

## Review Article

# Metformin: An Inhibitor of mTORC1 Signaling

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## Antidiabetic Drug-Metformin

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### Abstract

Recent progress in molecular medicine has identified the nutrient-sensitive kinase mechanistic target of rapamycin complex 1 (mTORC1) as the central regulator of protein and lipid synthesis, cell growth, proliferation, energy metabolism and autophagy. Age-related diseases of Western civilization such as obesity, diabetes mellitus, neurodegenerative diseases, and cancer are associated with enhanced mTORC1 signaling. According to the current opinion, metformin's primary mode of action is the alteration of cellular energy metabolism stimulating 5-AMP-Activated Protein Kinase (AMPK). However, the notion that AMPK primarily mediates metformin's anti-hyperglycemic action has recently been challenged, thrusting AMPK-independent effects into the focus of interest. We provide a new viewpoint on metformin's mode of action as an inhibitor of mTORC1. Metformin's insulin-lowering and AMPK-activating effects decrease RHEB-mediated stimulation of mTORC1. Independent of AMPK metformin inhibits mTORC1 in a RAG GTPase-dependent manner. Thus, metformin interferes with the two major pathways required for mTORC1 activation: 1) energy- and cell stress-mediated activation of AMPK attenuating the activity of the GTPase RHEB and 2) suppression of amino acid signaling down-regulating the activity of lysosomal RAG GTPases. Both RHEB- and RAG GTPase activation, which are required for mTORC1 activation at the lysosomal membrane, are thus suppressed by metformin. Metformin-induced suppression of mTORC1 subsequently decreases S6K1 activity and S6K1-mediated insulin resistance as well as AKT-FoxO1-mediated hepatic gluconeogenesis. Metformin represents an ideal, safe and cheap drug targeting the pathogenesis of mTORC1-driven anabolic and hyperproliferative diseases of civilization.

### ABBREVIATIONS

AA: Amino Acid; AAT: Amino Acid Transporter; AD: Alzheimer's Disease; AKT: Akt Kinase (protein kinase B); AMPK: Adenosine Monophosphate-activated Protein Kinase; AS160: AKT Substrate 160kD (TBC1 domain family member 4); ATF4: Activating Transcription Factor 4; AMP: Adenosine Monophosphate; ATM: Ataxia Teleangiectasia Mutated; ATP: Adenosine Triphosphate; BCAA: Branched-Chain Amino Acid; DDIT4: DNA Damage-Inducible Transcript 4; DDR: DNA Damage Response; 4-EBP-1: Eukaryotic initiation factor (eIF) 4E-Binding Protein 1; ER: Endoplasmic Reticulum; ERK: Extracellular signal Regulated Kinase; FGF21: Fibroblast Growth Factor 21; FoxO1: Forkhead Box O1 Transcription Factor; GAP: GTPase-activating protein; GCN2: General amino acid Control-non-derepressible 2; GEF: Guanine nucleotide Exchange Factor; GDH: Glutamate Dehydrogenase; GLUT4: Glucose Transporter 4; GDP: Guanosine Diphosphate; G6Pase: Glucose-6-Phosphatase; GTP: Guanosine Triphosphate; IGF-1: Insulin-Like Growth Factor-1; IRS: Insulin Receptor Substrate;  $\alpha$ KG:  $\alpha$ -Ketoglutarate; LAT1: L-Type Amino Acid Transporter 1; LEL: Late Endosomes and Lysosomes; LKB1:

Liver Kinase B1; Leu: Leucine; LeuRS: Leucyl-tRNA Synthase; MDM2: Mouse Double Minute 2 Homolog; MDMX: Mouse Double Minute 4 Homolog (p53-binding protein MDM4); mTORC1: Mechanistic (Mammalian) Target Of Rapamycin Complex 1; mTORC2: Mechanistic (Mammalian) Target Of Rapamycin Complex 2; OCT-1: Organic Cation Transporter-1; PAT: Proton-Assisted Amino Acid Transporter; PEPCK: Phosphoenolpyruvate Carboxykinase; PI3K: Phosphoinositol-3 Kinase; PPAR $\gamma$ : Peroxisome Proliferator-Activated Receptor- $\gamma$ ; PGC-1 $\alpha$ : Peroxisome Proliferator-Activated Receptor- $\gamma$  Co-Activator 1 $\alpha$ ; PTEN: Phosphatase and Tensin Homolog; RAG: RAS-Related GTP-Binding Protein; RAPTOR: Regulatory-Associated Protein of mTOR; REDD1: Regulated in DNA Damage and Development 1; RHEB: RAS-Homolog Enriched in Brain; RICTOR: Rapamycin-Insensitive Companion of mTOR; ROS: Reactive Oxygen Species; RSK: Ribosomal S6 Kinase 90kD; SeP: Selenoprotein P; Ser: Serine; S6K: Ribosomal protein S6 kinase 70kD; SREBP: Sterol Response Element Binding Protein; SOCS3: Suppressor OF Cytokine Signaling 3; STAT3: Signal Transducer AND Activator of Transcription 3; TBC1D7: TBC (Tre2-Bib2-Cdc16)1 Domain

family member 7; tRNA: Transfer-RNA; TSC1: Hamartin; TSC2: Tuberin; Tyr: Tyrosine; ULK-1: UNC51-Like Kinase 1; v-ATPase: Vacuolar H<sup>+</sup>-ATPase

## INTRODUCTION

The *Mechanistic Target of Rapamycin Complex 1* (mTORC1) signaling pathway couples energy and nutrient abundance to anabolic metabolism by sensing and simultaneously orchestrating pivotal signals such as cell energy, cell stress, nutrient and especially amino acid availability and growth factors such as insulin and insulin-like growth factor-1 (IGF-1) (Figure 1) [1-11]. It is the intention of this review to provide evidence that the major pharmacological action of metformin is the suppression of mTORC1.

