Research Article

Metformin Inhibits Mammalian Target of Rapamycin–Dependent Translation Initiation in Breast Cancer Cells

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Abstract

Metformin is used for the treatment of type 2 diabetes because of its ability to lower blood glucose. The effects of metformin are explained by the activation of AMP-activated protein kinase (AMPK), which regulates cellular energy metabolism. Recently, we showed that metformin inhibits the growth of breast cancer cells through the activation of AMPK. Here, we show that metformin inhibits translation initiation. In MCF-7 breast cancer cells, metformin treatment led to a 30% decrease in global protein synthesis. Metformin caused a dose-dependent specific decrease in cap-dependent translation, with a maximal inhibition of 40%. Polysome profile analysis showed an inhibition of translation initiation as metformin treatment of MCF-7 cells led to a shift of mRNAs from heavy to light polysomes and a concomitant increase in the amount of 80S ribosomes. The decrease in translation caused by metformin was associated with mammalian target of rapamycin (mTOR) inhibition, and a decrease in the phosphorylation of S6 kinase, ribosomal protein S6, and eIF4E-binding protein 1. The effects of metformin on translation were mediated by AMPK, as treatment of cells with the AMPK inhibitor compound C prevented the inhibition of translation. Furthermore, translation in MDA-MB-231 cells, which lack the AMPK kinase LKB1, and in tuberous sclerosis complex 2 null (TSC2$^{-/-}$) mouse embryonic fibroblasts was unaffected by metformin, indicating that LKB1 and TSC2 are involved in the mechanism of action of metformin. These results show that metformin-mediated AMPK activation leads to inhibition of mTOR and a reduction in translation initiation, thus providing a possible mechanism of action of metformin in the inhibition of cancer cell growth. [Cancer Res 2007;67(22):10804–12]

Introduction

mRNA translation is required for many cellular processes, including growth, proliferation, and differentiation. Initiation is the rate-limiting step of translation under most circumstances. Consequently, translation initiation is tightly regulated by a number of mechanisms. The rate-limiting step in translation initiation is thought to be the formation of the eukaryotic initiation factor 4F (eIF4F) complex, which mediates the recruitment of the 40S ribosomal subunit to the mRNA (1). eIF4F consists of eIF4E, which interacts with the 7-methylguanosine cap present at the 5′ end of all nuclear-transcribed cellular mRNAs, the helicase eIF4A, and the large scaffolding protein eIF4G (2). Assembly of the eIF4F complex is inhibited by a family of proteins known as the eIF4E-binding proteins (4E-BPs), which suppress translation by competing with eIF4G for binding to eIF4E (2). The binding of the 4E-BPs to eIF4E is regulated by phosphorylation (2, 3). The phosphorylation of the 4E-BPs is effected by the large serine/threonine kinase, mammalian target of rapamycin (mTOR), which controls cell growth and proliferation (4). It is thought that mTOR coordinates the latter processes by regulating mRNA translation initiation via phosphorylation of two of its major targets: the 4E-BPs (4E-BP1, 4E-BP2, and 4E-BP3) and the ribosomal protein S6 (rpS6) kinases (S6K1/S6K2). Hypophosphorylated 4E-BP1, the better characterized member of the 4E-BPs, binds to eIF4E and prevents formation of the eIF4F complex, thus inhibiting cap-dependent translation. Upon phosphorylation by mTOR, hyperphosphorylated 4E-BP1 is released from eIF4E, leading to an increase in cap-dependent translation (3, 5). Phosphorylation of S6K1 by mTOR enhances its kinase activity toward its downstream targets, including the 40S rpS6, eIF4B (6), and S6K1 Aly/REF-like target (7). The activity of mTOR is regulated by cellular energy levels, growth factors, nutrients, and oxygen levels (8). Dysregulation of mTOR can lead to increased cell growth, proliferation, and neoplasia. In fact, the pathways that regulate mTOR activity are often deregulated in human cancers. For example, mutational inactivation of the tumor suppressor PTEN is prevalent in a variety of cancers, leading to unrestrained mTOR activity (9, 10). This leads to an increase in translation of a subset of mRNAs that contribute to tumorigenesis. These mRNAs encode for growth factors, cell cycle activators, angiogenic factors, and inhibitors of apoptosis (11). Therefore, mTOR inhibition is considered as an anticancer therapy, particularly in the treatment of cancers that exhibit overactive mTOR signaling as a result of genetic lesions. Rapamycin, a specific inhibitor of mTOR (4), inhibits cap-dependent translation (12), and the growth of a variety of cancers, including those of the kidney, breast, and lung (13). Furthermore, rapamycin analogues are being explored as anticancer therapies in clinical trials (14, 15).

Metformin is a biguanide drug that is widely used for the treatment of type 2 diabetes (16, 17). The effectiveness of metformin as an antidiabetic drug is explained by its ability to decrease blood glucose by decreasing hepatic gluconeogenesis and stimulating glucose uptake in muscle (16, 18–20). Some of the beneficial effects of metformin have been linked to activation of the AMP-activated protein kinase (AMPK) in muscle, adipose tissue, and liver (17). AMPK is a heterotrimer serine/threonine protein kinase which is composed of a catalytic subunit, α, and two regulatory subunits, β and γ (21). AMPK regulates energy metabolism and is activated by an increase in the intracellular ratio of AMP/ATP. Activation of

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AMPK requires an allosteric change induced by AMP, as well as phosphorylation on Thr[172] within the catalytic domain of the α subunit (21, 22). LKB1 is the kinase responsible for phosphorylating AMPK (23, 24), and its activity is required for AMPK activation in response to energy stress in cell culture (25). LKB1 is a tumor suppressor whose inactivation leads to Peutz-Jeghers syndrome, a condition characterized by colorectal polyps and predisposition to malignant tumors of various tissues, including the testes, colon, and breast (26).

