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Regulation, Role and Therapeutic Targeting of AMPK in Prostate Cancer

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Abstract

The 5′-AMP-activated protein kinase (AMPK) is a master regulator of cellular homeostasis. Despite AMPK's known function in physiology, its role in pathological processes such as prostate cancer is enigmatic. However, emerging evidence is now beginning to decode AMPK's paradoxical role in cancer and therefore inform clinicians if and how AMPK could be therapeutically targeted. Here, we propose that it is the spatiotemporal regulation of AMPK complexes that govern the kinase's role in cancer. We hypothesize that different upstream stimuli will activate select subcellular AMPK complexes. This is supported by the distinct subcellular locations of the various AMPK subunits. Each of these unique AMPK complexes regulate discrete downstream processes that can be tumor suppressive or oncogenic. It is the weighted net function of these downstream signaling events, influenced by additional prostate-specific signaling, that determines AMPK's final biological output.

Keywords

5′-AMP-activated protein kinase (AMPK); androgen; androgen receptor (AR); calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2); metabolism; prostate cancer

Introduction

One of the most enigmatic signaling molecules in biology is the 5′-AMP-activated protein kinase (AMPK). AMPK is an evolutionarily conserved serine/threonine protein kinase that is a master regulator of cellular homeostasis[1]. Classically, AMPK has been defined by its ability to help cells adapt to various energetic stresses. In this context, activation of AMPK promotes ATP-generating catabolic processes while simultaneously inhibiting ATP-depleting anabolic processes[1]. As such, AMPK is absolutely required for embryonic growth

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A.S.K. and D.E.F. contributed equally to the manuscript. Both researched data for the manuscript, wrote the article and edited the manuscript prior to submission.

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and development[2,3]. While AMPK's function in basic physiology is established, its role in pathological processes such as cancer is far more confusing.

Initially, AMPK was described as a tumor suppressor due to its link to one of its major upstream regulators liver kinase B1 (LKB1), a *bona fide* tumor suppressor[4–6]. Correspondingly, a number of *in vitro* and *in vivo* studies have implicated an anti-cancer role for AMPK[7–20]. Conversely, recent reports implicate an oncogenic role for AMPK[21–45]. While some of these discrepancies may be attributable to the use of non-selective pharmacological modulators of AMPK, many of which have cellular effects independent of AMPK[34,45–55], molecular and genetic studies do indicate AMPK can have context-dependent roles[8,10,17–19,21,22,24,32–34,56–58]. This paradoxical role for AMPK is no different in prostate cancer.

As described in greater detail below, much of the initial interest in AMPK in prostate cancer came from retrospective clinical studies of the use of the antidiabetic drug metformin[59–62]. These studies suggested that metformin decreased the risk of cancer. Accordingly, several reports using pharmacological modulators of AMPK as well as genetic knockout of one of the catalytic subunits of AMPK, *PRKAA2*, support a tumor suppressive role for AMPK in prostate cancer[7,17,63–65]. However, more recent retrospective studies did not find any link between metformin use and decreased cancer risk[66–77]. In fact, some studies even suggested that increased metformin use correlated with more aggressive prostate cancers[66,71]. Importantly, the first prospective clinical trials directly testing the impact of metformin on prostate cancer have recently been completed and found limited efficacy ([78] and (NCT01433913)). Additional preclinical studies using pharmacological, molecular and genetic approaches have now identified an oncogenic role for AMPK in prostate cancer[29–31,33–35,37]. Further, levels of threonine-172 phosphorylated/activated AMPK (*discussed below*) and serine-80 of acetyl-CoA carboxylase (ACC), a canonical target of AMPK, were elevated in prostate cancer clinical samples compared to benign controls[7,33,37]. Phosphorylated/activated AMPK levels were found to also correlate with progression to the advanced/recurrent stages of the disease[33] and higher Gleason scores[7]. Collectively, these findings indicate a complicated role for AMPK that is likely context dependent. This confusion has undoubtedly frustrated clinicians and researchers and thus precluded subsequent drug development efforts.

Here, we propose a mechanistic explanation to assist in understanding how AMPK works in prostate cancer and therefore determine when AMPK is functioning in an oncogenic versus tumor suppressive capacity. Specifically, we hypothesize that there are different subcellular populations of AMPK that enable compartmentalized signaling (Figure 1). The location of these AMPK complexes is influenced by factors such as subunit composition (*reviewed below*). Each of these subcellular populations of AMPK will be associated with unique downstream cellular processes (Figure 2). Which AMPK populations are activated is determined by the spatial and temporal regulation of diverse upstream stimuli. It is the weighted net function of these downstream signaling events, influenced by additional prostate-specific signaling, that determines AMPK's final biological output. Below are descriptions of the factors that regulate AMPK activity, AMPK's known functions and current/emerging approaches for targeting AMPK signaling. It is our goal that the proposed

model can be leveraged to determine if, how and when AMPK could be therapeutically targeted.

Influence of AMPK's structure and subcellular location on downstream activity

The AMPK complex is a heterotrimer consisting of a catalytic α subunit and regulatory β and γ subunits[79,80]. The N-terminus of the α subunits contains the serine/threonine kinase domain as well as an activation loop that requires phosphorylation at a specific threonine residue, commonly referred to at Thr172 due to its position in the original rat sequence, by upstream kinases (*described below*) for full AMPK activation (activity increases > 100-fold)[81]. The C-terminus is needed for association with the β subunit. In addition, there is a central auto-inhibitory domain. The β subunit contains a central domain that allows it to interact with glycogen and a C-terminal domain that is needed for the association with the α and γ subunits. The γ subunit contains four tandem cystathionine β-synthase (CBS) motifs, three of which can bind nucleotides (site two can not). Site four appears to constitutively bind AMP, while sites one and three bind AMP, ADP or ATP in a competitive manner[82]. Binding of AMP and/or ADP promotes the phosphorylation of Thr172 on the α subunit by upstream kinases while also inhibiting the dephosphorylation of this same site by phosphatases[83–85]. These activating actions of AMP/ADP are antagonized by ATP. AMP, but not ADP, also causes an allosteric activation (reported to be 2–5 fold but the exact fold induction is still debated[86]) of the phosphorylated kinase[85]. In fact, this allosteric activation appears to be antagonized by ATP and ADP.

In total, there are two genes (*PRKAA1* and *PRKAA2*) that encode for the α1 and α2 catalytic subunits, two genes (*PRKAB1* and *PRKAB2*) encoding the β1 and β2 regulatory subunits and three genes (*PRKAG1*, *PRKAG2* and *PRKAG3*) encoding the γ1, γ2 and γ3 regulatory units[2]. The subunits are expressed to varying degrees in a cell-, tissue- and disease-specific manner[2]. In addition, splice variants of the subunits exist[87]. Thus, between the seven different subunits there are at a minimum 12 different AMPK complexes that can be formed.

An important aspect of AMPK that is often overlooked is the compartmentalization of AMPK signaling. Different isoforms of the various subunits can vary in their subcellular localization[88–91]. For example, the α1 subunit has a predominantly cytoplasmic localization while the α2 subunit can readily shuttle to the nucleus. Additional AMPK subunits have also been found to partly reside in the nucleus[92,93]. Further, the subcellular localization of subunits can be altered in response to different stimuli[90,94]. That said, there is evidence that under certain conditions α1 can also shuttle to the nucleus[95]. Whether this happens in the prostate and how this would occur are currently not known. Importantly, it has been demonstrated in yeast that the subcellular location of the β subunit directs the localization of the catalytic α subunit[91]. This effect has key functional consequences as it dictates which subset of downstream targets AMPK can interact with and therefore phosphorylate.

Relatively little is known regarding AMPK's role, if any, during the development of the prostate. Since AMPKα1/AMPKα2 double knockout mice are embryonic lethal at day 10.5

post conception, it is difficult to study prostate development in this context. To our knowledge, no one has developed a prostate-specific conditional AMPKα1/AMPKα2 double knockout mouse. Both global AMPKα1 and AMPKα2 single knockout mice have been created as well as knockouts of other AMPK subunits[2]. However, no prostate defects have been reported in any of these animals, but this could also be because the prostate was not examined. AMPKα1 global knockout mice are subfertile due to a decreased quality of spermatozoa[96]. A subsequent study using AMPKα1 conditional knockout mice identified defects in the Sertoli cells as the likely culprit[97]. Whether there were any defects in the prostate of the AMPKα1 global knockout mice and if these could then also contribute to the decreased fertility is not known. Global knockout of *Camkk2*, the predominant AMPK upstream kinase in the prostate (see "Regulation of AMPK" section), in mice did not lead to any overt morphological abnormalities in the prostate (unpublished observations). Taken together, it is not clear yet whether AMPK signaling is essential for normal prostate development. Conversely, a number of recent studies point to context-dependent roles for different AMPK complexes in prostate cancer.

