14 serines followed by a proline, a favourable consensus motif for mTORC1, as well as a NLS that enables nuclear shuttling of γ2-AMPK during energy stress [215]. Embedded in the γ2-NLS is S16, one of the proline-directed sites originally identified in a high-throughput study of murine pancreatic β-cells [216]. Phosphorylation of another serine—proline site, γ2-71, was found to be both insulin- and torin1-sensitive in adipocytes [217]. Mass spectrometry was used to identify four torin1-sensitive, proline-directed phosphorylation sites on γ2 (γ2-S113, γ2-S143, γ2-S162, γ2-S196) that have variable sensitivity to the mTORC1-specific inhibitor rapamycin (Figure 5A) [53]. Of these, γ2-S196 has a similar phosphorylation stoichiometry to α2-S345 in mammalian cells (∼40—60% [12,53]), with the sequences flanking both phospho-sites bearing some resemblance to one another (α2: $FYLASS^{345}PPSGS$; γ2: $IYASSS^{196}PPDTG$). Moreover, phosphorylation of γ2-S196 is modulated by insulin in adipocytes and glucose levels in pancreatic β-cells [216,217], altogether providing strong evidence it is an mTORC1 substrate.

The most well-studied aspect of γ2 physiology is its function in the heart. γ2-AMPK regulates intrinsic heart rate and is required for the salutary cardiac adaptations to endurance exercise training [218]. In the failing human heart, there is an upregulation of α1, β1 and γ2 expression, specifically the γ2-C variant, which due to its shorter NTE