Upon activation, AMPK phosphorylates a number of effector proteins leading to the activation of ATP generating pathways, such as glycolysis, and the inhibition of ATP-consuming pathways, such as cholesterol synthesis (21). AMPK regulates a variety of processes including cellular growth and proliferation, fatty acid synthesis, and mRNA translation (27). The latter process is very energy expensive, consuming up to 45% of total cellular energy (28). AMPK mediates its effects on mRNA translation through the inhibition of mTOR via phosphorylation and activation of tuberous sclerosis complex 2 (TSC2; ref. 29), a subunit of the TSC1/TSC2 (hamartin/tuberin) complex that negatively regulates mTOR signaling (30). Therefore, the inhibition of mTOR via AMPK activation represents a novel approach for the treatment of cancer. For example, activation of AMPK by a variety of compounds, such as 5′-aminoimidazole-4-carboxamide ribonucleoside (AICAR), caused an inhibition of breast, glioma, and prostate cancer cell proliferation (31). Furthermore, diabetics receiving metformin exhibited a decrease in cancer incidence (32).

Recently, we showed that metformin inhibited the growth of breast cancer cells through the activation of AMPK (33). To investigate the molecular mechanism underlying this process, we studied the effects of metformin on mTOR signaling and translation. Metformin inhibited translation initiation in breast cancer cells as indicated by the disruption of polysomes and a dose-dependent decrease in cap-dependent translation. The effect of metformin on translation was associated with mTOR inhibition and a decrease in the phosphorylation of S6K, rpS6, and 4E-BP1. Metformin failed to inhibit translation in cells lacking LKB1 or TSC2, highlighting these proteins as signaling components that are required for the mechanism of action of metformin.

Materials and Methods

Chemicals and reagents. All cell culture reagents were obtained from Invitrogen. Metformin (1, 1-dimethylbiguanide hydrochloride) was obtained from Sigma and dissolved in 1× PBS. The AMPK inhibitor, compound C (Calbiochem), was dissolved in DMSO (final concentration, 5 mmol/L), and cycloheximide (Calbiochem) was dissolved in water (final concentration, 10 mg/mL). All metformin and compound C treatments were carried out in DMEM containing 10% fetal bovine serum (FBS). Primary antibodies against phosphorylated AMPKα (Thr[172]), AMPKα, phosphorylated p70S6K (Thr[389]), p70S6K, phosphorylated rpS6 (Ser[240/244]), rpS6, and 4E-BP1 were purchased from Cell Signaling Technology. Horseradish peroxidase–conjugated antirabbit IgG and antimouse IgG were from Amersham Biosciences.

Cell culture. $TSC2^{-/-}$ and $TSC2^{+/+}$ mouse embryonic fibroblasts (MEF; a gift from Dr. Kun-Liang Guan, University of Michigan) were established from E10.5 $p53^{-/-}TSC2^{-/-}$ or $p53^{-/-}TSC2^{+/+}$ embryos (34). MCF-7 and MDA-MB-231 cells (American Type Culture Collection) and MEFs were maintained in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2.

Western blot analysis. Cells were washed once with cold PBS, collected, and lysed in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 mmol/L EGTA, 1% Triton, 10 μg/mL aprotonin, 10 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonylfluoride, and 0.5 mmol/L NaF. Cell lysates were clarified by centrifugation at 13, 000×g for 14 min at 4°C and assayed for total protein content (Bio-Rad). Cleared lysates (100 μg for AMPK blots, 50 μg for all other proteins) were resolved by 15% (4E-BP1) or 10% (all other proteins) SDS-PAGE, and separated proteins were transferred to a nitrocellulose membrane (Protran, Perkin-Elmer). Membranes were blocked in 5% nonfat milk in TBS containing 0.1% Tween 20 and probed with the appropriate primary and secondary antibodies. Immunoreactive proteins were visualized using enhanced chemiluminescence (Perkin-Elmer). After blotting for phosphorylated proteins, antibodies were removed with an acidic stripping buffer [200 mmol/L glycine, 500 mmol/L NaCl (pH 2.8)], and membranes were reprobed for the total level of each protein.

Metabolic labeling. MCF-7 and MDA-MB-231 cells were seeded in 24 well plates, and ^{35}S-methionine labeling was done as described previously (33). After labeling, the cells were washed with cold PBS and lysed in buffer containing 1% Triton, 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), and 1 mmol/L EGTA, and radioactivity incorporated into trichloroacetic acid (TCA, 5%) precipitable material was measured.

Cell growth assay. $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were seeded in 24-well plates (20,000 cells per well) in DMEM containing 10% FBS. Cells were treated with increasing doses (0–20 mmol/L) of metformin for 72 h. The media and metformin were changed every 24 h. After 72 h, the cells were washed once with 1× PBS, stained with 0.5% crystal violet (145 mmol/L NaCl, 0.5% formal saline, 50% ethanol) for 10 min and washed thrice with water. Crystal violet was eluted from cells with 33% acetic acid, and absorption of the supernatant from each sample was measured at 570 nm to evaluate cell growth.

Bicistronic reporter assay. The Renilla–hepatitis C virus internal ribosomal entry site–firefly luciferase reporter plasmid (pGL3/Rluc/HCVIRES/Fluc) was a gift from Martin Krüger (Medizinische Hochschule Hannover) and has been described previously (35, 36). For the luciferase reporter assays, MCF-7 cells were seeded in six-well plates and transfected with 0.5 μg per well of pGL3/Rluc/HCVIRES/Fluc using Lipofectamine and Plus reagent (Invitrogen). After transfection, cells were treated with metformin for 24 h and harvested using passive lysis buffer (Promega). Lysates were then assayed for Renilla and firefly luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) and a Berthold Technologies luminometer according to the manufacturer's instructions.