Unlike LKB1, the genes encoding AMPK are rarely mutated in cancer[98]. In contrast, the various subunits are more commonly elevated by amplifications and/or overexpression in human cancers. For example, the α1 subunit is the predominantly expressed catalytic subunit in prostate cancer[33,34,99]. While double catalytic subunit knockout mice ($\alpha1^{-/-}$ and $\alpha2^{-/-}$) are embryonic lethal[2], interestingly, mouse embryonic fibroblast (MEF) cells generated from these double knockout mice are resistant to oncogenic transformation[22]. In addition, spontaneous tumor formation has never been observed in α1- or α2-deficient mice, indicating that loss of AMPK itself is not sufficient to cause tumorigenesis[98]. However, deletion of α2 alone increased the growth of RAS-transformed MEFs[8]. In contrast, deletion of α1 alone decreased growth in the same cells[8]. While genetic deletion of the minor isoform, α2, slightly increased the incidence of prostatic intraepithelial neoplasia (PIN) in a fatty acid synthase (FAS)-transgenic model of mouse prostate hyperplasia[17], knockdown of either the predominant α1 alone or double knockdown of α1 and α2 decreased prostate cancer cell growth and migration[33,34]. Further, increased *PRKAA1* and decreased *PRKAA2* expression independently predict poor prognosis in prostate cancer patients[33,100]. Taken together, these results suggest that the two α catalytic subunits may have opposing actions in prostate cancer, with the α1 subunit acting more oncogenic while the α2 subunit appears to have tumor suppressive properties.

Like AMPKα1, AMPKβ1 may also have oncogenic roles. The gene encoding AMPKβ1, *PRKAB1*, was overexpressed in metastatic prostate cancers compared to primary tumors in four separate clinical cohorts[101]. Correspondingly, low *PRKAB1* expression predicts favourable clinical prognosis[100,102,103]. In addition, AMPKβ1 was identified in an unbiased RNAi screen as an essential component for prostate cancer cell survival[101]. These data are supported by work demonstrating increased *PRKAB1* expression in colorectal cancer lesions compared to matched benign tissues[104].

Genetic studies have revealed that the subunits are not necessarily redundant and therefore different AMPK complexes likely have unique activities[2]. Further, different stimuli can lead to changes in the heterotrimer composition[105–107]. Little is known regarding which

heterotrimer complexes are preferred in cancers and if their composition changes in response to oncogenic or tumor suppressive signals. The combined effect of elevated levels of the α1 and β1 subunits with their predominantly cytoplasmic subcellular localizations suggest extranuclear AMPK complexes may play a significant role in prostate cancer. Thus, the cytoplasmic kinases (*described below*) that rapidly activate these AMPK complexes likely would also have important roles in prostate cancer. To that end, multiple groups have developed new molecular tools to assess AMPK activity at different subcellular locations[108,109]. These key studies demonstrate that diverse upstream cues can activate unique subcellular AMPK populations. We hypothesize that each one of these unique subcellular AMPK complexes will be associated with, and can therefore regulate, a specific set of downstream signalling targets. As a result, depending on the duration and type of upstream signal, very different AMPK-mediated events would be elicited. Thus, the common oversimplification of general AMPK activity could lead to numerous misunderstandings and incorrect conclusions.

Regulation of AMPK

AMPK can be activated by both allosteric modulation and posttranslational modifications (Figure 3). While still a hotly debated area, the allosteric effects may pale in comparison to the regulatory effects of posttranslational modifications such as Thr172 phosphorylation[110–112]. Regardless, the most well-studied mechanism of AMPK activation is activation by AMP/ADP:ATP ratio. When the AMP/ADP:ATP ratio increases in the cell, AMP/ADP binds to the γ subunit of AMPK[3]. This causes a conformational change in the γ subunit that, in conjunction with a β-subunit myristoylation event[94], exposes the Thr172 site located on AMPK's α-catalytic subunit. Phosphorylation of this site then activates AMPK ~100-fold[81,113]. Thus, the phosphorylation of Thr172 is tightly regulated by upstream kinases and phosphatases.

The known Thr172-targeting kinases of AMPK are LKB1, TGF-β activated kinase-1 (TAK1) and calcium/calmodulin-dependent protein kinase kinase-2 (CaMKK2 or CaMKKβ). LKB1, which activates AMPK in response to energetic stress (ex. high AMP/ADP), is a tumor suppressor that is thought to be the dominant AMPK kinase in the body. However, several lines of evidence indicate that LKB1 is not the predominant AMPK kinase in the prostate. First, while LKB1 is a known tumor suppressor for many types of cancer, prostate cancer is not one of them[114]. In support of this, when LKB1 was deleted in $PTEN^{+/-}$ mice, there was an increase in tumor incidence for many cancer types but not prostate cancer[115]. Second, androgens were reported to decrease *LKB1* expression and subsequent AMPK phosphorylation in mouse 3T3-L1 cells[116]. This effect is in direct contrast to the increase in AMPK phosphorylation observed following androgen treatment in prostate cancer cells[34,35]. Third, *LKB1* is not highly expressed or regulated by AR in prostate cancer cell models[117]. Forth, in a study that did suggest *Lkb1* deficiency caused prostatic neoplasia in mice, the authors 1) deleted *Lkb1* using a mainly gastrointestinal track-specific driven Cre rather than a prostate-specific driven Cre and more importantly 2) observed continued high levels of Thr172-phosphorylated AMPK in *Lkb1*-deficient prostates[118]. This latter data led the authors to conclude that LKB1 was not an AMPK kinase in the prostate. To that end, LKB1 phosphorylates a number of other proteins and

hence, if LKB1 were to have antiproliferative effects, this could be due to AMPK-independent effects[119]. For example, LKB1 is known to phosphorylate and stabilize the tumor suppressor PTEN, one of the most commonly mutated/deleted tumor suppressors in prostate cancer[120].

TAK1 is thought to be another activator of AMPK; however, more in depth studies are needed to understand whether this occurs *in vivo* and requires LKB1[121,122]. *MAP3K7*, the gene for TAK1, is often lost during development of prostate cancer[123–125]. But these findings appear at odds with the increased Thr172-phosphorylated AMPK levels observed during the development of prostate cancer[7,33,123–125]. Alternatively, functional data indicate that TAK1's tumor suppressive effects are mediated through other stress kinases, namely p38 and c-Jun N-terminal kinase[126]. Taken together, these data suggest that TAK1 is not a major AMPK kinase in the prostate.

Roughly a decade ago, CaMKK2 was identified by three separate groups to be a Thr172-targeting AMPK kinase[127–129]. In 2011, we demonstrated that androgens, via AR, directly increased the expression of *CAMKK2*[34]. CaMKK2 phosphorylated and activated AMPK, leading to increased prostate cancer cell migration and invasion. The androgen response element which we identified and showed was responsible for AR-mediated expression of *CAMKK2*[34] is one of the most robust AR binding sites in castration-resistant prostate cancer (CRPC) tissue[130]. Interestingly, CaMKK2 can augment AMPK activity under starvation or nutrient-rich conditions, suggesting AR-CaMKK2 may potentiate AMPK activity independent of the environmental state. Later, two independent groups confirmed our findings and also demonstrated that CaMKK2 levels were elevated in clinical samples and track with disease progression[35,36]. These findings correspond with the clinical data demonstrating that levels of the Thr172 phosphorylated form of AMPK are increased in prostate cancer and further increased in the advanced stages of the disease[7,33,123–125]. Last year, Hu et al demonstrated that the tumor suppressive microRNA, miR-224, suppressed prostate cancer cell proliferation through decreasing *CAMKK2* expression[131]. Clinically, combined low *miR-224* and high *CAMKK2* expression correlated with advanced disease and shortened survival. Remarkably, in this study the authors showed a proliferative role for CaMKK2 even in AR-negative DU145 cells, indicating that in some of the most aggressive subtypes of prostate cancer CAMKK2 may be expressed and driving oncogenic processes independent of AR. Collectively, these studies suggest that CaMKK2 is the dominant AMPK kinase in prostate cancer. In addition, new oncogenic roles for CaMKK2 in other cancer types such as stomach, liver and brain have been observed[132–134]. While a promising target, additional work is needed to assess CaMKK2's i) functional role at different disease stages, ii) regulation, and iii) complete mechanism(s) of action (to understand potential side effects).