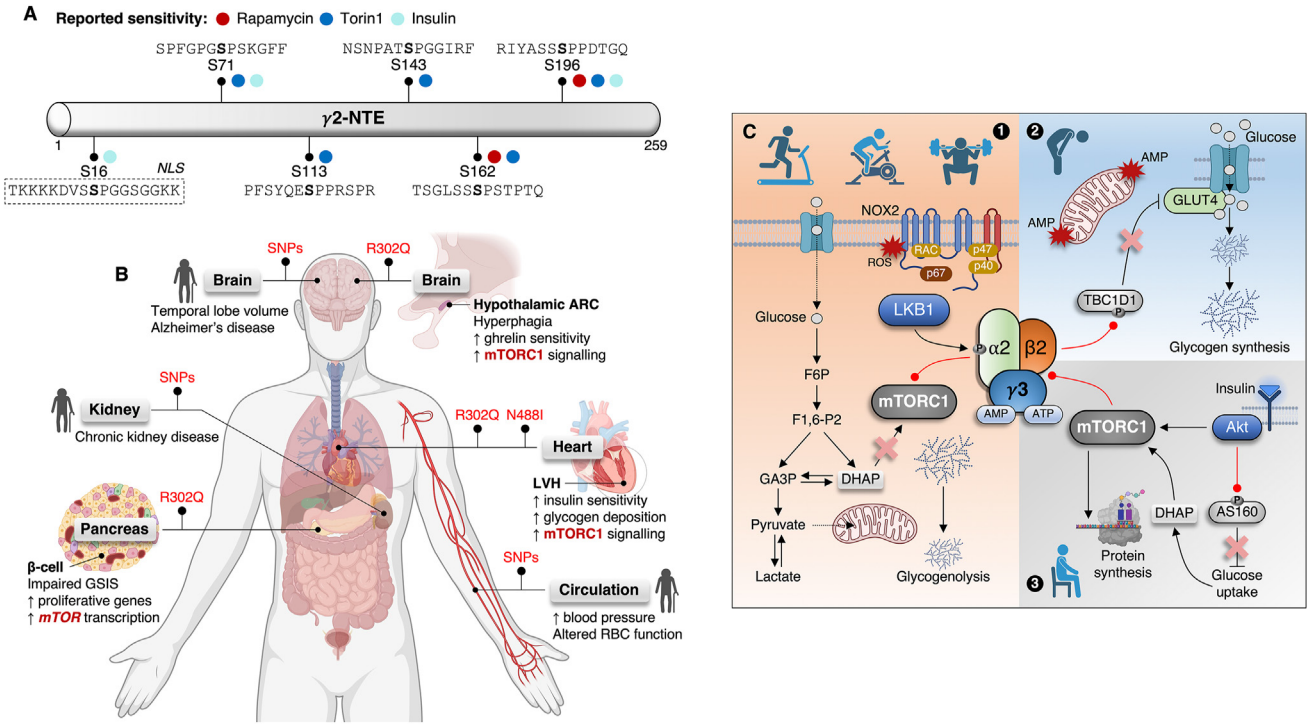

Figure 5: γ2- and γ3-AMPK in health and disease. A) The γ2-NTE is a hotbed of posttranslational modifications, especially phosphorylation, and home to 14 phospho-serines immediately followed by a proline residue, a favourable mTORC1 consensus sequence. Some of these sites, highlighted here, are sensitive to pharmacological mTORC1 inhibition (rapamycin, torin1), as well as insulin, a known activator of mTORC1 activity. Whilst the functional consequences of phosphorylation of these sites is completely unknown, they likely contribute to the complex interplay between γ2-AMPK and mTORC1 in both normal (healthy) and diseased tissue. **B**) γ2-AMPK activating mutations and SNPs are implicated in a variety of disease states, in some cases associating with elevated mTORC1 activity, such as in the brain, heart and kidneys. How γ2-AMPK and mTORC1 might collaborate, rather than antagonise one another, is completely unknown. **C**) α2β2γ3 is by far the most exercise-sensitive AMPK heterotrimer in human skeletal muscle and is activated by divergent forms of physical activity, which we summarise in the following hypothetical schema: ❶ Intense exercise necessitates rapid energy extraction from glucose, either from ROS-induced glucose uptake (mediated by the NOX2 complex) or intramuscular glycogen breakdown. Elevated glycolysis limits the production of the metabolite DHAP, an indirect activator of mTORC1, in turn relieving inhibitory phosphorylation of α2β2γ3 that activates these AMPK complexes, resulting in negative feedback and further downregulation of mTORC1. ❷ Immediately following the exercise bout, α2β2γ3 is required for insulin-independent glucose uptake and replenishment of glycogen stores, in part via phosphorylation of TBC1D1 and GLUT4 activation. ❸ With prolonged exercise recovery, insulin-dependent glucose uptake results in mTORC1 reactivation via Akt and DHAP production, and re-phosphorylation of α2β2γ3 by mTORC1. mTORC1 then takes advantage of freshly generated ATP to direct the energetically costly steps of protein synthesis. Where phosphorylation (Ⓟ) events are depicted in **C**, red lines ending with a circle denote inhibition of the target and black activation. For simplicity, Ⓟ symbols are omitted from the AMPK/mTORC1 negative feedback loop.

does not harbour the NLS [219]. In what might reflect a 'last-ditch' effort, γ2-C would direct AMPK toward cytoplasmic substrates implicated in nutrient utilisation to preserve cardiac function [220]. By contrast, cardiac ischemia-reperfusion in mice was shown to promote nuclear accumulation of γ2-AMPK, where it directly phosphorylated and inhibited the transcription initiation factor TIF-1A, thereby repressing ribosomal RNA transcription and endoplasmic reticulum stress to curtail reperfusion-induced injury [215]. Reperfusion reoxygenates the tissue, supplying nutrients to activate mTORC1 [221], which also has a role in preventing reperfusion-induced injury [222]. This begs the question of what factors are responsible for dispatching γ2-AMPK to the nucleus when mTORC1 itself is an indirect activator of TIF-1A and drives ribosomal biogenesis [223]. mTORC1 inhibition by rapamycin leads to TIF-1A nuclear export [223]; hence, one possibility is that γ2-AMPK and mTORC1 can simultaneously operate, but in physically separated cellular compartments. During reperfusion, mTORC1 may regulate alternate processes (e.g., autophagy [222]) as γ2 concomitantly supresses ribosome biogenesis.