In the past few years, the involvement of aberrant mTORC1 signaling in the onset and progression of ageing, obesity, type 2 diabetes mellitus, cancer and neurodegenerative diseases (type 3 diabetes) has been appreciated [12-18]. Enhanced mTORC1 signaling stimulates adipogenesis [19-22] and results in increased expression of the key adipogenic transcription factors peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) and sterol regulatory element binding transcription factor-1 (SREBP1) [10,23-27]. mTORC1 suppresses lipolysis, stimulates lipogenesis, and promotes fat storage [28], thus plays a pivotal role in adipogenesis [29]. The major mTORC1 downstream target, the ribosomal S6 kinase 1 (S6K1), plays a critical role in early adipocyte differentiation [30]. In fact, absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity [31].

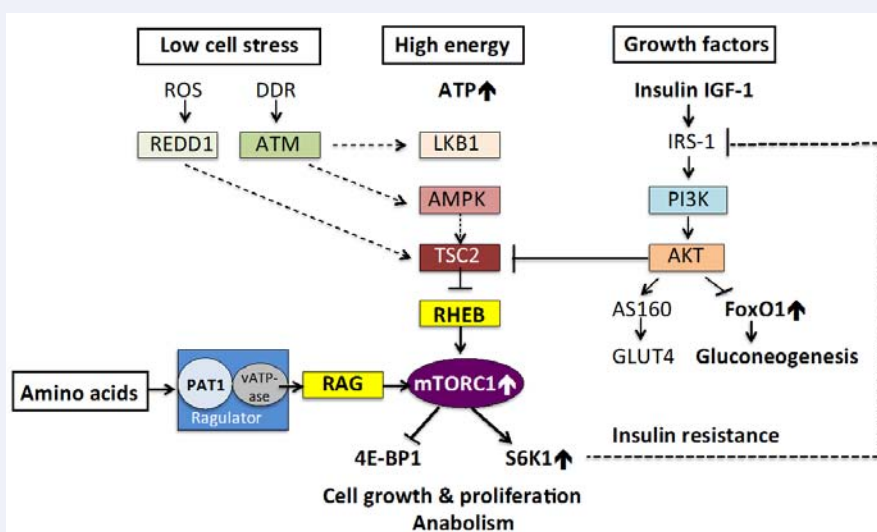
Over-stimulation of mTORC1 signaling by excess food and high amino acid intake appears to be the crucial factor underlying the diabetes epidemics [12,32]. mTORC1 signaling is involved in pancreatic  $\beta$ -cell growth,  $\beta$ -cell mass regulation, insulin synthesis and secretion [33-37]. The mTORC1-S6K1 signaling axis controls, at least in part, glucose homeostasis, insulin sensitivity, adipocyte metabolism, body mass and energy balance, tissue and organ

size, and aging [38,39]. Notably, S6K1-mediated phosphorylation of insulin receptor substrate-1 (IRS-1) plays a central role for the induction of insulin resistance [40-50]. Postnatal stimulation of mTORC1 by ablation of tuberous sclerosis complex 2 (TSC2) in  $\beta$ -cells of mice resulted in a biphasic response with increased  $\beta$ -cell mass and insulin secretion in young but progressive  $\beta$ -cell apoptosis, insulin deficiency and hyperglycemia in adult mice [51].

mTORC1 is a master regulator of protein and lipid synthesis, nucleotide synthesis and cell cycle progression that couples nutrient availability to cell growth and cancer [52-59]. Dysregulation of multiple elements of the mTORC1 pathway (PI3K amplification/mutation, PTEN loss of function, AKT over expression, and S6K1-, 4EBP1- and eIF4E over expression) have all been reported in many types of cancer [60-66].

mTORC1 plays a role in amyloid- $\beta$ - and tau-induced neurodegeneration [67,68]. Increased phosphorylation of S6K and eIF4E in postmortem human Alzheimer's disease (AD) brains compared to age-matched control patients suggests higher mTORC1 activity in AD brains [69-72]. Remarkably, inhibition of mTORC1 by rapamycin abolished cognitive deficits and reduced amyloid- $\beta$  levels in a mouse model of AD [73]. Insufficient autophagy by increased mTORC1 activity may contribute to amyloid- $\beta$  accumulation and formation of tau oligomers and insoluble aggregates, whereas appropriate autophagy enhances the clearance of soluble and aggregated forms of amyloid- $\beta$  and tau proteins [74]. The frequently observed comorbidity of insulin resistance, type 2 diabetes mellitus and AD (type 3 diabetes) [75] appear to derive from a common underlying mechanism, i.e., increased mTORC1 signaling.

Aging is defined as an accumulation of cellular damage over time, promoting disease and death. Over-activated mTORC1 signaling, which suppresses autophagy [76,77], results in increased accumulation of damaged proteins and cell constituents, thereby promotes the process of aging. In fact,



**Figure 1** Illustration of over-stimulated mTORC1 signaling during nutrient overload, high cellular energy and excess amino acid availability. Over-activation of S6K1 results in the inhibition of IRS-1 with decreased AKT activity resulting in reduced glucose uptake (insulin resistance) and increased FoxO1-mediated hepatic gluconeogenesis.

inhibition of mTORC1 signaling extends lifespan in yeast, worms, flies and mice [78-88].

Taken together, enhanced mTORC1 signaling appears to represent the molecular interface connecting metabolic stress with aging and age-related metabolic diseases [14,89].