AMPK can also be allosterically activated in two ways. First, AMPK can be allosterically activated by the binding of AMP, but not ADP, to the γ subunit. This direct allosteric activation by AMP does not require the β subunit myristoylation[94]. Second, AMPK can be pharmacologically activated by the binding of drugs such as A-769662 to the β subunit[135]. Though drugs like A-769662 function in part by inhibiting the dephosphorylation of Thr172, they also allosterically activate AMPK. As such, this type of activation does not necessarily

require Thr172 phosphorylation of the α subunit but does typically involve the autophosphorylation of Ser108 in the β subunit, which is often required for full AMPK activity[92]. It is not known whether AMPK can be activated in the absence of Thr172 phosphorylation in response to endogenous signaling.

There are other ways in which AMPK could be regulated in prostate cancer. For example, DNA damaging agents like ionizing radiation and some chemotherapies activate AMPK via ataxia telangiectasia mutated (ATM), an initiator of the DNA damage response[136–141]. While there is still debate regarding whether LKB1 is required for this genotoxic response, this will likely have consequences for therapeutic resistance[137,138,140–142]. Additionally, reactive oxygen species (ROS) can increase AMPK activity[38,143,144]. This is significant because oxidative stress is one of the hallmarks of aggressive prostate cancers[145]. Although the exact mechanisms through which this occurs are still being elucidated (ex. could also include ATM[142,146]), this may function as a survival signal for cancer cells coping with the harsh tumor microenvironment[38]. Conversely, it has been reported that protein kinase B (Akt) can phosphorylate AMPK and reduce AMPK's activation by LKB1[147]. This would seemingly be an important feedback mechanism in prostate cancer where most advanced stages exhibit elevated phosphoinositide 3-kinase (PI3K)-Akt signaling[100]. However, it is unclear if this type of regulation occurs in prostate cancer where a) levels of activated AMPK remain high even in the presence of increased Akt[7,33,37] and b) LKB1 does not appear to be the major AMPK kinase (*described above*).

AMPK-regulated processes

One of the most pressing questions regarding AMPK signaling is "Which downstream proteins does AMPK target in cancer?" Presumably not all AMPK-modulated processes are oncogenic and/or tumor suppressive. Identification of the specific cascades that are modulated by AMPK would facilitate our understanding of whether AMPK was having oncogenic or tumor suppressive effects and therefore if AMPK should be modulated therapeutically. Further, parsing out the exact downstream processes that are true oncogenic drivers would highlight potential new therapeutic targets. Listed in Tables 1 and 2 are previously described direct[18,24,26,93,95,148–203] and indirect[27,198,204–211] AMPK targets, respectively. A number of the targets have been described in greater detail in several excellent reviews[1,98,117,119,212,213]. At this time it is not known whether many of these are regulated in prostate cancer or if they have a pathogenic role in the disease. Certainly more work is needed to elucidate their regulation and role. An important point to remember is that rarely does the regulation of signaling pathways, such as those modulated by AMPK, occur in isolation. Most cancer signaling networks are influenced by additional oncogenic cascades such as PI3K-Akt or, as is typically the case in prostate cancer, AR. Below is a description of some of the known AMPK targets that likely play a role in many prostate cancers and how they could be influenced by other events such as AR signaling.

AMPK has been classically defined as a master regulator of cellular metabolism in which the activation of AMPK by energetic stress leads to an overall increase in catabolic processes. These catabolic reactions serve to breakdown nutrients for the generation of ATP. Simultaneously, AMPK shuts down a diverse range of anabolic processes to conserve ATP

levels. Hence, many of AMPK's direct actions lead to an inhibition of proliferation as a way to deal with the energetic stress. This would be consistent with a role for AMPK as an initial tumor suppressor. For example, AMPK can phosphorylate wild-type p53 on serine-15 to potentiate its activity as a tumor suppressor, increasing p21 levels and causing a G1/S cell cycle arrest[193,194]. But while sustained AMPK causes wild-type p53-mediated cellular senescence, transient AMPK activation promotes cell survival following glucose starvation, consistent with a context-dependent, oncogenic role for AMPK[194]. Likewise, AMPK has been shown to phosphorylate and potentiate the transcriptional activity of FOXO3[179,180]. FOXO3 is a transcription factor that often functions as a tumor suppressor, but can also help manage metabolic stress[214–216]. Further, increased AMPK activity led to the phosphorylation of threonine-198 of the cell cycle inhibitor p27[26]. While phosphorylation caused a stabilization of p27, it enabled survival during starvation and/or metabolic stress through the induction of autophagy. However, it is not clear whether this signaling cascade would be present in prostate cancer since it required LKB1 and, as described above, LKB1 does not appear to be the dominant AMPK kinase in the prostate. In addition, the existence of mutations and/or deletions in several of these tumor suppressors such as p53, suggest that many of these tumor suppressive signals may not even exist in advanced prostate cancer.

One of the first described activities of AMPK is its ability to regulate lipid metabolism[164,217,218]. This occurred by the phosphorylation and inhibition of several proteins such as acetyl Co-A carboxylase 1 (ACC1), ACC2, 3-hydroxy-3-methyl-glutaryl-CoA (HMGCR), and the lipogenic transcription factors sterol regulatory element binding proteins-1 and -2 (SREBP-1 and 2) and hepatocyte nuclear factor 4A (HNF4A)[164,217–220]. The exact biological effect is determined by which specific proteins are targeted. For example, phosphorylation/inhibition of ACC1 blocks *de novo* fatty acid synthesis, while inhibition of the related isoform ACC2 increases fatty oxidation. Recently, AMPK was demonstrated to promote cancer under conditions of matrix detachment or glucose deprivation by inhibiting ACC1 and ACC2, resulting in the maintenance and production of pro-tumorigenic NAPDH levels, respectively[32]. Regardless, it is not clear how AMPK signaling through any of these downstream targets such as ACC1 will impact prostate cancer. This is because many of the inhibitory effects of AMPK, such as AMPK-mediated phosphorylation and inhibition of ACC1, are overridden by AR signaling. Increased lipogenesis is one of the hallmarks of prostate cancer[221]. AR increases the expression of several enzymes involved in *de novo* lipogenesis including fatty acid synthase, ATP-citrate lyase, HMGCR, ACC and farnesyl diphosphate synthase through the increased expression of the *SREBF1* (encodes SREBP-1), *SREBF2* (encodes SREBP-2) and *SCAP*, which further activates the SREBPs[221,222]. Thus, when AR signaling is present, as is the case in most prostate cancers, cells can simultaneously maintain AMPK signaling and pro-tumorigenic lipogenesis.

Prostate cancer is metabolically unique compared to many other cancer types. Relative to benign prostate, prostate cancer exhibits increased fatty acid and glucose oxidation[223–225]. This enhanced TCA cycle flux paradoxically occurs despite the above-described accumulation of intracellular lipid levels. Increased TCA cycle flux is now know to be in part caused by decreased levels of zinc in the transformed prostate cell[223,226–228]. The decreased zinc leads to a derepression of the enzyme aconitase, facilitating the forward

metabolism of substrates through the cycle. In prostate cancer, this process can also be augmented by the AMPK-mediated induction of peroxisome-proliferator-activated receptor γ coactivator 1α (PGC-1α), a master regulator of mitochondrial biogenesis[33,172]. Importantly, this entire cascade can be activated by AR[33]. The precise mechanism of AMPK's induction of PGC-1α expression is not known and may involve both direct and indirect effects[33,172,204]. There may also be other mechanisms by which AR-mediated AMPK signaling increases fatty acid oxidation in prostate cancer[28,32,178,192]. Curiously, there are additional AMPK targets that are known to regulate mitochondrial turnover (ex. mitophagy) and function (ex. fragmentation), suggesting that AMPK signaling may simultaneously promote the breakdown of old mitochondria and the synthesis of new mitochondria, perhaps improving cellular function[189,201,213]. To what degree this occurs in prostate cancer is currently not known.

In addition to alterations in mitochondrial metabolism, AMPK can modulate other aspects of sugar metabolism. For example, AMPK can directly phosphorylate two of the four isoenzymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate 2-phosphatase (PFKFB), an enzyme that represents the rate-limiting step of glycolysis[40,173,174,229,230]. These phosphorylation events on PFKFB2 and PFKFB3 increase their kinase activity and thus promote forward flux through glycolysis. In prostate cancer, there is likely an additional level of regulation because AR signaling increases the expression of *PFKFB2*[231]. PFKFB2 has also been demonstrated to be phosphorylated and activated at the same AMPK-target site in response to oncogenic PI3K-Akt signaling[100,232]. While AR-AMPK-PFKFB signaling is thought to occur in prostate cancer[35], the extent each isoform is stimulated by AR and/or AMPK, how the isoforms are regulated, and their functional roles are incompletely defined.