To determine the effect of compound C on the bicistronic reporter, MCF-7 cells were transfected with pGL3/Rluc/HCVIRES/Fluc and incubated for 18 h, upon which time they were treated with 20 μmol/L compound C or an equal volume of vehicle (DMSO) for 30 min. Cells were then treated with metformin for 7 h, and lysates were harvested and assayed for luciferase activity as described above. Compound C treatment was maintained during the 7 h incubation with metformin. Ratios of Rluc/Fluc activity were calculated as a measure of cap-dependent translation.

Polysome analysis. Cells were cultured in 15-cm Petri dishes and treated with 20 mmol/L metformin for 24 h. Cells were washed with cold PBS containing 100 μg/mL cycloheximide and collected by centrifugation at 1,000 rpm for 10 min at 4°C. Cell pellets were lysed in hypotonic lysis buffer [5 mmol/L Tris-HCl (pH 7.5), 2.5 mmol/L $MgCl_2$, 1.5 mmol/L KCl, 100 μg/mL cycloheximide, 2 mmol/L DTT, 0.5% Triton X-100, and 0.5% sodium deoxycholate], and cellular debris was removed by centrifugation at 13,000×g for 2 min at 4°C. Lysates were loaded onto 10% to 50% sucrose density gradients [20 mmol/L HEPES-KOH (pH 7.6), 100 mmol/L KCl, 5 mmol/L $MgCl_2$] and centrifuged in a Beckman SW40 rotor at 35,000 rpm for 2 h at 4°C. After centrifugation, gradients were fractionated at a rate of 2.5 mL/min (25 fractions, 12 drops per fraction) and the absorbance at 254 nm was continuously recorded (chart speed, 30 cm/h) using an ISCO fractionator (Teledyne ISCO).

Statistical analysis. Error bars for all data represent the SD from the mean. Statistical significance was assessed using a two-sample Student's t test.

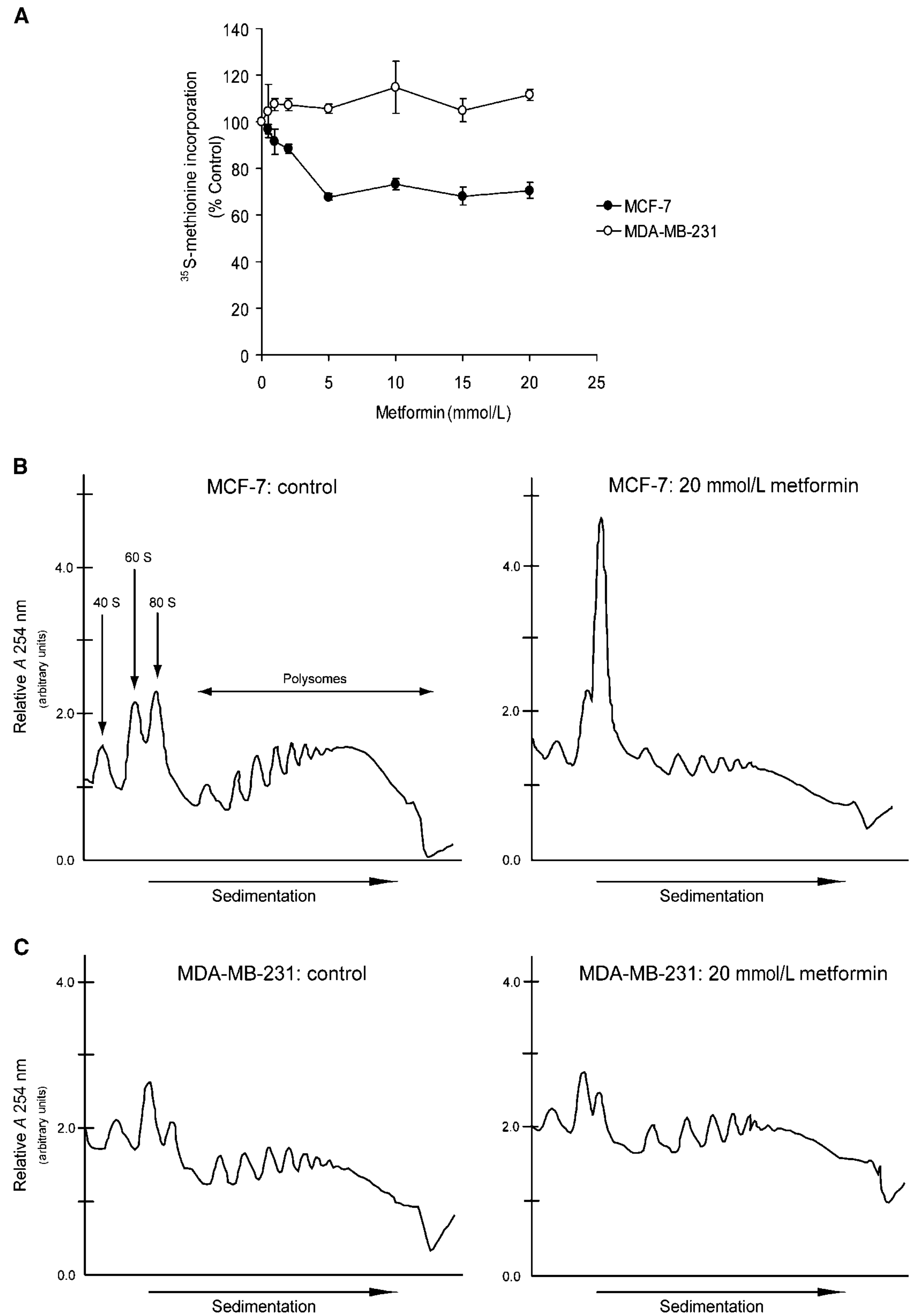

Figure 1. Effect of metformin on protein synthesis and polysome distribution in breast cancer cells. *A,* MCF-7 and MDA-MB-231 cells were incubated with the indicated doses of metformin for 24 h and ^{35}S-methionine protein labeling mix (20 μCi/mL) for 45 min. Cells were harvested after labeling and radioactivity incorporated into TCA precipitable material was measured. Protein synthesis levels are displayed as a percentage of the value obtained in the absence of metformin. *Points,* average of three separate replicates; *bars,* SD from the mean. MCF-7 (*B*) or MDA-MB-231 (*C*) cells were treated with 20 mmol/L metformin or an equal volume of PBS for 24 h. Cell lysates were sedimented on 10% to 50% sucrose gradients. Gradients were fractionated and absorbance (*A*) at 254 nm was continuously recorded. Polysome profiles were analyzed three separate times. Representative profile for each cell type and treatment.