According to the current opinion, attenuation of mTORC1 signaling either by dietary restriction and/or pharmacological intervention should be effective in the prevention of age-related diseases [90]. A well-tolerated and cheap drug for widespread use, which would effectively attenuate over-stimulated mTORC1 signaling, is highly desirable. In this review, we will provide evidence that the synthetic biguanide metformin (*N,N*-dimethylimido-dicarbonimidic diamide) perfectly corrects over-activated mTORC1 signaling. Naturally occurring biguanides originating from the French lilac (*Galega officinalis*) have been used for the treatment of diabetes in folk medicine for centuries. To understand the impact of metformin on mTORC1 pathways, a brief introduction into recent concepts of canonical mTORC1 signaling may be helpful.

### Canonical mTORC1 Signaling

mTOR is a multi-domain protein of approximately 300 kDa exhibiting a serine/threonine protein kinase domain at its C-terminus related to phosphoinositol-3-kinases (PI3Ks). In mammalian cells two functionally different mTOR complexes exist: mTORC1 and mTORC2, respectively [1-5]. Among other functional proteins, mTORC1 contains the partner protein RAPTOR, which interacts with substrates for mTORC1-mediated phosphorylation. mTORC1 controls the G<sub>1</sub>/S transition and G<sub>2</sub>/M progression of the cell cycle [53]. In contrast to mTORC2, which contains the partner protein RICTOR, only mTORC1 plays a special role in sensing cellular nutrients, amino acids, energy (ATP) levels, and oxygen stress (ROS), which are all important signals for the regulation of cell growth and proliferation.

### AMPK-Mediated Regulation of mTORC1

LKB1 and AMP-activated protein kinase (AMPK) are critical regulators of mTORC1 [91,92]. The serine/threonine kinase LKB1 represents the major kinase phosphorylating the AMPK activation loop ( $\alpha$ -subunit of AMPK) under conditions of energy stress [93,94]. AMPK plays a key role in energy-dependent regulation of mTORC1. AMPK is activated during energy-deficient conditions, when AMP levels rise. AMPK phosphorylates TSC2 and RAPTOR, thereby suppressing mTORC1 [95]. AMPK-mediated inhibition of mTORC1 activates autophagy by initiating the kinase ULK-1 [96,97]. In response to glucose deprivation, hexokinase-II, which catalyzes the first step of glycolysis, binds to mTORC1 through its TOS motif, thereby decreasing mTORC1 activity [98,99].

The mTORC1 pathway is also regulated by oxidative stress that down-regulates mTORC1 signaling [1]. Ataxia Teleangiectasia Mutated (ATM) is another kinase of the PI3K family that responds to oxidative stress (ROS) and DNA damage by phosphorylating key substrates such as LKB1. This results in AMPK-mediated activation of TSC2 suppressing mTORC1 [100-103]. ATM plays a central role in maintaining genomic stability [104,105].

Most functions of mTORC1 are inhibited by rapamycin,

a triene macrolide antibiotic synthesized by *Streptomyces hygroscopicus* [2]. Growth factor signals (insulin and IGF-1) are integrated by the tuberous sclerosis protein TSC1 (hamartin) and TSC2 (tuberin) that regulate RHEB (RAS-homolog enriched in brain), one essential activator of mTORC1 [95,106-110]. In its GTP-bound form, RHEB directly activates mTORC1. The RHEB-specific GTPase-activating protein (GAP) is the TSC2 protein, which functions as a heterotrimer with its binding partners TSC1 and TBC1D7 [110]. Growth factor signaling via TSC2 phosphorylation reduces the inhibitory function of the TSC1/TSC2/TBC1D7 complex towards RHEB, which results in activation of RHEB and finally of mTORC1.

### Amino Acid-Mediated Regulation of mTORC1

Amino acids, especially leucine, glutamine and arginine, play a most important role for the activation of mTORC1 [3-9]. Amino acids activate mTORC1 even in the absence of insulin but not *vice versa* [7,111,112]. The activation of mTORC1 depends on two major pathways: 1) the upstream activation of RHEB by signals derived from growth factors and 2) by the amino acid-dependent translocation of inactive mTORC1 to active RHEB localized in lysosome compartments [113-115]. Insulin and IGF-1 signaling, via activated AKT as well as other growth-related kinases such as ERK and RSK, phosphorylate TSC2 and thereby suppress the inhibitory function of the TSC1/TSC2/TBC1D7 complex towards RHEB. The TSC complex associates with the lysosome in a RHEB-dependent manner, and its dissociation in response to insulin requires AKT-mediated TSC2 phosphorylation. Loss of the PTEN tumor suppressor results in constitutive activation of mTORC1 through the AKT-dependent dissociation of the TSC complex from the lysosome [116]. These recent findings provide a unifying mechanism by which independent pathways affecting the spatial recruitment of mTORC1 and the TSC complex to RHEB at the lysosomal surface serve to integrate diverse growth signals [116]. The inhibition of either TSC1 or TSC2 leads to activation of RHEB and ultimately of mTORC1 [109,116-117].

Amino acid uptake into the cell is crucial for mTORC1 signaling. Nicklin [118] suggested that cellular export of glutamine is required for cellular leucine uptake and subsequent leucine-mediated mTORC1 activation. Intracellular glutamine is required for preloading the SLC7A5/SLC3A2 bidirectional amino acid transporter, which drives the efflux of glutamine and influx of leucine for leucine-mediated mTORC1 activation [118,119]. Remarkably, in response to amino acid depletion, mTORC1 activity is rapidly abolished [19]. Amino acid starvation even impairs binding of mTORC1 to RHEB [120]. Of all essential amino acids, leucine exerts the greatest effects on mTORC1 signaling [3,4,19,111]. Notably, from all animal proteins, milk proteins provide the highest amounts of the essential branched-chain amino acids (BCAAs) leucine, isoleucine and valine [121].