As previously discussed, constitutive γ2-AMPK activity causes pathological cardiac hypertrophy that in humans is associated with a range of γ2 missense mutations, the most common being R302Q and N488I (corresponding to the AMP-binding γ1-R70 and γ1-N256 in the CBS3/4 linker, respectively) [224]. These, and other γ2-CBS domain mutations, increase basal AMPK activity but disrupt nucleotide exchange, rendering AMPK largely insensitive to AMP [218,225–228]. In some instances, the same mutation decreases or has no effect on basal activity [101,102,227–229]. One interpretation is that enhanced γ2-AMPK activity is not intrinsically linked to a given mutation but is rather a secondary product of altered cellular bioenergetics. It is particularly noteworthy that the two most prominent γ2 mutations, R302Q and N488I, are associated with elevated cardiomyocyte mTORC1 signalling and growth and proliferation in cultured cells and *in vivo* [230,231], revealing broader implications for conditions in which interplay between these two normally antagonistic kinases accounts for disease pathogenesis.

8.1. γ2 and mTORC1 control appetite and pancreatic function

AMPK expressed in the hypothalamic region of the brain controls appetite. Hypothalamic AMPK is activated by a fall in global energy levels and secretion of the orexigenic gut hormone ghrelin to drive food-seeking behaviour [232–234]. Conversely, feeding and signalling via the anorexigenic, adipocyte-derived hormone leptin, inhibits

hypothalamic AMPK and promotes satiety [232,235]. Changes in the methylation status of the *PRKAG2* gene are associated with extreme eating disorders (e.g., hypomethylation; binge eating) [236,237], and human γ2-R302Q carriers have increased adiposity and altered glucose homeostasis [213]. Mice expressing the corresponding γ2-R299Q mutation are characterised by hyperphagia-induced obesity arising from hypersensitivity to ghrelin and activation of orexigenic agouti-related peptide (AgRP)/neuropeptide Y (NPY)-expressing neurons in the hypothalamic arcuate nucleus (ARC) [213].

Two overrepresented transcriptional pathways in the ARC of γ2-R299Q mice are mitochondrial oxidative phosphorylation and paradoxically, mTOR signalling and the enrichment of ribosome biosynthesis genes [213]. In fact, phosphorylated ribosomal protein S6, a readout of mTORC1 activity and marker of ARC ghrelin signalling [238—240], is higher in mutant γ2 versus wild-type mice in response to fasting [213]. Notwithstanding some inconsistencies over the importance of mTORC1 in controlling feeding behaviour in AgRP/NPY-expressing neurons [239—243], these findings give the impression that mutant γ2-AMPK facilitates mTORC1 signalling, enabling each kinase to be operational in unison. In neighbouring pro-opiomelanocortin (POMC)-expressing ARC neurons, mTORC1 has a vastly different role. mTORC1 mitigates the inhibitory action of leptin on food intake [244,245], in part by promoting α2-S491 phosphorylation and inhibition of AMPK [9]. It is unclear if mTORC1 is inhibiting POMC-expressed γ2-AMPK, as mice with α2 solely deleted in POMC neurons encounter age-related increases in bodyweight and adiposity due to reduced daily energy expenditure rather than increased appetite for which γ2 is responsible [246]. Furthermore, mice with POMC neurons genetically modified to constitutively activate mTORC1, have a near-identical hyperphagic obese phenotype to γ2-R299Q mice [242,247].

Similar patterns emerge in the pancreas. γ2-R299Q mice have impaired pancreatic glucose-stimulated insulin secretion (GSIS) associating with enrichment of so-called "disallowed" genes involved in cell proliferation and repression of genes required for β-cell maturation [213]. β-cell-specific deletion of the gene *Tsc1*, which hyperactivates mTORC1, generates a strikingly similar transcriptional profile culminating in dysregulated GSIS [248]. γ2-R299Q mice also have an increased rate of *mTOR* transcription in β-islets [213], providing another clue that γ2 complexes and mTORC1 work in tandem during disease pathogenesis. Joint loss of α1 and α2 in pancreatic β-cells relieves repression of these disallowed genes, otherwise mirroring the transcriptional consequences of both γ2-AMPK *and* mTORC1 hyperactivity [213,249]. As such, it has been surmised that reciprocal antagonism between mTORC1 and AMPK in general determines the proliferative capacity of β-cells (immature phenotype), or their insulin-responsiveness (mature phenotype) [248]. Such functional relationships, not just in the pancreas and brain, but possibly other cells and tissues, are all but abolished when either kinase is inexhaustibly active. One possibility is that γ2-AMPK and mTORC1 somehow participate in a positive, rather than negative, feedback loop in this setting. How this is accomplished remains a mystery, and delineation of the functional consequences of mTORC1-mediated phosphorylation of the γ2-NTE will be revealing, since γ2 could almost be invoked as some kind of "rogue element" shying away from the classical negative feedback loop that characterises AMPK/mTORC1 crosstalk [250,251].