Results

Metformin inhibits translation initiation in MCF-7 breast cancer cells. We recently showed that metformin reduced general protein synthesis in MCF-7 cells (33). Consistent with our previous report, treatment of MCF-7 cells with metformin led to a reduction in general translation with a maximal inhibition of 30% (Fig. 1*A*). LKB1, an upstream kinase of AMPK (24), is required for AMPK activation in response to metformin in the liver as well as energy stress in cell culture (20, 25). To show that LKB1 is required for the translation inhibition caused by metformin, protein synthesis was examined in MDA-MB-231 cells, which do not express LKB1 mRNA or protein (37). MDA-MB-231 cells were treated with increasing doses of metformin (0–20 mmol/L) for 24 h, and protein synthesis was assessed by incorporation of ^{35}S-methionine into TCA precipitable material. As expected, metformin treatment had no effect on protein synthesis in MDA-MB-231 cells (Fig. 1*A*). Thus, in MCF-7 breast cancer cells, LKB1 is required for the inhibition of translation by metformin.

Next, we examined the step at which metformin inhibits translation. To this end, a polysome profile analysis was done. Treatment of MCF-7 cells with 20 mmol/L metformin caused a shift from large to small polysomes and an increase in 80S ribosomes, demonstrating a decrease in translation initiation (Fig. 1*B*). As expected, metformin had no effect on the polysome profile of MDA-MB-231 cells (Fig. 1*C*).

Metformin activates AMPK and inhibits mTOR signaling. We next examined the effects of metformin on the activity of mTOR, a downstream effector of AMPK and a major regulator of translation initiation. We hypothesized that metformin would inhibit mTOR signaling in MCF-7 cells, but signaling would remain intact in MDA-MB-231 cells. To test this hypothesis, we used Western blot analysis to assess the phosphorylation status of two direct downstream targets of mTOR, S6K, and 4E-BP1, as a measure of mTOR activity. The activation of AMPK was also evaluated by monitoring the phosphorylation of AMPK on Thr^{172}. Phosphorylation on Thr^{172}, which lies in the catalytic domain of the AMPK α subunit, is required for activation of AMPK (21, 23). In MCF-7 cells, metformin treatment led to a dose-dependent increase in the phosphorylation of AMPK on Thr^{172} (Fig. 2*A*). Metformin treatment also caused a strong decrease in the phosphorylation of S6K and its target, rpS6. We also examined the phosphorylation of 4E-BP1. Three isoforms of 4E-BP1 were detected (Fig. 2*A*), which represent differentially phosphorylated forms of the protein, with the slowest migrating band γ corresponding to the hyperphosphorylated form and the fastest migrating band α corresponding to the hypophosphorylated form of the protein (3, 12). Metformin treatment caused a shift in the phosphorylation state of 4E-BP1 from the γ to the α form (Fig. 2*A*). Notably, metformin treatment had no affect on the phosphorylation of AMPK, S6K, rpS6, or 4E-BP1 in MDA-MB-231 cells (Fig. 2*B*), indicating that LKB1 is absolutely required for AMPK activation and inhibition of mTOR activity by metformin.

TSC2 is necessary for the inhibitory effects of metformin. The TSC1/TSC2 complex functions as the GTPase-activating protein (GAP) of the small GTPase Ras homologue enriched in brain (Rheb; ref. 38). Rheb exists in two states: the GTP-bound form of Rheb activates mTOR, whereas the GDP-bound form of Rheb cannot activate mTOR (38). AMPK phosphorylates TSC2 to enhance its GAP activity, leading to an increase in GDP-bound Rheb and consequently mTOR inhibition (29). To study the role of TSC2 in the mechanism of action of metformin and to further delineate