Amino acids play a pivotal role in the translocation of inactive mTORC1 to lysosomal compartments enriched in activated RHEB [113,114]. The spatial regulation of inactive mTORC1 by amino acids is mediated by an active RAG heterodimer and is of crucial importance for amino acid sensing and activation of mTORC1 [122]. The pentameric Ragulator complex acts as a scaffold for the RAG GTPases and mTORC1 at the lysosomal membrane. According to the recent opinion, RHEB and RAGs come together

at the lysosome to activate mTORC1 [116,123].

Amino acid accumulation in the lysosomal lumen generates an activating signal that is transmitted in a vacuolar H<sup>+</sup>-ATPase (v-ATPase)-dependent fashion to activate the guanine nucleotide exchange factor (GEF) activity of Ragulator towards RAGA. Upon RAGA-GTP loading, mTORC1 is recruited to the lysosomal surface where it interacts with RHEB and becomes activated [122]. Thus, mTORC1 integrates insulin, IGF-1, energy-, and ROS-derived signals to RHEB. In parallel mTORC1 activation requires sufficient amino acid signals for complex assembly allowing efficient activation of mTORC1 [124].

Proton-assisted amino acid transporters (PATs) localized on late endosomes and lysosomes (LEL) interact with RAGs and are required for mTORC1 activation [125]. PAT1 (SLC36A1) is expressed at the luminal surface of the small intestine and is commonly found in lysosomes of many cell types [126]. PAT1 has a relative low affinity ( $K_m$  1-10 mM) for its substrates, which include zwitterionic amino and imino acids, heterocyclic amino acids, and amino acid-based drugs and derivatives [126].

The v-ATPase interacts with the activated RAG/Ragulator complex to control amino acid-dependent mTORC1 activation, which is regulated by the rapid accumulation of extracellular amino acids in LELs [12,127]. Thus, in response to amino acids these molecules form a signaling complex that has been called the 'nutrisome' [12,125]. Cycling of protons through this nutrisomal engine induces conformational changes that may activate mTORC1. Importantly, signaling from the insulin receptor and subsequent activation of the PI3K/AKT/RHEB cascade promotes shuttling of PATs from the cell surface to LEL membranes, hence increasing PAT-dependent mTORC1 activation [125,127]. In addition, the accumulation of amino acids in the LEL lumen presumably involves transport into intracellular endosomal compartments via currently unknown amino acid transporters (AATs) or potentially via endocytosis. Cytoplasmic leucine, which is brought into cells via the heterodimeric amino acid transporter CD98 [128], has been shown to play a key role in activating mTORC1 in some cultured cells and may be important in this process. Influx of leucine or other amino acids into the LEL system may ultimately allow the amino acid substrates of PAT1 to accumulate in the LELs through amino acid exchange mechanisms, leading to PAT1-mediated activation of the nutrisome [125].

Leucyl-tRNA synthetase (LeuRS) acts as a cytosolic amino acid sensor [129,130]. LeuRS also plays a critical role in amino acid-induced mTORC1 activation by sensing intracellular leucine concentration and initiating mTORC1 activation by binding to and activating RAG GTPase [129,130]. LeuRS acts as a GTPase-activating protein (GAP) for RAGD, enhancing the GTP-bound form of RAGA/B crucial for amino acid-mediated mTORC1 activation [130]. Furthermore, Duran [131] suggested that leucine stimulates mTORC1 indirectly through its effects on glutaminolysis. Glutamine in combination with leucine increased GTP charging of exogenously expressed RAGB, promoting mTORC1 activation by enhancing glutaminolysis and the production of  $\alpha$ -ketoglutarate [131]. In contrast, the eIF2 $\alpha$  (eukaryotic initiation factor 2 $\alpha$ ) kinase GCN2 (general amino acid control-non-derepressible 2) senses the absence of one or more amino acids by virtue of direct binding to

uncharged cognate tRNAs [132].

These recent insights into regulation of mTORC1 underline that the activation status of AMPK and the availability of amino acids determines the magnitude of mTORC1 signaling.

### Metformin by Activation of LKB1-AMPK Inhibits mTORC1

The primary target of metformin action is the liver. Hepatocytes abundantly express organic cation transporter-1 (OCT-1), the predominant transporter for cellular metformin uptake [133-136]. OCT-1 expression plays also an important role for the antiproliferative action of metformin in cancer cells [137]. Once taken up by the cell, the major action of metformin is believed to alterate cellular energy metabolism associated with direct and indirect stimulation of AMP-Activated Protein kinase (AMPK) [138]. AMPK is a serine/threonine protein kinase that acts as a sensor of cellular energy status [139]. AMPK represents a heterotrimeric complex composed of a catalytic subunit (AMPK- $\alpha$ ) and two regulatory subunits (AMPK- $\beta$  and AMPK- $\gamma$ ) [139]. Metformin-induced inhibition of complex I of the mitochondrial electron transport chain reduces ATP production and increases AMP levels [140-144]. This results in 5'-AMP-mediated activation of Liver Kinase B1 (LKB1) that phosphorylates and activates the catalytic  $\alpha$ -subunit of AMPK [145-147]. Loss of LKB1 in intestinal tumors from *LKB1*<sup>-/-</sup> mice is accompanied by an increase in mTORC1 signaling, as detected by the phosphorylation of its major downstream targets p70 ribosomal S6 kinase (S6K) and Eukaryotic translation Initiation Factor (eIF) 4B binding protein 1 (4E-BP1) [148]. LKB1 is thus the major upstream activating kinase of AMPK in the liver [149]. It has been demonstrated that metformin requires LKB1 in the liver to lower blood glucose levels [149]. Recently, Zhang [150] reported that metformin has a stronger binding ability to the  $\gamma$  subunit of AMPK than to  $\alpha$  subunit. AMPK-mediated down regulation of mTORC1 results from phosphorylation and activation of TSC2 [95,151], the negative regulator of RHEB [95,106-110]. Furthermore, AMPK phosphorylates and inhibits RAPTOR, a positive regulator and substrate processor of mTORC1 [95].