8.2. γ2 and mTORC1 are implicated in aging

Dietary energy restriction and rapamycin are both recognised for their ability to retard pathophysiological processes associated with aging [252,253]. For this reason, mTORC1 has been pinned as one of the foremost pro-aging factors in mammalian systems and AMPK a promising therapeutic counterbalance [254,255]. For nearly two decades, activation of AMPK has been linked to longevity in model organisms [256—260], whereas age-associated reductions in mitochondrial function manifest from reduced potency of AMPK, contributing to dysregulated lipid metabolism and susceptibility to diseases like type 2 diabetes [261]. However, the role AMPK has in promoting longevity seemingly depends on the actions of the γ isoform. Fasting regimens upregulate γ2 expression in adipose tissue of both humans and model organisms [262,263], yet its levels increase with aging, at which point γ2 expression becomes indifferent to changes in nutritional status [262]. When discerning the part played by AMPK γ isoforms in aging, γ1 presents itself as a longevity molecule; introduction of the γ1-R70Q mutant in the killifish model organism, which as previously mentioned corresponds to γ2-R302Q, improves metabolic health parameters and leads to longevity [262]. In that investigation, expression of *PRKAG1* in humans was identified as a marker of healthy aging [262]. Underscoring the intrinsic pro-metabolic health/anti-aging properties of γ1 is the finding that transgenic mice bearing an activating D316A mutation are protected from diet-induced obesity [264].

A growing list of studies have identified relationships between *PRKAG2* single nucleotide polymorphisms (SNPs) and cognitive impairment, type II diabetes, and diabetes-related traits in middle-aged and older individuals [265—268]. Genome-wide association studies have also identified *PRKAG2* as a gene located in susceptibility loci for chronic kidney disease [269—272], as well as loci influencing blood pressure and red blood cell function [273—275]. The former is in keeping with γ2 mutations associated with cardiac dysfunction causing kidney injury and morphological abnormalities in mice and humans [225,276]. Additionally, SNPs in *PRKAG2* were found to be one of the top genetic determinants influencing temporal lobe volume [277]. *PRKAG2* expression is elevated in the brains of individuals with Alzheimer's disease and its protein content correlates with toxic β-amyloid deposits [278]. A mouse model of Alzheimer's disease demonstrated γ2 is heavily glycated in hippocampal extracts, yet this coincides with a parallel reduction in α-T172 phosphorylation that can be overturned by arginine supplementation [279]. It is unclear whether γ2 action herein is causative or compensatory, analogous to its mobilisation in the injured heart [215]; regardless, the data from human studies make a compelling case for γ2 being implicated in disease aetiology.

Rare variants of *PRKAG2* have been associated with human longevity [280], and a recent investigation demonstrated that as a target of metformin, *PRKAG2*-induced lowering of glycosylated haemoglobin (a risk factor for type II diabetes) was associated with a reduction in biomarkers of aging [281]. Taken altogether, the weight of evidence portrays γ2 as both friend and foe in health and disease. Its more nefarious side, caused by missense mutations/SNPs (and even amplification in cancer; Supplementary Table 1) in many respects collaborates with mTORC1 en route to compromised longevity (Figure 5B), whereas γ2 fit for purpose (i.e., nucleotide sensing) has a *function* that preserves organismal health. γ1 and γ2 operating opposingly as anti- and pro-aging isoforms is a novel proposition that warrants further investigation to unravel the precise feedback mechanisms controlling γ2-AMPK/mTORC1 and relationships to physiological processes.

9. THE CURIOUS CASE OF γ3

Of all the AMPK subunit isoforms, arguably the most inscrutable is γ3. α2β2γ3 is probably the only physiologically relevant γ3-containing AMPK heterotrimer, found chiefly in skeletal muscle alongside α1β2γ1 and α2β2γ1, but also in brown adipose tissue [282—285]. In

humans, α1, α2, β2 and γ1 expression are comparable between skeletal muscle fibre types, whereas γ3 is predominantly found in fast type II (glycolytic) skeletal muscle fibres [58,286], with congruent observations in rodents [287,288].