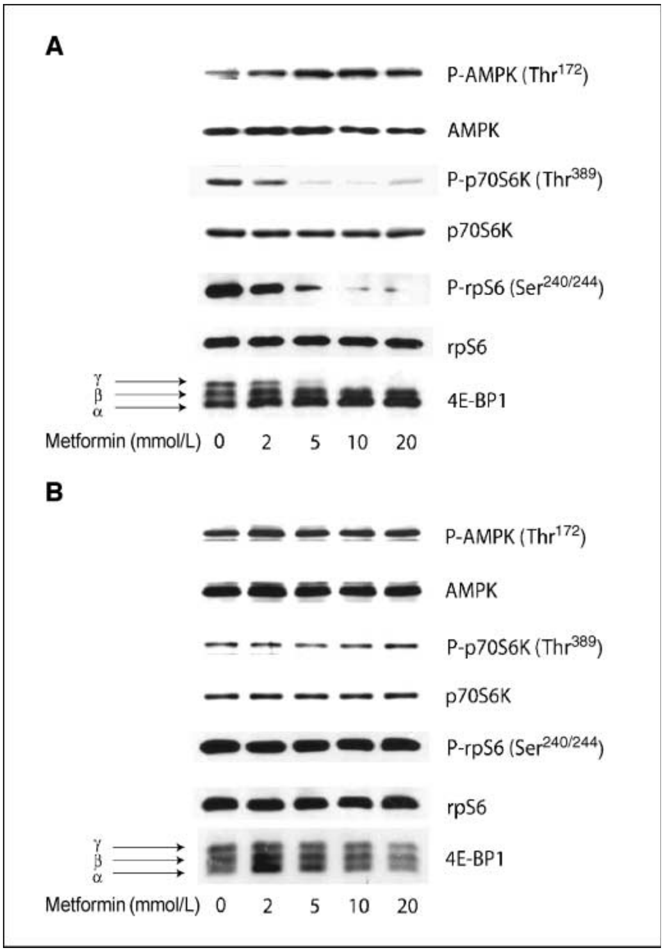

Figure 2. Metformin activates AMPK and induces dephosphorylation of S6K and 4E-BP1 in MCF-7 but not MDA-MB-231 cells. Western blot analysis of metformin-treated MCF-7 (*A*) and MDA-MB-231 (*B*) cells. Cells were treated with the indicated doses of metformin for 24 h in DMEM containing 10% FBS. Cells were harvested, and lysates were resolved by 15% (4E-BP1) or 10% (all other proteins) SDS-PAGE. Immunoblot analysis was carried out using antibodies against phosphorylated AMPKα (Thr^{172}), AMPKα, phosphorylated p70S6K (Thr^{389}), p70S6K, rpS6, phosphorylated rpS6 ($Ser^{240/244}$), and 4E-BP1.

the role of mTOR in metformin-mediated translation inhibition, we examined the effect of metformin on established $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs (34). Treatment of TSC2 $^{+/+}$ MEFs with 20 mmol/L metformin caused an inhibition of translation initiation, as indicated by the shift from large to small polysomes and a concomitant increase in the amount of 80S ribosomes (Fig. 3*A*). In contrast, metformin treatment had no effect on the polysome profile of $TSC2^{-/-}$ MEFs (Fig. 3*B*). The response of $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs to metformin was also examined by Western blot analysis of AMPK, S6K, rpS6, and 4E-BP1. In $TSC2^{+/+}$ MEFs, metformin caused an increase in the phosphorylation of AMPK on Thr^{172} and a decrease in the phosphorylation of S6K and rpS6. In addition, metformin treatment led to dephosphorylation of 4E-BP1 as shown by a shift from hyperphosphorylated 4E-BP1 to the hypophosphorylated form (Fig. 4*A*). $TSC2^{-/-}$ MEFs were responsive to metformin as indicated by an increase in AMPK Thr^{172} phosphorylation. However, metformin failed to inhibit mTOR signaling in these cells, as S6K, rpS6, and 4E-BP1 remained phosphorylated in the presence of increasing doses of the drug (Fig. 4*B*). These results paralleled those found in MCF-7 and MDA-MB-231 breast cancer cells, wherein LKB1 status determined metformin sensitivity. To correlate the effect of metformin on translation and mTOR signaling to cell growth, TSC2$^{+/+}$ and TSC2$^{-/-}$ MEFs were treated with metformin for 72 h and cell growth was assessed by a crystal violet cell growth assay. Consistent with its effects on translation, metformin inhibited the growth of TSC2$^{+/+}$ MEFs by 53% but did not affect TSC2$^{-/-}$ MEFs (Fig. 4*C*).

Metformin inhibits cap-dependent translation. Because inhibition of mTOR inhibits cap-dependent translation, we wished to examine whether metformin specifically inhibits cap-dependent translation. To this end, we used a bicistronic reporter mRNA composed of two cistrons encoding Renilla and firefly luciferase separated by the HCV IRES (Fig. 5*A*). Translation of the Renilla cistron is cap-dependent, whereas translation of the firefly cistron is controlled by the HCV IRES and therefore occurs in a cap-independent manner and acts as an internal control for transfection efficiency (35). Transfected MCF-7 cells were treated with increasing doses of metformin (0–20 mmol/L) for 24 h. Metformin caused a dose-dependent decrease in the expression of the Renilla luciferase, with a maximal inhibition of 44% at a dose of 20 mmol/L (Fig. 5*B*). Metformin treatment led to a decrease in the Rluc/Fluc ratio with a maximal reduction of 41% at a dose of