Beyond its role in glycolysis, AMPK may also have a more general function in glucose uptake. While not yet shown, it is possible that AMPK-mediated processes identified in other tissues may have relevance in prostate cancer. For example, AMPK is known to induce the translocation of the glucose transporter, GLUT4, in muscle and fat[153,156,233,234]. This occurs through the direct phosphorylation and regulation of TBC1D1 and TBC1D4/AS160, molecules that control vesicle trafficking. Additionally, AMPK has been reported to increase GLUT1 levels through a variety of mechanisms[209–211]. But like with the regulation of PFKFB2, other oncogenic cues can influence glucose uptake, and as such need to be considered. To that end, PI3K-Akt signaling can increase glucose transporter translocation and function in other cancers[235]. Similarly, GLUT1 levels can be stimulated by MYC, another commonly amplified oncogene in prostate cancer[100,235]. In addition, both PI3K-Akt and Myc can increase the expression of *HK2*, the first step of glycolysis and hence may further augment glucose uptake and metabolism[236,237].

Autophagy is a cellular recycling process that is increased following AMPK activation. Similar to AMPK, initial research first defined autophagy as a tumor suppressive process[238,239]. Also like AMPK, a number of recent studies have identified an oncogenic role for autophagy, particularly in the late stages of the disease[240,241]. These findings extend to prostate cancer where autophagy has been implicated in disease progression[30,242–244]. Despite initial indications of a functional role for autophagy in prostate cancer, it is still not

clear how this process is used during the different stages of the disease or how it is regulated. For example, there are discrepancies regarding AR's regulation of autophagy[30,242,243,245]. This is somewhat surprising given AR's robust induction of AMPK in prostate cancer[33–36,130]. The discrepancies may be due to variations in the stimuli duration (ex. sustained versus transient), use of indirect or non-selective modulators of AMPK and autophagy such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), metformin, chloroquine, etc.) and treatment conditions. Regarding the last point, most experiments that are performed to examine the effects of hormones are done using media that contains charcoal-stripped serum, a condition commonly referred to as "androgen-starved". While the charcoal-stripping of serum indeed removes most androgens, it also removes many other steroid hormones and peptide growth factors that could affect AMPK and autophagy. To that end, it is worth noting that switching AR-negative PC-3 prostate cancer cells from regular serum-containing media to the "androgen-starved", charcoal-stripped serum containing media activates AMPK and autophagy in an AR-independent manner (unpublished data). Clearly, additional studies are needed using more sophisticated molecular and genetic approaches to help resolve these discrepancies.

AMPK can directly increase autophagy through the phosphorylation of ULK1 and possibly ULK2[188,189,246]. Recently, AMPK was also shown to increase autophagy via the direct phosphorylation of VPS34 and Beclin-1[169]. Moreover, AMPK can indirectly increase autophagy through decreasing mTOR signaling, an inhibitor of autophagy. This inhibition of mTOR, and thus derepression of autophagy, is thought to occur predominantly through two mechanisms. First, AMPK can directly phosphorylate the mTOR adaptor protein raptor, inducing the binding of 14-3-3 to raptor and inhibiting the mTORC1 complex[171]. Second, AMPK can directly phosphorylate TSC2 to potentiate its repressive effects on mTORC1[170]. AMPK may also regulate mTOR signaling through the phosphorylation and inhibition of upstream components of the PI3K-Akt-mTOR pathway such as insulin receptor substrate-1[177]. However, as described above, this later regulation may not be prevalent in prostate cancer given the propensity of high PI3K/Akt signaling in advanced prostate cancers. In addition, it may be difficult to predict AMPK's effects on mTOR signaling in prostate cancer given the unusual crosstalk between the two pathways.

In contrast to what has been described in conventional physiology, AMPK and mTOR signaling can simultaneously occur in prostate cancer[33,34,247,248]. Thus, caution should be used when extrapolating results from basic biology, or even other cancer types, regarding AMPK and mTOR in prostate cancer. Why some crosstalk that occurs in other tissues does not occur in prostate cancer is not entirely clear but recent evidence is beginning to address how this may mechanistically occur. In prostate cancer, AR signaling increases the expression of a splice variant of TSC2, termed TSC2A, that cannot block mTOR signaling[249]. This results in a unique situation in which the cancer cell can concurrently activate two normally opposing signaling pathways. Hence, it may be possible that prostate cells can enjoy the pro-cancer benefits of both pathways.

While AMPK is defined as a master regulator of cellular metabolism, new findings indicate AMPK may have several non-metabolic roles that could impact processes of pathological importance in cancer. For example, AMPK appears to have an important role in mitosis

through several mechanisms including targeting components of the mitotic spindle assembly, regulating the breakdown and assembly of the Golgi apparatus, and modulating the cytoskeleton[159,198,207,250–252]. While these effects may at first appear contrary to the induction of tumor suppressive factors such as p53 (*described above*), collectively these events may function as a protective checkpoint, assuring that cell cycle progression does not occur prematurely. This type of "cautious" signaling would be in contrast to other reports of more direct oncogenic roles for AMPK such as acting as an essential downstream effector of oncogenic HRasV12 or Pten deletion that functions to directly phosphorylate and inhibit the tumor suppressor retinoblastoma (Rb) protein[24]. To what extent this occurs in advanced prostate cancer, where Rb is often mutated or lost, is unknown[253].

Finally, one of the emerging areas of interest is AMPK's actions in the nucleus. While early reports of AMPK functions largely pertained to cytoplasmic AMPK activity, it is now evident that this kinase has additional roles in transcription and epigenetics. To that end, AMPK can regulate the activity of several transcription factors and transcriptional coregulators such as HNF-4α, cAMP-response element binding protein (CREB), FOXO3, PGC-1α, CRTC2 and class IIa histone deacetylases[33,162,172,178,179,199,200,203,219,254–258]. In addition, AMPK was reported to interrupt the association of nuclear receptors with the coactivator p300[93,185]. This could have important implications in prostate cancer given the key role of AR in the disease. Correspondingly, AMPK was reported to inhibit AR activity[15]. However, this study appears to be in direct contrast to data from Karacosta et al indicating that CaMKK2 potentiates AR activity in prostate cancer[36]. In our hands, we have been unable to detect either agonistic or antagonistic effects of CaMKK2 and/or AMPK on AR activity with the caveat that we have only explored a limited gene set (unpublished data). Hence, differences between groups may be attributable to variations in the subsets of AR-target genes being regulated. Certainly, this is an area that needs further investigation.

The predominant view of those supporting a context-dependent role for AMPK suggest that AMPK first acts as a tumor suppressor early in tumorigenesis and then later shifts towards a more oncogenic role in the advanced stages of the disease, contributing to therapy resistance and cancer reoccurrence[3,98,119,259–268]. Early on, AMPK would be activated in response to inhibitory mutations in tumor suppressor genes or gain-of-function events in known oncogenic pathways. This would lead to the classic AMPK-mediated catabolic functions including inhibition of oncogenic mTOR signaling and/or lipogenesis as well as regulation of the cell cycle. As the tumor evolves, the cancer cells encounter various stresses such as hypoxia, matrix detachment and starvation in addition to chemotherapies that also increase cellular stress. At this advanced stage, AMPK is hypothesized to drive cancer progression by promoting metabolic plasticity, resistance to cellular stress and thus, cell survival.

While the early/tumor suppressive and late/oncogenic paradigm could indeed be true for a number of cancers, prostate cancer may be unique. Contrary to many other cancers, clinical data suggest that AMPK activity is increased in both early and late disease stages[7,33,37,268]. In addition, as described above, many of the classic tumor suppressive functions of AMPK are overridden in prostate cancer by other canonical signaling pathways. For example, AR's ability to increase mTOR signaling and lipogenesis in the presence of AMPK signaling (*described above*), may negate two of the major tumor suppressive networks. However, it

should be noted that prostate cancer is an extremely heterogeneous disease[269]. As such, broad generalizations may lead to inaccuracies. Future studies are required to better characterize AMPK's disease stage-specific roles and whether these roles vary amongst prostate cancer subtypes.

Targeting AMPK

Several strategies are currently being employed to modulate AMPK activity in cancer. However, it is unclear whether agonists or antagonists should be used given AMPK's context-dependent role (oncogenic versus tumor suppressive). Further, the important roles of AMPK in numerous physiological processes may ultimately preclude AMPK itself from ever being a viable therapeutic target since alteration of this key signaling molecule will likely have multiple side effects. Described below are efforts to activate or inhibit AMPK activity and special considerations that will need to be considered moving forward.

Interest in targeting AMPK started when retrospective studies of diabetic patients taking the biguanide drug metformin reported decreased risks of a variety of cancer types including prostate cancer[59–62]. These were extremely exciting reports since metformin was an ideal candidate drug for repurposing as it is cheap, widely available, easy to use and safe at the concentrations used to treat diabetes. But as described above, recent retrospective as well as new prospective studies have called into question the anticancer effects of metformin in prostate cancer ([66–78] and (NCT01433913)). Debate continues in part because metformin's potential anticancer mechanism of action is still not clear.