### Metformin-Induced ATF4/REDD1-Mediated mTORC1-Inhibition

Inhibition of mitochondrial complex I activity by metformin enhanced the expression of fibroblast growth factor 21 (FGF21), an endocrine hepatic hormone that exhibits anti-obesity and anti-diabetes effects [152]. A strong dose-dependent increase in FGF21 expression has been observed in rat and human hepatocytes treated with metformin [153]. Both increased FGF21-expression as well as mTORC1 inhibition has been associated with increased lifespan [154-156]. The starvation hormone FGF21 induces hepatic fatty acid oxidation and ketogenesis and increases insulin sensitivity. Metformin induced expression of FGF21 is mediated through activating transcription factor 4 (ATF4) [152]. It has recently been demonstrated in hepatocytes that tetracyclines also induce ATF4, which was associated with mTORC1 inhibition [157]. Regulated in DNA damage and development 1 (REDD1) also known as DNA damage-inducible transcript 4 (DDIT4) functions to repress signaling through mTORC1 in response to diverse stress conditions such as increased endoplasmic

reticulum stress. Notably, ATF4 facilitates the transcription of the REDD1 gene [158,159]. REDD1 inhibits mTORC1 by stabilizing the TSC1-TSC2-TBC1D7 inhibitory complex [160].

### Metformin via p53/REDD1-Induction Inhibits mTORC1

The tumor suppressor protein p53, which induces Sestrin1/2 [161], and p63, required for the induction of REDD1 and activation of the TSC complex [162,163], inhibit mTORC1 signaling [164]. REDD1 is another target of p53 and mirrors the tissue specific pattern of the p53 family member p63 [162]. REDD1 suppresses mTORC1 activity by releasing TSC2 from AKT-mediated association with inhibitory 14-3-3 proteins [163], which leads to inhibition of mTORC1, a physiological mechanism in response to hypoxia [164].

Recent evidence underlines that p53 suppresses carcinogenesis by inhibiting mTORC1 [165]. Importantly, p53 is another target of AMPK. AMPK induces phosphorylation of p53 on Ser15, which induces AMPK-dependent cell-cycle arrest [166,167]. Critical for the control of p53 function are its main negative regulators Mdm2 and Mdmx [168]. Recently, He [169] demonstrated that metformin-activated AMPK results in phosphorylation and inactivation of Mdmx, leading to p53 stabilization and activation. AMPK-mediated phosphorylation of Mdmx on Ser342 enhanced the association between Mdmx and 14-3-3. This inhibits p53 ubiquitylation stabilizing and activating p53 [169]. p53-induced Sestrin1 and Sestrin2 activate AMPK, which phosphorylate TSC2 thereby inhibiting mTORC1 [161]. Thus, metformin-activated AMPK and AMPK-mediated p53 activation and Sestrin1/2 expression functions as a feed forward loop inhibiting mTORC1 signaling [170]. It has recently been demonstrated that REDD1 expression is increased by metformin even when AMPK was down-regulated [171]. Remarkably, in LNCaP prostate cancer cells, REDD1 induced mTORC1 inhibition in response to metformin [171].

### Metformin-Induction of ATM Suppresses mTORC1

The ATM gene is mutated in the human genetic disorder ataxia-telangiectasia [172]. ATM is a 350 kDa protein is a member of the PI3K super family. At the G1/S interface ATM plays a central role in radiation-induced activation of p53. ATM binds to p53 in a complex fashion and activates p53 in response to breaks in DNA by Ser15-phosphorylation [172]. AMPK, the major pharmacologic target of metformin, is one of the downstream targets of ATM [173-175]. In rat hepatoma cells, inhibition of ATM reduced metformin-stimulated phosphorylation of AMPK, suggesting that ATM plays a therapeutic role for the action of metformin [176,177].

By activating the ATM-mediated DNA Damage Response (DDR), metformin might sensitize cells against further damage, thus mimicking the precancerous stimulus that induces an intrinsic barrier against carcinogenesis. It has been proposed that metformin might function as a tissue sweeper of pre-malignant cells before they gain stem cell/tumor initiating properties [178]. Recent evidence indicates that variations of *ATM* alter the glycemic response to metformin in patients with type 2 diabetes [179]. The *ATM* rs11212617 variant has been associated with successful glycemic response to metformin in patients with type

2 diabetes and insulin resistant HIV-infected patients [179,180].