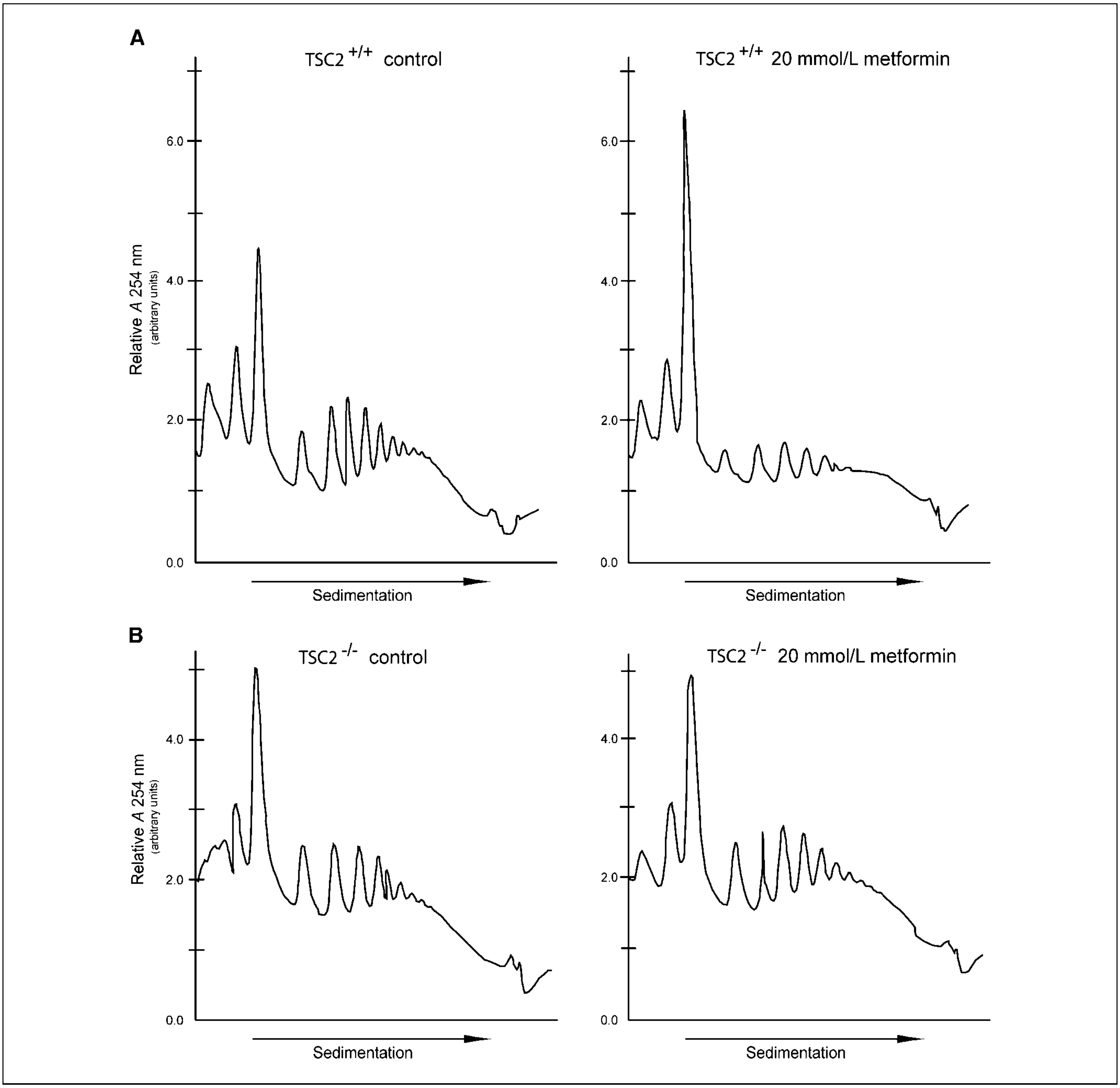

Figure 3. Effect of metformin on polysome distribution in TSC2$^{+/+}$ and TSC2$^{-/-}$ MEFs. TSC2$^{+/+}$ (*A*) or TSC2$^{-/-}$ (*B*) MEFs were treated with 20 mmol/L metformin or an equal volume of PBS for 24 h. Cell lysates were then prepared and sedimented on 10-50% sucrose gradients. Polysome profiles were generated and analyzed as described for Fig. 1. Polysome profiles were analyzed three separate times. Representative profile for each cell type and treatment.