In general, it is thought that the effects of metformin on cancer can be through two mechanisms categorized under indirect or direct effects. The most obvious indirect effect would result from metformin's ability to reduce circulating insulin levels, a known mitogen and anti-apoptotic signal for some cancers[270]. What is not known in this regard is whether the metformin-induced changes in insulin levels would be significant enough, particularly in nondiabetics, to alter tumor biology[271]. In contrast, numerous preclinical studies have shown direct tumor suppressive effects in *in vitro* and *in vivo* models[272,273]. One of the major concerns with ongoing clinical trials was the question of whether a high enough concentration of metformin could reach the tumor cell to have direct anti-tumor effects. This depends both on the bioavailability of the drug and cellular uptake. Doses of metformin that are used to treat diabetic patients achieve plasma concentrations in the portal vein, where the drug is first absorbed and shuttled to, between 40–70 μM[274]. After liver uptake (note, metformin is not metabolized in animals or humans and is eliminated by the kidneys unchanged), systemic plasma concentrations drop to ~10–40 μM[275]. The micromolar concentrations used to treat diabetics are clearly enough to decrease glucose production in the liver, likely functioning in part through AMPK. However, there is recent debate even in this regard[276,277]. An initial concern in the field was that the majority of *in vitro* studies required higher concentrations of metformin (i.e. millimolar range) to inhibit the respiratory chain complex 1 and have tumor suppressive effects[278]. This would present a problem for the model that metformin has direct tumor suppressive effects. In this case, high enough drug concentrations could not reach the tumor and would indicate any potential anti-cancer effects would have to be mediated through an indirect mechanism. If one did want direct

tumor suppressive effects, new clinical trials using higher metformin doses would have to be considered. However, this might defeat one of the major benefits of repurposing metformin, its very safe clinical profile at the doses used to treat diabetics as higher doses may result in dangerous side effects such as lactic acidosis. Regardless, two brief letters were recently (April 2016) published back-to-back in *Cell Metabolism* that suggest the current doses being used in preclinical animal models may be sufficient to model effective clinical doses[279,280].

Dowling et al used liquid chromatography-mass spectrometry to demonstrate that in a xenograft model of colorectal cancer, mice given drinking water with metformin dissolved into it at a concentration of 5 mg/ml (comparable to what has previously been used in similar studies), plasma and, surprisingly, tumor concentrations of metformin reached ~30 μM, a concentration that was sufficient to increase AMPK activity (assessed by AMPK Thr172 phosphorylation)[279]. It was not reported whether metformin-treated mice also exhibited decreased tumors in this experiment. To achieve similar phospho-AMPK Thr172 levels in cell culture, HCT116 colon cancer cells had to be treated with 10–20 mM (>300 fold increase) metformin. These results indicate that metformin is much more readily taken up *in vivo* than *in vitro*. Interestingly, samples taken from nondiabetic breast cancer patients given metformin exhibited considerably lower plasma metformin levels (~2.8 μM) than those from diabetic patients (up to 25 μM), suggesting higher doses may be needed for anti-cancer efficacy particularly in non-diabetic patients. These results again call into question a role for metformin's reported direct antitumor effects in previous clinical studies. If high enough concentrations could not be reached in patient tumors, how could the reported beneficial effects of metformin be due to direct actions on the tumor?

Chandel et al performed a similar study using the HCT116 colon cancer xenograft model but with a 1.25 mg/ml solution of metformin[280], a concentration the investigators previously had shown to inhibit tumor growth in this model (a functional role for AMPK was never tested). Here, they detected both plasma and tumor metformin concentrations in the range of 3.2–12.4 μM, levels that can be achieved in routine diabetes treatment[281]. In addition, it was noted that due to its cationic nature, metformin is predicted to accumulate 100- to 500-fold in the mitochondria due to the membrane potential. Collectively, these results indicate that the doses currently being used in ongoing clinical trails are reasonable. However, this still leaves the question that if doses currently given to diabetic patients are sufficient to block mitochondrial activity in patient tumors, then why did the previous epidemiological studies not consistently demonstrate anticancer effects? More importantly, why was no survival benefit observed following the first formal blinded clinical trail of metformin even though plasma drug levels were in the micromolar range[282]? Certainly further work is needed in this area. It will be particularly important to determine whether isolated mitochondria from treated patients indeed do have significantly enriched metformin levels and whether additional biological factors like the expression of membrane transporters influence drug efficacy.

The hydrophilic nature of metformin likely prevents it from passively diffusing through plasma membranes. Hence, cellular uptake is controlled by cationic transporters such as OCT1, OCT2 and OCT3 (encoded by the genes *SLC22A1*, *SLC22A2* and *SLC22A3* respectively)[283]. OCT3/*SLC22A3* is highly expressed in the prostate indicating that

metformin uptake would not be an issue[284]. But interestingly, low *SLC22A3* expression is a strong predictor of poor prognosis in prostate cancer patients[285–288]. Whether changes in *SLC22A3* expression could in part explain some of the differences between the contrasting retrospective analyses of metformin's effects on prostate cancer remains to be determined. One would predict that tumors with low OCT3 levels would have decreased sensitivity to metformin. As risk allele variants associated with *SLC22A3* expression are known[285,286,288,289], these could be screened prior to selection of patients for future clinical trials.

Despite the controversies surrounding the clinical studies, enthusiasm for metformin is still high due to preclinical studies demonstrating that the drug has potent therapeutic effects across a broad range of cancers[290]. Researchers have hypothesized that the drug's anticancer effects could be mediated through AMPK because of work demonstrating that metformin can increase AMPK activity in cells[20,220,291–293]. Metformin, like other biguanides such as phenformin, can indirectly activate AMPK by inhibiting complex I of the electron transport chain[51,63,276,278,294,295]. This subsequently causes cellular energy stress, increasing the AMP/ATP ratio that could lead to an activation of AMPK complexes throughout the cell. Further studies pointed to an additional metformin-initiated, genotoxic activation of AMPK[296]. But the exact mechanism of this effect has been called into question[297,298]. Like metformin, a number of other activators of AMPK, including synthetic (ex. phenformin, AICAR, rosiglitazone, 2-deoxyglucose, etc) and natural (ex. salicylate, resveratrol, berberine, etc) compounds, can inhibit cancer cell growth, migration and/or invasion[1,7,20,293,299,300]. In contrast, AICAR was able to rescue the inhibition of prostate cancer cell proliferation caused by the molecular or pharmacological inhibition of CaMKK2[35]. It is important to note that many of these compounds such as metformin and AICAR have been demonstrated to be highly nonspecific and clearly have tumor suppressive (and possibly oncogenic) properties independent of AMPK[45,47,49,50,53,54,276]. It is reasonable to speculate then that additional stress signaling pathways may be responsible for the decreased cellular growth following the onset of stress. In fact, induction of cell death by several AMPK "activators" can be exacerbated by knocking out AMPK, indicating that AMPK is often turned on in response to these drugs as a last ditch survival effort[21–23,26,28–31,53,170,178]. In this regard, use of these compounds in combination with inhibitors of AMPK could be warranted. While drugs like metformin may indeed have tumor suppressive effects under some contexts, this may be due more to systemic effects rather that direct targeting of the cancer cell itself. Genetic studies using conditional knockout and transgenic animals will be essential to parse out these mechanisms of action.

To avoid some of the uncertainties inherent to using indirect activators of AMPK, direct activators of AMPK have been identified and shown to have tumor suppressive properties in several cancers[11,12,17,47]. The exact AMPK binding sites for some of these compounds (ex. OSU-53, PT1) are incompletely defined[11,13,14,301]. Further, the off-target effects of these drugs are unclear[55]. One of the more recently developed direct activators, MT 63–78, was able to decrease the proliferation of prostate cancer cells *in vitro* as well as suppress tumor growth *in vivo*[17]. MT 63–78, similar to A-769662 and salicylates, allosterically activates AMPK by directly binding to the β1 regulatory subunit[17,300,302]. These results would appear at odds with the above-described clinical data and functional studies that suggest the β1

subunit is oncogenic[101,104]. This apparent conundrum may be due to differences in the duration of stimuli. For example, transient pulses of AMPK activity may only lead to the phosphorylation and modulation of the most sensitive downstream AMPK targets. Conversely, a sustained robust activation of AMPK, such as in the presence of pharmacological activation by MT 63–78 may hyperactivate a greater number of AMPK complexes, therefore modulating a larger set of downstream targets. In this latter case, the broad activation of downstream targets would have tumor suppressive effects such as increasing cellular stress.