Active (α2-T172-phosphorylated) human α2β2γ3 expressed in bacteria has a substantially higher Michaelis–Menten constant (K_M) for ATP (~9-fold) than α2β2γ1 [113], indicating that in addition to the γ2-NTE, the γ3-NTE may likewise interact with the kinase domain. Various reports have shown that the basal activity of α2β2γ3 can vary in comparison (both higher and lower) to the α2β2γ1 complex [139,282,287,289]. Despite a higher affinity of γ3-CBS3 for AMP than γ1 [113], and an ability to associate with AMP mimetics (i.e., ZMP) [290], substantial evidence demonstrates AMP has negligible-to-mild allosteric activating effects toward γ3-AMPK [18,99,113,120,291–293]. This points to altered interactions of the nucleotide-sensing α-linker with the CBS domains of γ3. In addition, although occupying distinct regions on the γ-subunit to AMP, the AMP mimetic C2 also fails to allosterically activate γ3-AMPK [72]. AMP does, however, promote LKB1 phosphorylation of α-T172 and protects it from dephosphorylation in γ3 complexes, but the LKB1-promoting effect is weaker in comparison to γ1 [120]. Considering γ1- and γ3-CBS3 domains share only 60% sequence conservation (Table 1), in the absence of any structural and/or biophysical data it remains unclear what role unique or poorly conserved regions in γ3, like the NTE and CBS3, have in modulating AMPK activity.

9.1. γ3 activation by AICAR and exercise

α2β2γ3 is unequivocally the most exercise-sensitive AMPK complex, activated in humans by both short-duration/high-intensity exercise (ranging from 30 s maximal effort sprints to 20 min at ~80% peak O_2 consumption ($\dot{V}O_{2peak}$)), and longer, sub-maximal intensity exercise bouts (>30 min, ~65% $\dot{V}O_{2peak}$) [58,282,294,295]. In contrast, α2β2γ1 has been shown to be inhibited by sprint exercise [282]; α2β2γ1 activation commands a longer exercise duration (>60 min) at moderate intensities, yet the magnitude of its response still pales in comparison to α2β2γ3 (e.g., rapid and >10-fold α2β2γ3 activation versus slower, and maximum ~3.5-fold α2β2γ1 activation) [58,294]. The same can otherwise be said for α1β2γ1, but the overall activating effect of exercise is generally very weak [294], with the majority of studies assaying α1 skeletal muscle immunocomplexes (presumably representing α1β2γ1) either reproducing this mild effect [296], or showing no change in activity at all [111,112,297,298]. Some inconsistencies exist, including activation [299] and inhibition [282] of α1-AMPK immediately following an identical sprint protocol. Regardless, in support of α2β2γ3 being the major exercise-sensitive AMPK heterotrimer, following a period of habitual exercise training, AMPK activity declines in response to acute exercise, which can be partly explained by reduced γ3 expression [285,300–302].

Skeletal muscle α2β2γ3 is responsible for glucose uptake and replenishment of energy stores in the recovery period from exercise, rather than during the exercise bout itself [295]. The latter is mostly AMPK-independent, involving cytosolic reactive oxygen species (ROS) production by NADPH oxidase 2 (NOX2) and GLUT4 translocation to the plasma membrane in a manner dependent on the small GTPase, Rac1 [303]. In a similar vein, α2β2γ3 is required for glucose disposal into glycolytic muscle in response to AICAR, but not ADaM site activators [283,284,293]. That role is fulfilled by α2β2γ1 in both muscle fibre types [18]. AICAR-induced activation of skeletal muscle AMPK (presumably occurring via ZMP-induced α-T172 phosphorylation) is reminiscent of exercise, in that it more potently activates α2β2γ3 than other AMPK complexes [284,304]. Furthermore, γ1-AMPK has been shown to be upregulated by AICAR with shorter (40 min) but not longer (1 h) incubations in glycolytic muscle [284], coinciding with findings in isolated hepatocytes and adipocytes (where γ1 is a major isoform) in which AICAR-induced AMPK activity occurs rapidly (15 min) yet declines thereafter (1 h) despite elevated ZMP [68].