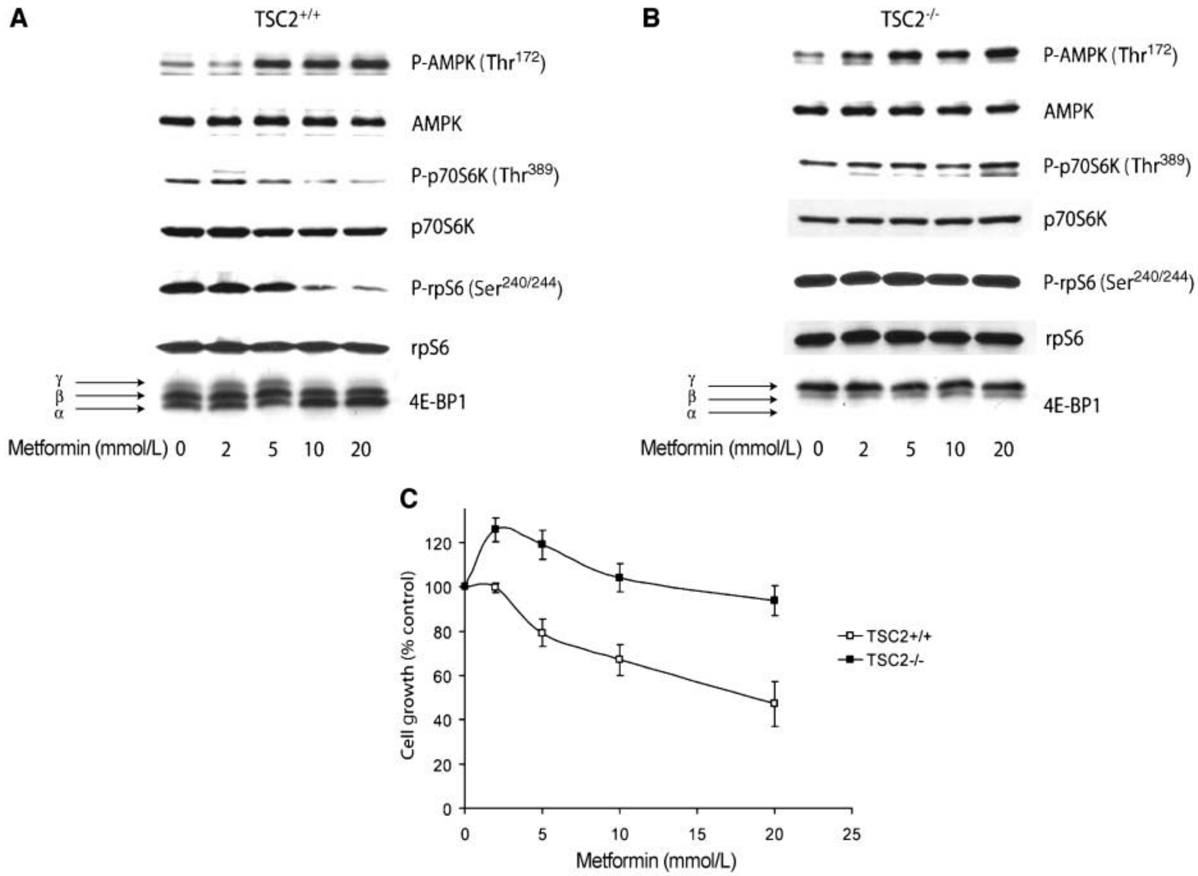

Figure 4. Metformin inhibits mTOR signaling and cell growth in $TSC2^{+/+}$ but not $TSC2^{-/-}$ MEFs. Western blot analysis of metformin-treated $TSC2^{+/+}$ (*A*) and $TSC2^{-/-}$ (*B*) MEFs. Cells were treated with the indicated doses of metformin for 24 h in DMEM containing 10% FBS. Cells were harvested, and lysates were resolved by 15% (4E-BP1) or 10% (all other proteins) SDS-PAGE. Immunoblot analysis was carried out using antibodies against phosphorylated AMPKα (Thr^{172}), AMPKα, phosphorylated p70S6K (Thr^{389}), p70S6K, rpS6, phosphorylated rpS6 ($Ser^{240/244}$), and 4E-BP1. *C,* $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were treated with the indicated doses of metformin for 72 h and stained with crystal violet. Crystal violet was eluted with acetic acid, and cell growth was assessed by measuring absorption of the supernatant from each sample at 570 nm. Values are displayed as a percentage of the control (no metformin). *Points,* average of three separate replicates; *bars,* SD from the mean.

20 mmol/L, indicating an inhibition of cap-dependent translation (Fig. 5*D*). In contrast, firefly luciferase expression was unaffected, indicating that metformin does not inhibit HCV IRES–driven translation of the firefly cistron (Fig. 5*C*). To ensure that the effects of metformin were specifically mediated by AMPK, experiments were repeated in the presence of compound C, a potent and specific small molecule inhibitor of AMPK (17). Pretreatment of cells with 20 μmol/L compound C completely prevented the inhibition of cap-dependent translation caused by metformin treatment (Fig. 5*E*). Taken together, these data show that metformin inhibits specifically cap-dependent translation because of the suppression of mTOR activity.

Discussion

Because metformin is a drug used for the treatment of type 2 diabetes, the majority of studies focusing on the effects of metformin have been carried out in tissues involved in insulin signaling and metabolism, such as muscle, adipose tissue, and liver (20, 39, 40). However, the effects of metformin on other tissues or cells in culture have not been well characterized. Our previous work showed that breast cancer cells are sensitive to metformin, which acted as a growth inhibitor (33). In the present study, we investigated the basis for this growth inhibition.

Treatment of MCF-7 breast cancer cells with metformin caused a 30% reduction in global protein synthesis and a 41% decrease in cap-dependent translation. The most likely explanation for the partial inhibition of translation is that metformin preferentially inhibits the translation of a subset of mRNAs. It is believed that high levels of eIF4F are required for efficient translation of mRNAs containing highly structured 5′ untranslated regions (41). Treatment of cells with metformin led to inhibition of mTOR and a decrease in the phosphorylation of 4E-BP1. Hypophosphorylated 4E-BP1 inhibits translation initiation by binding to eIF4E with high affinity. The binding of 4E-BP1 to eIF4E prevents the formation of the eIF4F complex, which is the rate-limiting step in translation initiation (1). Rapamycin, a specific inhibitor of mTOR, also inhibits cellular translation initiation as a result of dephosphorylation of 4E-BP1 (12) and has been shown to decrease the translation of a subset of cellular mRNAs (42, 43). Treatment of NIH 3T3 cells with rapamycin caused a 2-fold reduction in global protein synthesis and a 42% reduction in cap-dependent translation (12).

Furthermore, treatment of Swiss mouse 3T3 cells with rapamycin caused a shift of some mRNAs, particularly those encoding elongation factors and ribosomal proteins, from large to small polysomes and monosomes (44). Rapamycin treatment also caused a similar increase in 80S ribosomes as we observed with metformin (45).

AMPK activation can lead to an increase in eukaryotic elongation factor 2 phosphorylation, which inhibits the translocation step of elongation (46). However, the mRNA shift in the polysome profile of metformin-treated cells indicates that translation initiation is the primary target of this drug. In addition, metformin did not affect HCV-driven translation of a firefly reporter cistron (Fig. 5*C*) but inhibited cap-dependent translation of a Renilla reporter (Fig. 5*B*). Therefore, it is unlikely that the effects of metformin on translation were due to a reduction in elongation.

Translational inhibition by metformin is dependent on the tumor suppressor LKB1 (Fig. 6). Treatment of MCF-7 cells with metformin led to an increase in the phosphorylation of Thr^{172} on AMPK, whereas no increase in phosphorylation was observed in MDA-MB-231 cells that do not express LKB1 mRNA or protein (37). These results support previous studies showing that LKB1 is required for metformin-mediated AMPK activation (20, 25, 33).

AMPK mediates its inhibitory effect on mTOR through TSC2. In contrast, it was also reported that AMPK can inhibit mTOR directly through phosphorylation on Thr^{2446} after activation with AICAR, dinitrophenol, or nutrient deprivation (47). However, our results clearly show that the regulation of mTOR via TSC2 is dominant over any potential direct effects that AMPK may have on mTOR because metformin failed to inhibit translation in $TSC2^{-/-}$ MEFs. The resistance of $TSC2^{-/-}$ MEFs to metformin could not be explained by a failure of these cells to respond to the drug, because AMPK was activated in response to metformin treatment as indicated by an increase in phosphorylation on Thr^{172}. These results show that TSC2 is the sole mediator of AMPK inhibitory activity of mTOR signaling and mRNA translation (Fig. 6).