In contrast to studies demonstrating AMPK activity blocks prostate cancer cell growth, antagonists of AMPK such as compound C inhibit prostate cancer cell proliferation and migration[29–31,34,37]. In addition to compound C, other inhibitors of AMPK have been identified. These include the kinase inhibitors sunitinib and midostaurin that have potent anticancer effects *in vitro* and *in vivo* in a variety of cancers[303,304]. However, similar to many of the AMPK agonists, these drugs have well known pleiotropic effects and hence likely also possess AMPK-independent tumor suppressive properties[48,50,305,306].

Part of the rationale for the use of AMPK activators in cancer is AMPK's known ability to inhibit oncogenic cellular processes such as *de novo* lipogenesis and mTOR signaling. However, inhibitors of lipogenesis and mTOR already exist raising the question "Could we instead target the specific oncogenic, downstream processes of AMPK signaling?" Furthermore, AMPK agonists may not have a strong effect in prostate cancer where AR and PI3K-Akt are commonly hyperactivated and increase lipogenesis and mTOR signaling and thus may override the inhibitory effects of AMPK on these processes. Conversely, inhibition of AMPK may augment lipogenesis and/or mTOR signaling. In this regard, combined treatment with inhibitors of AMPK and lipogenesis and/or mTOR may have utility. However, it remains to be determined whether such an approach would have severe side effects given the important roles of these processes throughout the body.

A key step that needs to occur in our understanding of AMPK's actions in prostate cancer is the identification of the downstream targets of AMPK that are the drivers of the disease. Elucidation of these pathways may reveal better, and more selective, therapeutic targets. Alternatively, if we can identify the tissue- or disease-specific regulators of AMPK, these may also yield viable new targets. CaMKK2, as an upstream kinase of AMPK, may represent one such target. As highlighted above, CaMKK2 is increased in prostate cancer, has a restricted expression profile, and mediates several oncogenic processes. A cell-permeable inhibitor of CaMKK2, STO-609, has been available since 2002[307]. Correspondingly, STO-609 treatment reduced androgen-sensitive prostate cancer proliferation, migration and invasion as well as the growth of castration-resistant tumors[34,35]. Therefore, STO-609, or an STO-609-like compound with better pharmacokinetic and pharmacodynamic properties, may have therapeutic value in prostate cancer.

Conclusions

To understand AMPK's role in prostate cancer, one must first identify 1) the type of stimulus (i.e. how AMPK is activated), 2) the composition of the heterotrimer(s) and 3) the downstream driver pathways that are being modulated. All three are interconnected. The type and duration of the stimulus will dictate which subcellular AMPK complexes are activated and for how long. The amount of AMPK complexes at each subcellular site will be influenced by the subunit composition. The location of the complexes may also be regulated by posttranslational modifications and/or additional anchoring mechanisms. At this time, it is poorly understood what determines the distribution of the complexes. Each distinct AMPK complex is going to be associated with a set of targets that can be phosphorylated when that particular complex is activated. One should also be cognizant that many of these downstream processes are influenced by other oncogenic signals such as AR and/or Myc. For instance, even though increased AMPK may lead to the phosphorylation of ACC1, an event known to block *de novo* lipogenesis, in most prostate cancers AR signaling likely overrides this blockade by increasing the expression of an entire network of enzymes involved in lipogenesis. Taken together, the upstream cues determine which specific AMPK complexes are activated and therefore what downstream biological processes will be modulated. An example would be the activation of AMPK by prolonged energetic stress (ex. metformin treatment) in contrast to the controlled activation of a subpopulation of AMPK by an upstream kinase such as CaMKK2. High levels of AMP caused by prolonged energetic stress would lead to the robust activation of a large number of AMPK complexes throughout the cell, modulating most of the known AMPK targets and processes. Compare this to the activation by CaMKK2, a major upstream kinase of AMPK in prostate cancer (*described above*). CaMKK2's predominant cytoplasmic localization and requirement for a direct association with AMPK to phosphorylate and activate the protein indicates CaMKK2 will only increase the activity of a smaller, restricted set of AMPK complexes located primarily in the cytoplasm. This is exacerbated in prostate cancer where the α1 isoform of the catalytic subunit, which is more cytoplasmic[88,89], is the predominant form[33,34,99]. Interestingly, to date the majority of AMPK's tumor suppressive effects have been associated with its nuclear functions (ex. p53, p21 and p27 induction, p300 inhibition, etc). Hence, stimuli that favor the activation of non-nuclear AMPK complexes may favor the induction of more oncogenic processes.

Given AMPK's ubiquitous and diverse roles throughout the body, we suspect that broadly targeting AMPK may not be a viable option in cancer. Further, the use of direct AMPK activators or inhibitors will likely suffer from the counterproductive activation of some AMPK-mediated oncogenic pathways and impairment of other AMPK-mediated tumor suppressive signals. We propose that the elucidation of downstream AMPK-mediated processes will 1) uncover the driver signaling events and 2) highlight new therapeutic targets. Alternatively, identification and targeting of the prostate cancer-specific upstream cascades that favor the activation of these downstream oncogenic events could have greater overall efficacy because it would impact multiple AMPK-mediated, pro-cancer processes. In addition, the tissue and disease-specific nature of the upstream signal would offer a unique therapeutic target to prostate cancer, potentially mitigating side effects. Finally, while the

model of AMPK signaling outlined above is for prostate cancer, it is possible that the molecular concepts described here could be extended to explain AMPK actions in other cancers, diseases and even non-disease states.

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Key points

- AMPK is a heterotrimer complex that can come in at least 12 different versions. The different AMPK complexes can have unique subcellular locations and activities.
- Diverse upstream signals regulate different AMPK subcellular complexes.
- While first identified as a master regulator of metabolism, AMPK may have numerous roles beyond metabolism.
- AMPK can have context-dependent effects in cancer.
- CaMKK2 appears to be the dominant upstream AMPK kinase in the prostate.
- Most small molecule modulators of AMPK have known off-target effects.
- Given its ubiquitous expression and varied roles throughout the body, directly targeting AMPK may present numerous on-target side effects.