### Metformin Inhibits Amino Acid-Mediated mTORC1 Activation

In addition to metformin's inhibitory effects on mTORC1 by AMPK-TSC2-mediated suppression of RHEB, metformin inhibits mTORC1 in a RAG GTPase-dependent manner [181]. The mechanism by which metformin inhibits RAG GTPases resulting in suppressed mTORC1 signaling has not yet been clarified. Similar to amino acid withdrawal of cells treatment with the biguanide phenformin caused mTOR to disperse from perinuclear aggregates and to diffuse throughout the cytoplasm [181]. Proton-assisted amino acid transporters (PATs) localized on late endosomes and lysosomes (LEL) interact with RAGs and are required for mTORC1 activation [125,182,183]. PAT1 has been identified as an essential mediator of amino acid-dependent mTORC1 activation involved in the function of the PAT1/RAG/Ragulator complex [125]. The mTORC1-regulatory role of the PATs is conserved in humans [184]. Activation of mTORC1 in starved HEK-293 cells stimulated by amino acids requires PAT1 and PAT4, and is elevated in PAT1-overexpressing cells. Importantly, in HEK-293 cells, PAT1 is highly concentrated in intracellular compartments, including endosomes, wherein mTOR shuttles upon amino-acid stimulation [184]. PAT1 and mTOR co-localize at the surface of the same intracellular compartments [184]. Therefore it has been proposed that PATs modulate the activity of mTORC1 not by transporting amino acids into the cell but by modulating the intracellular response to amino acids [184]. PAT1 and RAGC form part of a putative amino acid-sensing complex [184]. Knockdown of PAT1, PAT4, or mTOR in serum- and nutrient-starved cells reduced amino acid-dependent mTORC1 signaling following refeeding [184]. Conversely, over-expression of PAT1 in starved cells enhanced the sensitivity of the TORC1 response to amino acids during refeeding [184]. Notably, siRNA knock down of *PAT1* inhibits mTORC1 activation [184]. Thus, suppression of PAT1 attenuates mTORC1 signaling. Intriguingly, Metzner [185] demonstrated that PAT1 accepts guanidine derivatives such as the anti-diabetic compound  $\beta$ -guanidinopropionic acid, and its derivatives guanidinoacetic acid, and guanidinobutyric acid as substrates. However, metformin in excess amounts (10 mM) only exhibited a modest PAT1 inhibition of L-[<sup>3</sup>H]proline uptake in Caco-2 cells. To our knowledge no study investigated the effect of biguanides on PAT4 activity, which is also involved in the regulation of mTORC1 [186]. Future studies using different metformin concentrations and various lysosomal PAT1- and PAT4 expressing cell systems might be promising to elucidate the potential inhibitory effect of metformin and related biguanides on the regulation of PAT/RAG/Ragulator/v-ATPase nutrison-mediated mTORC1 signaling.

### Metformin and mTORC1/FoxO1-Regulated Gluconeogenesis

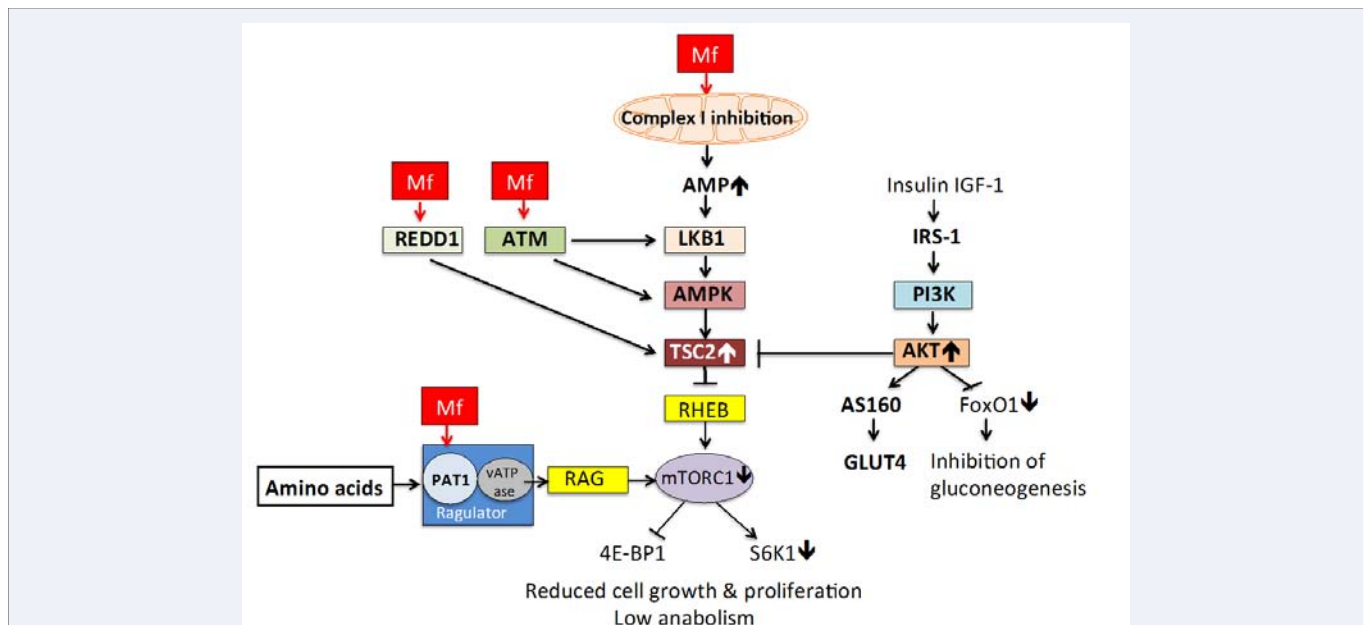
For about a decade AMPK was assumed the prime mediator of metformin's anti-hyperglycemic action [138,145,146]. However, the suggested role of metformin's mode of action stimulating AMPK-mediated inhibition of hepatic gluconeogenesis has been challenged. Genetic loss of function studies demonstrated that metformin lowered glucose production in liver of transgenic mice

that lacked either AMPK or its upstream activator LKB1 [187]. Recently, Li [188] identified mTORC1 as an essential component in the insulin regulated pathway of hepatic lipogenesis but not gluconeogenesis. mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis [188,189]. Gluconeogenesis is primarily controlled by the Fork head box class O1 transcription factor (FoxO1), which after insulin-induced AKT-mediated phosphorylation is extruded from the nucleus [190]. Nuclear FoxO1 stimulates the expression of key gluconeogenic genes, such as phosphoenolpyruvate carboxykinase (*PEPCK*) involved in the net glucose output from the liver [191]. Glucose-6-phosphatase (G6Pase), the enzyme that catalyzes the final step of gluconeogenesis, also plays a key role in the control of blood glucose levels. Onuma [192] observed a correlation between FoxO1a and FoxO3a binding and the inhibition of basal G6Pase catalytic subunit gene transcription by insulin. FoxO1a and FoxO3a share an identical binding specificity for G6Pase insulin response sequences 1 (IRS1) and IRS2 [192]. Notably, AMPK directly phosphorylates FoxO transcription factors at six regulatory sites that enhance FoxO transcriptional activity [193]. FoxO1 is the transcription factor of starvation and is upregulated by nutrient restriction [190,194]. Metformin exerts comparable effects to that of nutrient restriction [195]. In adipocytes, metformin increased nuclear FoxO1 expression and induced FoxO1-dependent lysosomal acid lipase as well as lipid droplet degradation through lipophagy [195]. Hepatic gluconeogenesis is absolutely required for survival during prolonged fasting or starvation, whereas during phases of nutrient abundance insulin suppresses hepatic gluconeogenesis. Peroxisome proliferator-activated receptor- $\gamma$  co-activator 1 $\alpha$  (PGC-1 $\alpha$ ), a transcriptional co-activator, binds and co-activates FoxO1 in a manner inhibited by AKT-mediated phosphorylation [196]. FoxO1