9.2. γ3 and mTORC1 crosstalk

Recent phosphoproteomics analyses of human skeletal muscle subjected to three different modes of exercise (prolonged endurance, sprint and resistance-based) has proven valuable in understanding the regulation of α2β2γ3 [305]. Immediately following exercise there is a clear diminution of mTORC1 signalling and α2-S377 phosphorylation alongside elevated α-T172, α2-S491 and β2-S108 phosphorylation, signifying an active α2β2-containing enzyme [305]. Because endurance and sprint exercise bouts were virtually identical to aforementioned α2β2γ3-activating protocols that elicit either minor activatory (endurance) or inhibitory (sprint) responses from α2β2γ1 [282,294], suggests this phosphorylation profile symbolises α2β2γ3 [305]. Whilst the analysis did not detect phospho-α2-S345 (potentially due to poor sequence coverage of the area surrounding α2-S345 in their analysis), its phosphorylation has been detected in human muscle and C2C12 myotubes by western blot analysis [8].

Dihydroxyacetone phosphate (DHAP) is a glycolytic intermediate cleaved from fructose-1,6-bisphosphate by the enzyme aldolase, where it serves as a precursor for lipid synthesis or is isomerised and resumes catabolism through the lower segment of glycolysis. DHAP is also an indirect activator of mTORC1, and its depletion switches off mTORC1 independently of AMPK [82]. Accelerated glycolytic flux accompanying high-intensity exercise would constrain DHAP production, inhibiting mTORC1 and its phosphorylation of α2β2γ3. We also predict this accounts for AICAR's potency toward α2β2γ3. Like exercise, AICAR induces glycolysis (as evidenced by a spike in blood lactate after *in vivo* administration) [306], and is anticipated to potentiate glycolytic flux via allosteric regulation of AMP-sensitive glycolytic enzymes [69,307]. Altogether, mTORC1 is potentially a major regulator of α2β2γ3, either due to cellular colocalization, or ostensibly via the γ3-NTE serving to increase effective substrate (i.e., α2-S345/S377) availability. Supporting the latter proposition, the basal phosphorylation stoichiometry of α2-S377 is almost 10% higher in the α2β2γ3 versus α2β2γ1 complex expressed in HEK293T cells [53]. Feedback between γ3-AMPK and mTORC1 is perhaps best exemplified by mice expressing kinase-dead α2 that experience a diminished muscle protein synthetic response to contractions owing to a glycogen deficit [308]. In humans, marked α2-AMPK activity following high-intensity resistance-based exercise precedes stimulation of muscle protein synthesis [309], which is mTORC1-dependent [310]. Therefore, α2β2γ3-dependent post-exercise glucose uptake and glycogen replenishment would be leveraged to generate ATP in preparation for the energetically costly steps of protein translation (Figure 5C). Whilst this might not be the case for mice [311], a lack of inter-species conservation in γ3 would presumably result in regulatory differences.

9.3. Caveats of murine studies of γ3

One caveat associated with detailed studies of γ3 is the use of mouse models, either via knockouts or introduction of point mutations [283,284,293,295,311–313]. This is because at the entire sequence level, mouse versus human γ3 has the lowest level of similarity between all three γ isoforms (γ1 = 97%, γ2 = 94%, γ3 = 84%). If the sequences of CBS domains alone are considered, this conservation improves by roughly 5% for γ2 and 13% for γ3. The largest degree of inter-species sequence divergence is evident for the γ-NTE; 65%,

86% and 66% similarity for γ1, γ2 and γ3, respectively. Since differential regulation of γ2 and γ3 complexes probably arise as a result of their respective NTEs, studies assessing murine versions of these proteins, namely γ3, should be interpreted with some caution in relation to our understanding of human physiology. In that regard, a future, "humanised" γ3 mouse model (conserving the γ3-NTE) would be illuminating, particularly in the context of exercise-induced substrate utilisation.