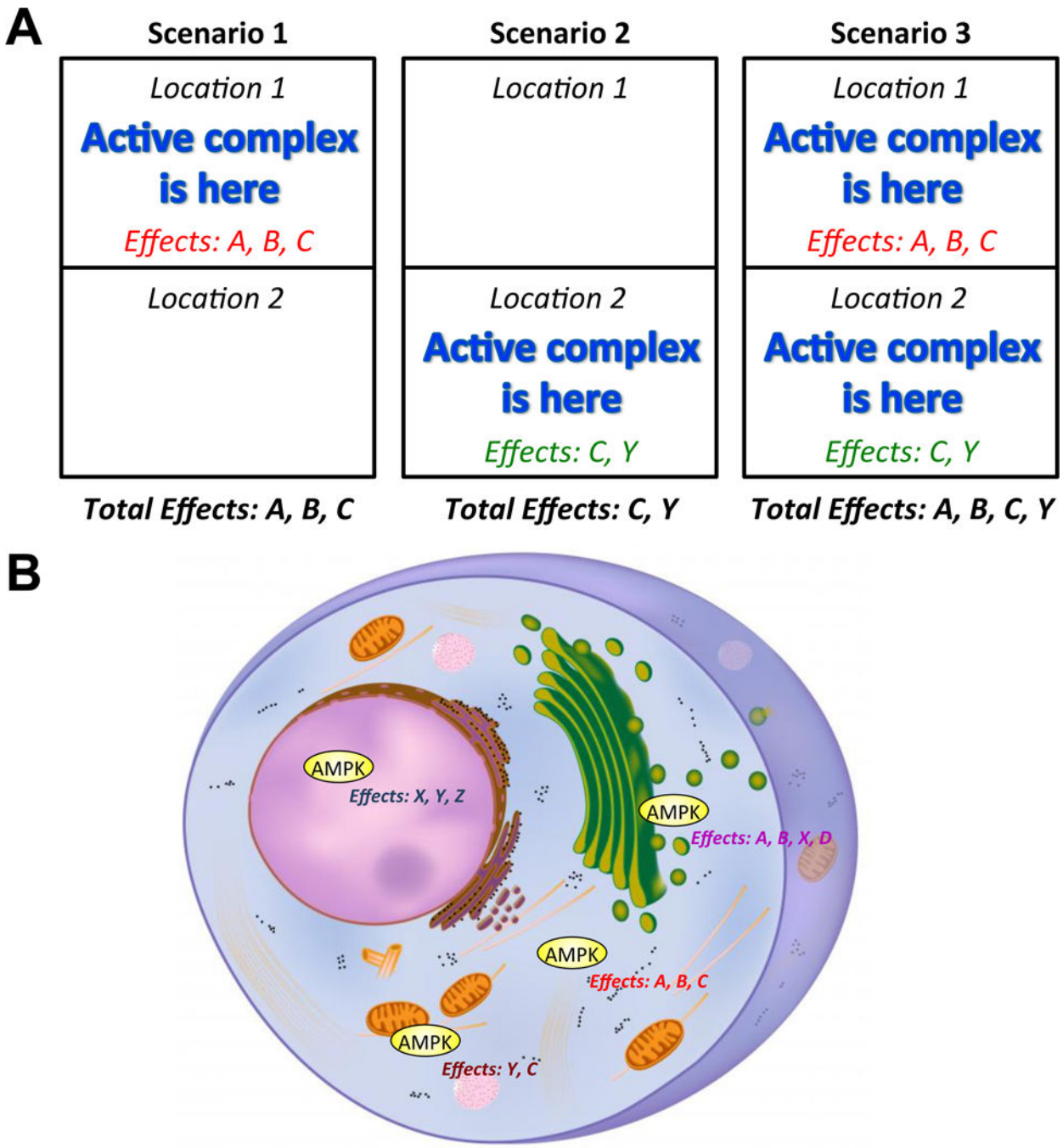

Figure 1. Compartmentalized signaling

A, Example of compartmentalized signaling. In Scenario 1, a particular signaling complex is activated at a specific location (Location 1). At Location 1 are the parts needed to generate certain effects (A, B, C) that are also known to be regulated by the described active complex. Thus, when the active complex is at this site (Location 1), the associated downstream processes are regulated to produce effects A, B and C. Processes regulated by the active complex but located elsewhere, such as at Location 2 that could produce effects C and Y, will not be altered in this scenario. In contrast, when the complex is activated at Location 2

and not 1 such as in Scenario 2, then only effects C and Y are produced and not effects A and B. In Scenario 3, the complex is activated everywhere and hence, all known processes controlled by the active complex will be regulated, producing a broad range of effects (A, B, C, Y). **B**, Regarding AMPK-mediated cellular effects, there are different AMPK complexes located throughout the cell (ex. cytoplasmic versus nuclear). Depending on which of these complexes is activated (could be more than one), the net effect AMPK has on a cell will be the summation of the actions of all of the activated subcellular populations of AMPK and their associated downstream effector processes.

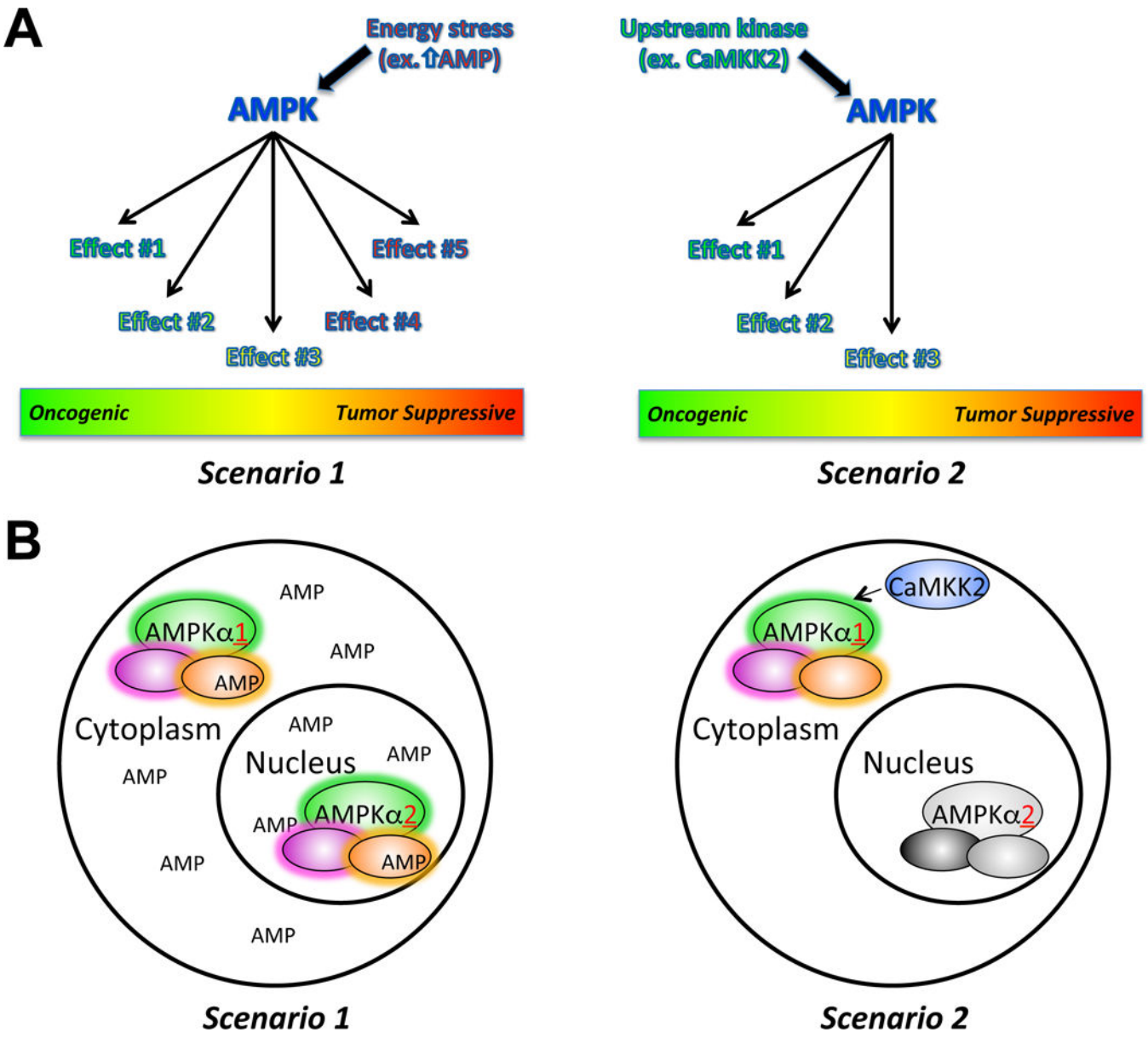

Figure 2. Upstream stimuli determine the differential regulation/activation of AMPK-mediated downstream effects

A, Depending on the particular upstream cue (ex. energy stress (*Scenario 1*) or phosphorylation by an upstream kinase (*Scenario 2*)), different subpopulations of AMPK can be activated (or inactivated). The net phenotypic effect of each type of AMPK activation will be the summation of all the regulated downstream pathways, shifting the balance between oncogenic and tumor suppressive AMPK signaling. In *Scenario 1*, all downstream AMPK targets (both oncogenic and tumor suppressive) are activated. Here, the tumor suppressive functions could dominate. In *Scenario 2*, there is a more selective activation of AMPK complexes that favor the induction of oncogenic downstream processes. **B**, The type of upstream stimuli and thus manner in which cellular AMPK complexes are activated is likely influenced by both the location of upstream cues and AMPK complexes, which can be

influenced by amongst other aspects the subunit composition, as well as the duration of signal. In this regard, in *Scenario 1*, a persistent energetic stress such as high AMP (or ADP) levels would be able to activate the majority of AMPK complexes. In contrast, an upstream kinase with a more restricted location such as CaMKK2 (*Scenario 2*) could only phosphorylate/activate local AMPK complexes, perhaps for a limited duration. This would lead to a restricted set of downstream processes that AMPK could regulate.