function is required for the robust activation of gluconeogenic gene expression in hepatic cells and in mouse liver by PGC-1 $\alpha$  demonstrating that FoxO1 and PGC-1 $\alpha$  interact in the execution of a program of insulin-regulated gluconeogenesis [196].

Thus, there appears to be a contradiction as metformin on the one hand increases FoxO1 expression in adipocytes but on the other hand apparently suppresses FoxO1-mediated hepatic gluconeogenesis. As metformin's suppressive effect on hepatic gluconeogenesis is independent of LKB1 and AMPK [187], but strongly dependent on the degree of AKT-mediated FoxO1-phosphorylation, metformin's primary target in the regulation of hepatic gluconeogenesis appears to be mTORC1-S6K1 signaling. Nutrient and amino acid excess results in hepatic mTORC1-overactivation enhancing the activity of S6K1. S6K1-mediated phosphorylation of IRS-1, the adaptor protein of key downstream effects of the insulin receptor, dampens the activity of AKT thereby increases nuclear FoxO1-levels that promote hepatic gluconeogenesis. Thus, S6K1-mediated phosphorylation of S6K1 leads to insulin desensitization [31]. Notably, hyperactive mTORC1 signaling is an essential event in the development of hepatic insulin resistance in the presence of excess amino acids [197]. Exposure of HepG2 cells to excess amino acids reduced AMPK phosphorylation and impaired the insulin-stimulated phosphorylation of AKT Ser473 and IRS-1 Tyr612 [197]. Metformin inhibited mTORC1 signaling, thereby prevented hepatic insulin resistance induced by excess amino acid intake [197].

Metformin's major mode of action thus appears to be the inhibition of mTORC1 decreasing the activity of S6K1 resulting in enhanced insulin sensitivity. Suppression of S6K1 reduces FoxO1-mediated hepatic gluconeogenesis and insulin resistance



**Figure 2** Metformin (Mf) inhibits complex I of the mitochondrial electron transport chain associated with an increase in AMP levels that induce LKB1/AMPK-mediated activation of TSC2 inhibiting mTORC1. Furthermore, metformin via stimulation of REDD1 and ATM enhances AMPK/TSC2-mediated mTORC1 inhibition. Furthermore, metformin inhibits amino acid-mediated activation of RAG GTPases at the lysosomal surface. Thus, metformin attenuates both RHEB- and RAG-mediated activation pathways of mTORC1. Reduced mTORC1 activity downregulates S6K1 and thereby improves AKT-mediated glucose uptake and suppresses FoxO1-mediated gluconeogenesis.

of peripheral tissues by increased AKT-mediated translocation of glucose transporter-4 (GLUT4) to the plasma membrane [198,199]. This mechanism explains the beneficial clinical effects of metformin treatment on glucose homeostasis (Figure 2).

This concept is supported by recent studies exploring the expression of the liver-derived secretory protein Selenoprotein P (SeP). SeP is regulated similarly to G6Pase. G6Pase-expression is stimulated by PGC-1 $\alpha$  and FoxO1a [200]. SeP encoded by *SEPP1* in humans induces insulin resistance in type 2-diabetes [201]. Remarkably, metformin suppresses *SEPP1* expression by activating AMPK and subsequently inactivating FoxO3a in hepatocytes [202]. Treatment with metformin reduced *SEPP1* promoter activity in a concentration- and time-dependent manner. Computational analysis of transcription factor binding sites conserved among the species resulted in identification of the FoxO-binding site in the metformin-response element of the *SEPP1* promoter. Metformin did not affect FoxO3a expression, but it increased its phosphorylation and thereby decreased its nuclear localization. This effect is compatible with the proposed mode of metformin action centering on inhibition of mTORC1-S6K1-signaling, which explains increased AKT-mediated FoxO-phosphorylation and nuclear extrusion by metformin-mediated suppression of mTORC1 (Figure 2).

### Metformin Modifies mRNA Translation and mTORC1 Signaling

Recently, Larsson [203] presented data of a genome-wide analysis of translational targets of canonical mTOR inhibitors compared with metformin. Metformin exerted profound effects on the translome and perturbed the translome to an extent comparable to canonical mTOR inhibitors such as rapamycin and PP242 [203]. Metformin suppressed translation of limited subsets of functionally related mRNAs that encode proteins involved in cell-cycle control, metabolism, mRNA translation, and RNA processing. Importantly, metformin's antiproliferative activity could be explained by selective translational suppression of mRNAs encoding cell-cycle regulators via the mTORC1/4E-BP pathway [203].

## DISCUSSION AND CONCLUSION

Metformin interferes with cellular pathways that sense ROS signals and DNA damage responses, cellular energy homeostasis and amino acid availability finally converging in restrained mTORC1 signaling. In comparison to allosterical, natural or synthetic mTORC1 inhibitors such as rapamycin [204], resveratrol, curcumin, caffeine, epigallocatechin gallate, silymarin and others plant-derived polyphenols [205] and synthetic active-site mTORC1 inhibitors (TORkinibs) [206,207], metformin is a unique drug that attenuates the two major independent pathways required for efficient activation mTORC1. Metformin 1) suppresses RHEB-mediated signals integrating growth factors (insulin, IGF-1), cellular energy status (AMPK), ROS-status and ER stress (ATM, REDD1), and 2) suppresses amino acid-mediated PAT1/Ragulator-RAG-signaling (Table 1).