10. NOVEL INSIGHT FROM GENETIC KNOCKDOWN STUDIES OF AMPK

Individual genetic deletion of α1, α2, β2, γ1 or γ3 have all been shown to trigger appreciable loss of other subunit isoforms they would be expected to form heterotrimers with [262,283,284,314–317]. This is because unbound subunits of AMPK are susceptible to degradation [118,318]. Most of these studies were undertaken in skeletal muscle and will be the main focus of discussion here. In mice, β1-containing AMPK complexes make up only a small proportion of the entire heterotrimer population [287], explaining why global and muscle-specific loss of β1 has no effect on α1 and α2 stability in this tissue [110,316]. Despite no evidence of forming AMPK heterotrimers in human skeletal muscle [285], β1 and γ2 are still expressed in this tissue [58,291,300,302,319]. β1 is located mainly in oxidative type I human muscle fibres [58], while the mRNA encoding γ2 is the most abundant of all three γ isoforms in human primary muscle cells originating from satellite cells, which are involved in muscle regeneration [288]. Of interest is the γ2-3B splice variant that has been detected in oxidative skeletal muscle fibres, albeit in mice [320]. Serine 25, unique to γ2-3B, was found to be a regulated phospho-site in human skeletal muscle following endurance exercise [305], suggesting this modification is not inconsequential. Basic arginine residues line the P−2 and P−3 positions, making this site a good fit for a basophilic (and exercise-sensitive) kinase like protein kinase A (PKA) [172]. β1-S108 phosphorylation has also been detected in human skeletal muscle but without regulation by exercise [305]. Thus, either these phospho-species of β1 and γ2-3B are markers of AMPK in non-muscle cells (or immature muscle stem cells like satellite cells) detected in the muscle homogenate, or as somehow stable subunit isoforms, they are being directly targeted by upstream kinases in the muscle tissue.

βγ complexes can nucleate in the absence of an α isoform [56,321,322]. The first known function of a βγ heterodimer was recently assigned to an AMP-bound βγ complex that sequestered the PPP6C phosphatase targeting T56 of the eukaryotic translation elongation factor 2 (eEF2) [321]. Phosphorylation of eEF2 inhibits the energy-demanding elongation step of protein translation, and by preventing its dephosphorylation, this βγ heterodimer was thought to preserve cell viability under stress [321]. In that study, β1γ1 and β1γ2 dimers were detected in mouse liver, and β2γ1 and β2γ3 dimers in mouse skeletal muscle [321]. However, these findings could not be reproduced by a separate group, casting doubt over its physiological relevance [322]. While the latter authors did find a β2γ3 dimer in muscle from mice genetically lacking α1 and α2, it was undetectable in corresponding wild-type tissue [322]. At present, methodological differences have been asserted to account for these inconsistencies [323], which evidently will require further and independent scrutiny. The β-subunit scaffold (α-γ-SBS) contains a major hydrophobic patch (β1: 237–270; β2: 239–270) that would require another hydrophobic binding partner to assist in overall complex folding in the absence of interactions with a usual α-subunit. Complete loss of the α-subunit caused by γ1 knockdown in the killifish model does not perturb γ2 content [262], and neither is γ3 abundance meaningfully disrupted (maximum 20% reduction in expression) in both chronic and inducible muscle-specific α1/2-dKO mice, contrasting the more than 90% corresponding loss of γ1 [317]. However, in both instances, either β1 (killifish) [262] or β2 (muscle) [317] expression is abolished. Questions that arise are whether there are elements in the γ2/3-NTEs that strengthen an interaction with a given β isoform to make these isoforms more amenable to dimerization, or even encourage association with non-AMPK proteins, or is it simply a case of γ2 and γ3 being less susceptible to degradation than other subunit isoforms? Complicating matters further, the α-subunit can also form dimers with either β or γ when co-expressed in mammalian COS7 cells [324].