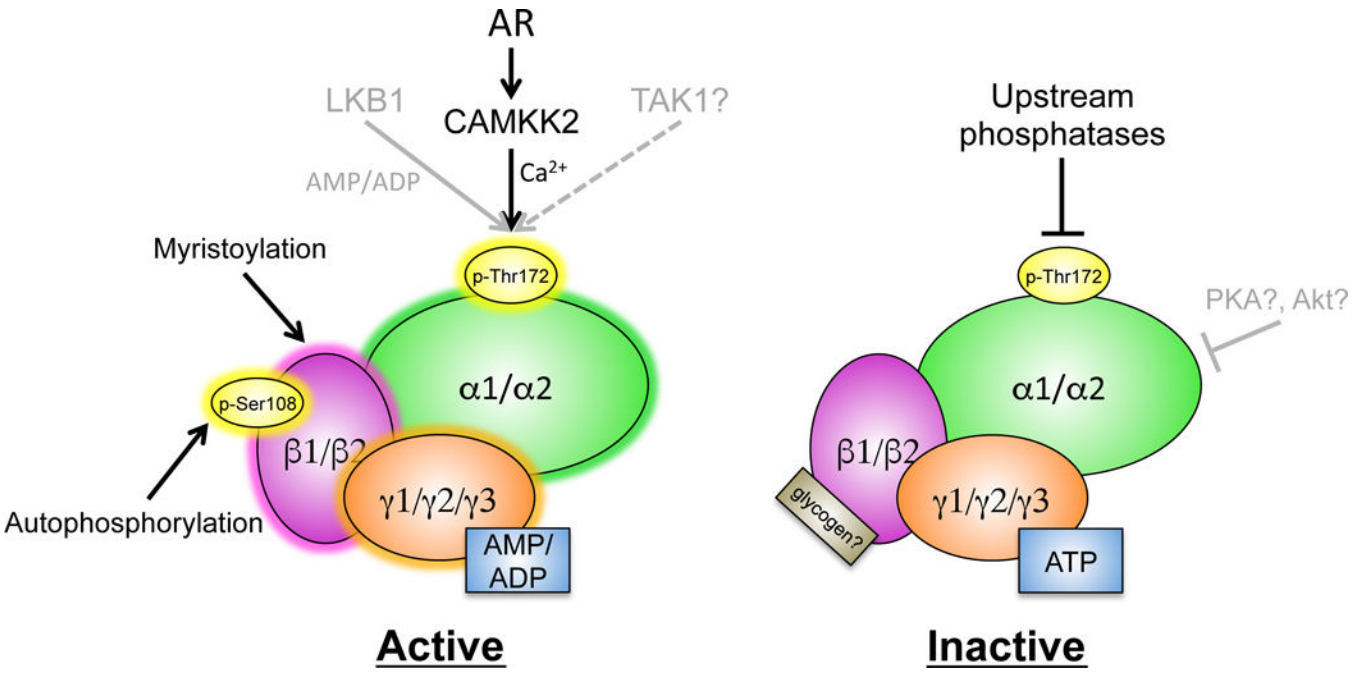

Figure 3. Proposed regulation of AMPK in prostate cancer

AMPK can be activated by multiple posttranslational modifications as well as energetic stress (ex. high AMP or ADP levels). In the prostate, the dominant upstream kinase of AMPK is CAMKK2, a calcium-dependent kinase whose expression is directly controlled by AR signaling. In contrast, AMPK can be inactivated by upstream phosphatases that, to date, are still ill-defined in the prostate. Further, inhibitory phosphorylation events caused by other kinases have been described but it is unclear if these modifications occur in prostate cancer. Additionally, high levels of ATP are known to inhibit AMPK. However, the inhibition of ATP may be overridden when CAMKK2 is highly expressed.

Table 1

Direct AMPK Targets

List of previously validated direct targets of AMPK.

AMPK Target	Target Site (Human)	Functional Consequence	References
ACC1	S80	Inhibits ACC enzymatic activity leading to inhibition of *de novo* lipogenesis	Carlson & Kim (1973), Davies et al. (1990), Ha et al. (1994), Munday et al. (1988)
ACC2	S222	Inhibits ACC enzymatic activity leading to promotion of fatty acid oxidation	Winder et al. (1997), Chen et al. (2000), Dzamko et al. (2008), Steinberg et al. (2010)
AKAP1	S107	Facilitates mitochondrial respiration	Hoffman et al. (2015)
AMOTL1	S793	AMPK phosphorylates AMOTL1 to stimulate Lats kinase which inhibits YAP	DeRan et al. (2014)
Beclin-1	S91, S94	Induces autophagy	Kim et al. (2013)
BRAF	S729	Promotes the association of BRAF with 14-3-3 proteins and disrupts its interaction with the KSR1 scaffolding protein	Shen et al. (2013)
CKIe (Clock)	S389	Increases CKIe activity	Um et al. (2007)
CLIP-170	S312	Results in CLIP-170 localizing closer to the distal end of the microtubules (cell polarity), modulating cell migration	Nakano & Takashima (2010)
CREB	S133	Increases CREB transcriptional activity and expression of downstream target genes	Thomson et al. (2008)
CRY1 Clock)	S71	Targets CRY1 toward ubiquitin mediated degradation	Lamia et al. (2009)
CRTC2/TORC2	S171	Induces 14-3-3- interaction, blocks nuclear translocation and association/activation of CREB	Koo et al. (2005)
eEF2K	S398	Activates eEF2K and blocks translation elongation by inactivating eEF2; can also induce autophagy	Browne et al. (2004), Hong-Brown et al. (2008), Leprivier et al. (2013), Xie et al. (2014)
FOXO3	S413, S588	Increases transcriptional activity of FOXO3	Greer et al. (2007), Bodur et al. (2015)
GBF1	T1337	Suppresses GEF activity of GBF1 resulting in disassembly of the Golgi apparatus	Miyamoto et al. (2008)
GFAT1/GFPT1	S261	Inhibits enzymatic activity of GFAT1, decreasing flux through the hexosamine biosynthetic pathway	Li et al. (2007), Eguchi et al. (2009)
HDAC4/5/7	S259, S498	Induces 14-3-3 binding, cytoplasmic sequestration and inhibition of HDACs	McGee et al. (2008), Mihaylova et al. (2011)
H2B	S37	Increases transcription of genes involved in cell survival	Bungard et al. (2010),
HMGCR	S872	Inactivates HMGCR and thus inhibits cholesterol synthesis	Clarke & Hardie (1990)
HNF4	S304	Represses transcriptional activity of HNF4α	Hong et al. (2003)
IRS1	S794	Context-dependent regulation of PI3K-Akt signaling	Jakobsen et al. (2001), Qiao et al. (2002), Tzatsos et al. (2007)
MFF	S155, S172	Induction of mitochondrial fission	Toyama et al. (2016), Ducommun et al. (2015)
PAK2	S20	Promotes PAK2 activity, leading to increased phosphorylation/inhibition of MRLC	Banko et al. (2011)
PFKFB2	S466	Increases PFKFB2 kinase activity and glycolysis	Marsin et al (2000)
PFKFB3	S461	Increases PFKFB3 kinase activity and glycolysis	Marsin et al (2002)
p27	T198	Stabilizes p27, inducing autophagy-mediated cell survival	Liang et al. (2007)
p300	S89	Inhibits its ability to interact with nuclear receptors	Leff et al. (2003), Yang et al. (2001)

AMPK Target	Target Site (Human)	Functional Consequence	References
p53	S15	Increases p53 activity, promotes expression of p21 and cell cycle arrest	Imamura et al. (2001), Jones et al. (2005)
PGC-1α	T177, S538	Induction of PGC-1α and mitochondrial biogenesis	Jager et al. (2007)
PPP1R12C	S452	Induces 14-3-3 binding and inhibition of PPP1R12C, leading to increased phosphorylation/inhibition of MRLC	Banko et al. (2011)
Rb	S811	Inhibition of Rb followed by subsequent increased proliferation	Dasgupta et al. (2009), Rios et al. (2013)
RPTOR	S722, S792	Induces 14-3-3 binding and inhibition of mTOR	Gwinn, D.M., et al. (2008)
SIRT1	T344	Disrupts the interaction between SIRT1 and its inhibitor DBC1	Lau et al. (2014)
SREBP1	S396	Inhibits transcriptional activity of SREBP1	Li et al. (2011)
TBC1D1	S237, T596	Promotes 14-3-3 binding and glucose transporter (ex. GLUT4) trafficking	Chen et al. (2008), Chavez et al. (2008), Pehmoller et al. (2009)
TBC1D4/AS160	T642, S704	Promotes 14-3-3 binding and glucose transporter (ex. GLUT4) trafficking	Treebak et al. (2006), Kramer et al. (2006), Treebak et al. (2010)
TSC2	T1227, S1345	Enhances TSC2 activity	Inoki et al (2003)
ULK1	S317, S467, S556, S778	Activates ULK1, promotes autophagy	Kim et al. (2011), Egan et al. (2011)
VPS34	T163/S165	Inhibits the non-autophagy Vps34 complex	Kim et al. (2013)
YAP	S94	Disrupts YAP-TEAD interaction and leads to inhibition of YAP	Mo et al. (2014)

Table 2

List of notable indirect targets of AMPK.

AMPK Indirect Target	Functional Consequence	References
GLUT1	Induction of GLUT1 expression, increased glucose uptake	Barnes et al. (2002), Yun et al. (2005), Wu et al. (2013)
MRLC	Changes in cell shape, induction of cell polarization, proper spindle pole assembly and mitosis	Lee et al. (2007), Bultot et al. (2009), Banko et al. (2011), Thaiparambil et al. (2012)
PRODH/POX	Increases PRODH activity, increasing flux through the pentose phosphate pathway, increasing autophagy and promoting cell survival under conditions of nutrient stress	Pandhare et al. (2009)
SIRT1	Enhances SIRT1 activity by increasing NAD+ levels	Canto et al. (2009)
SREBP2	Reduces levels of SREBP2 and SREBP2 downstream targets HMGCR and HMGCS, decreasing *de novo* cholesterol synthesis, ameliorates the SREBP2 up-regulation induced by thyroid-stimulating hormone	Liu et al. (2015)