Is it thus conceivable that metformin functions as a unique mTORC1 inhibitor that attenuates the progression of mTORC1-driven diseases of civilization associated with increased food intake [208,209], obesity [210-217], insulin resistance and type 2 diabetes [32,138,145,218,219], hepatic steatosis [220], polycystic ovary syndrome [221,222], acne [223-225], dyslipoproteinemia [226], atherosclerosis and cardiovascular diseases [227,228], cancer [203,229-248], and neurodegenerative diseases [249,250]. Metformin in a pharmacological way attenuates both exaggerated nutrient signaling of Western diet associated with enhanced insulin/IGF-1 signaling (the RHEB-axis of mTORC1 activation) and nutritional overload of essential BCAAs (the RAG-axis of mTORC1 activation) [251-253]. Notably, the mTORC1 inhibitor metformin counteracts the anabolic signaling of milk [254], which has been identified as an endocrine postnatal signaling system promoting mTORC1-mediated postnatal growth [255]. From all animal proteins, milk proteins transport the highest amounts of essential BCAAs [121]. Elevated plasma levels of essential BCAAs significantly correlate with insulin resistance and the risk of type 2-diabetes [50,256,257]. Not only carbohydrate- and lipid overload but also amino acid excess induces mTORC1 hyper-activation resulting in S6K1/IRS-1- and STAT3/SOCS3-mediated insulin resistance [44,45,258-266] (Figure 2). In this regard, metformin by down-regulating mTORC1-S6K1 signaling compensates the adverse effects of persistent hyperalimentation,

**Table 1:** Metformin-mediated effects that inhibit mTORC1 signaling.

Metformin targets	Pathways suppressing mTORC1 signaling	Reference
Inhibition of complex I of the mitochondrial electron transport chain	Decrease in ATP, increase in AMP, activation of LKB1, LKB1-mediated activation of AMPK, activation of TSC2 $\rightarrow$ mTORC1 inhibition; AMPK-mediated inhibition of RAPTOR $\rightarrow$ mTORC1 inhibition; AMPK-mediated inactivation of Mdmx, p53 stabilization, p53-induced expression of Sestrin1 and Sestrin 2, AMPK activation $\rightarrow$ mTORC1 inhibition	[95,140-149, 151] [95] [169]
Induction of cellular stress transcription factor ATF4	ATF4-mediated expression of REDD1, TSC2 stabilization $\rightarrow$ mTORC1 inhibition	[157-160, 164]
Activation of ATM	ATM-mediated activation of AMPK $\rightarrow$ mTORC1 inhibition	[176,177]
Inhibition of RAG GTPase	Possible interference with PAT1 function (?), disintegration of the nutrisome complex (PAT1/RAG/Ragulator/v-ATPase) $\rightarrow$ mTORC1 inhibition	[184-186]
Modification of the translome	Suppression of components of the mTORC1/ 4E-BP pathway $\rightarrow$ mTORC1 inhibition	[203]

**Abbreviations:** mTORC1: Mechanistic Target of Rapamycin Complex 1; AMP: Adenosine Monophosphate; ATP: Adenosine Triphosphate; LKB1: Liver kinase B1; AMPK: AMP-activated protein Kinase; TSC2: Tuberin; RAPTOR: Regulatory-Associated Protein of mTOR; ATF4: Activating Transcription Factor 4; REDD1: Regulated in DNA Damage and Development 1; ATM: Ataxia Teleangiectasia Mutated; PAT1: Proton-Assisted amino acid Transporter 1; RAG: RAS-related GTP-binding protein; v-ATPase: Vacuolar H<sup>+</sup>-ATPase; 4E-BP: Eukaryotic initiation factor (eIF) 4E-Binding Protein.

high milk, milk protein and milk fat consumption associated with anabolic nutrient signaling.

Metformin has been used for decades to improve glucose homeostasis and its major mode of action had focused on its role to activate AMPK. Now, we begin to understand that metformin's pivotal mode of action is the attenuation of mTORC1-S6K1 signaling, a regulatory key node of cellular metabolism that in response to excess nutrients orchestrates the signaling pathways of anabolism. Accumulating evidence underlines that persistently increased mTORC1 signaling is the common promoter of diseases of civilization [12-18].

Suppression of mTORC1 explains metformin's effectiveness in diabetes and cancer. Down-regulation of S6K1 explains IRS-1/PI3K/AKT/FoxO1-mediated suppression of hepatic gluconeogenesis as well as AKT-AS160-mediated translocation of GLUT4 that reduces insulin resistance.

Metformin compensates the adverse metabolic effects of Western sedentary lifestyle with overnutrition that promotes mTOR-driven age-related diseases. Thus, metformin exerts preventive and therapeutic effects for common metabolic diseases of civilization for which „less TOR is more“[90].

Metformin apparently normalizes the magnitude of mTORC1 signaling in cancer cells with genetic aberrations of signaling components of the mTORC1 pathway such as over-activated AKT of PTEN loss of function mutations. Metformin treatment appears to lower mTORC1 signaling to a level of a vegan low-protein diet [234]. Taken together, metformin by targeting mTORC1 signaling acts at the molecular interface connecting metabolic stress, aging, obesity, cardiovascular and neurodegenerative diseases and cancer.

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BCM searched the literature and wrote the manuscript. GS provided further scientific informations, discussions and literature references. Both authors read and approved the final manuscript.

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