One controversial idea already put forward is that βγ dimers can associate with an alternate protein kinase, such as one of the AMPK-related protein kinases [139], a family comprised of 13 members (including AMPK) that are all phosphorylated on a conserved activation loop threonine by LKB1 [325]. We are familiar with only one study undertaken in HEK293 cells that found no association of β1, β2 and γ1 with any AMPK-related kinase [326]. Although in that investigation, one of the family members NUAK2, which is sensitive to contraction and regulates myocyte survival [327,328], co-associated alongside AMPK with the HSP90/Cdc37 co–chaperone complex [326]. In addition to protein folding and stabilisation, HSP90/Cdc37 complexes facilitate transitions of protein kinases to interact with different effector molecules [329]. Therefore, a relevant direction for the field will be to 1) determine whether βγ dimers can form in normal cells and tissues (i.e., non-genetically modified), 2) if they have non-α-subunit complex partners, and 3) pinpoint what is responsible for the stability and regulation of single subunit isoforms (e.g., γ2-3B in skeletal muscle) and if it is due to dimerization or even interactions with proteins other than AMPK.

11. CONCLUSIONS AND PERSPECTIVES

AMPK is an ancient and fundamental regulator of cellular and whole-body metabolism that couples the availability of individual nutrients and metabolites with organismal growth potential. One of the biggest obstacles to understanding the regulation and function of AMPK is disentangling the precise, individual actions of heterotrimeric complexes nucleated by differing subunit isoforms, for which there are a multitude of possible combinations that vary depending on cell and tissue type, age, and disease states. In the present review, we have shed light on several key and unresolved areas of AMPK isoform biology that can be summarised as follows:

Firstly, α1-AMPK complexes have higher basal activity than α2 and are more amenable to α-T172 phosphorylation-independent activation by ADaM site ligands. α2 complexes display greater AMP sensitivity than α1 and are more heavily regulated by LKB1 and mTORC1. We suspect some of these differences account, at least in part, for the tumour promoter and suppressor functions of α1 and α2, respectively. Next, the activity of β1-AMPK is preferentially regulated by the myristoyl-switch and movement on and off cellular membrane-bound organelles, while the propensity for β2 complexes to infiltrate the nucleus ostensibly reconciles their oncogenicity in some cancers. Lastly, the unique γ2- and γ3-NTEs, suspected of directly interacting with the kinase domain, are emerging as major regulatory hotspots for these γ isoforms. The γ2-NTE is home to a multitude of phosphorylation sites, many of which are putative mTORC1 substrates. However, in contrast to the classical negative feedback loop, γ2-AMPK and mTORC1 enjoy a peculiar relationship, in which they seem to cooperate with one another in a variety of diseases and potentially throughout aging. The γ3-NTE may be a critical regulatory node fine-tuning AMPK activity in

response to physical exercise (e.g., affecting colocalization with mTORC1), and future studies should now consider either alternatives to rodent models, or even genetically engineered "humanesque" γ3 in mice to tease out mechanisms of preferential γ3-AMPK activation and control of glucose uptake by exercise and AICAR.

Like any comprehensive review, we are left with a host of questions. One concerns the contentious and current hot topic of whether AMPK isoforms can assemble into functional dimers, or even interact with novel, non-AMPK binding partners. Current evidence for AMPK forming dimers in normal cells and tissues and assuming physiologically relevant roles is inconclusive. Nevertheless, unbound monomers of AMPK are inherently unstable. Thus, observations of γ2 and γ3 expression persisting in the face of complete loss of their binding clientele, not to mention expression and phospho-regulation of γ2 in human muscle where it does not form heterotrimers, makes a reasonable case for their incorporation into AMPK dimers, or even formation of unorthodox protein complexes. Reinforcing our earlier point, this is an extremely fertile area of research and could uncover completely unexpected roles for AMPK and associated biological and physiological systems.

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CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

William J. Smiles: Writing — review & editing, Writing — original draft, Conceptualization. **Ashley J. Ovens:** Writing — review & editing, Writing — original draft. **Jonathan S. Oakhill:** Writing — review & editing, Writing — original draft. **Barbara Kofler:** Writing — review & editing, Writing — original draft, Conceptualization.

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Images of protein structures were rendered with PyMol and figure schematics were created using selected images obtained from BioRender.com. Sequence alignments were performed using Clustal Omega and secondary structure predictions were performed using the Chou-Fasman method (http://cib.cf.ocha.ac.jp/bitool/MIX/).

DECLARATION OF COMPETING INTEREST

The authors declare there are no competing interests associated with this manuscript.

DATA AVAILABILITY

Data will be made available on request.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2024.102042.

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