Mutation or inactivation of LKB1 or TSC1/TSC2 leads to the development of Peutz-Jeghers syndrome (PJS) and TSC, respectively. TSC is characterized by the formation of hamartomas in a variety of tissues and an increased risk for the development of brain, skin, and renal cancer (10, 29). In addition, loss of TSC2 can lead to dysregulation of hypoxia inducible factor-1α and increased angiogenesis, which plays a critical role in tumor progression (10). PJS is characterized by gastrointestinal hamartomatous polyps and a predisposition to cancers of the colon and breast due to the importance of LKB1 in the regulation of epithelial cell polarity (37, 48). Because metformin failed to inhibit translation in cells lacking LKB1 or TSC2, it is unlikely that metformin will inhibit the growth of such cancers. However, these tumors, particularly those which lack LKB1, may respond to other AMPK activators, such as AICAR, because AICAR activates AMPK and inhibits the growth of $LKB1^{-/-}$ MEFs (31).

Metformin has previously been reported to cause an inhibition of protein synthesis in cardiac myocytes stimulated with phenylephrine (49). Our study shows that metformin inhibits the initiation step in translation via mTOR, a major regulator of cellular growth and proliferation. Furthermore, we show that metformin requires two major tumor suppressors, LKB1 and TSC2, to mediate its effects on translation (Fig. 6). We also provide an analysis of the activity of metformin in the context of cancer as opposed to the tissues involved in insulin signaling and metabolism.

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway that regulates mTOR is dysregulated in a large number of human cancers, which leads to an increase in mTOR activity resulting in enhanced mRNA translation and increased cellular proliferation. As a result, the PI3K/Akt/mTOR signaling pathway is a prime target for anticancer therapies. The inhibition of translation via

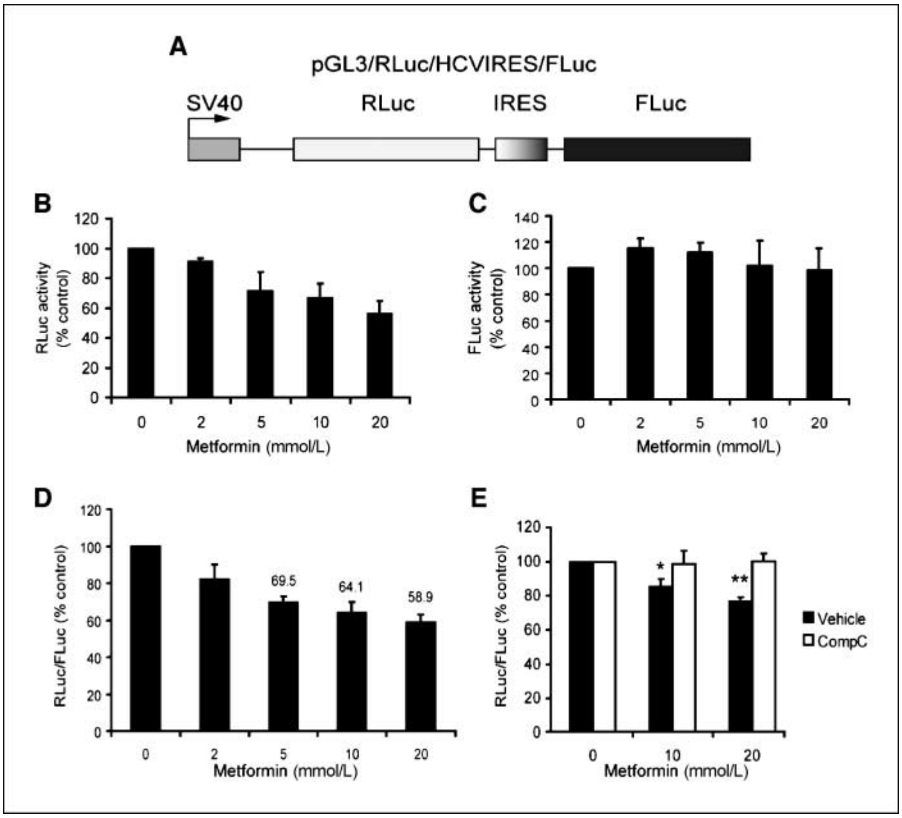

Figure 5. Inhibition of cap-dependent translation by metformin. *A,* a diagram of the pGL3/Rluc/HCVIRES/Fluc bicistronic reporter construct. *B* and *C,* MCF-7 cells were transfected with the bicistronic reporter construct and treated with increasing doses of metformin. After 24 h, cell lysates were prepared and assayed for Renilla (*B*) and firefly luciferase (*C*) activity; values are expressed as a percentage of the control (vector alone). *D,* luciferase activity values are expressed as Rluc/Fluc ratios and as a percentage of the control (vector alone, no metformin). Renilla luciferase activity averaged 9.0×10^4 relative light units, and firefly luciferase activity averaged 1.7×10^4 relative light units for control samples (vector alone, no metformin). For samples treated with 20 mmol/L metformin, Renilla luciferase activity averaged 5.2×10^4 relative light units, and firefly luciferase activity averaged 1.65×10^4 relative light units. *Columns,* average of four separate replicates; *bars,* SD from the mean. *E,* MCF-7 cells were transfected with the bicistronic reporter and incubated for 18 h. After 18 h, cells were pretreated with 20 μmol/L compound C or vehicle (DMSO) for 30 min, followed by treatment with metformin for 7 h. Cell lysates were then prepared and assayed for luciferase activity. Values are expressed as Rluc/Fluc ratios and as a percentage of the control (vector alone, plus compound C or vehicle). *Columns,* average of three separate replicates; *bars,* SD from the mean. Difference between compound C plus 10 mmol/L metformin and vehicle plus 10 mmol/L metformin was significant; *, $P < 0.05$. Difference between compound C plus 20 mmol/L metformin and vehicle plus 20 mmol/L metformin was significant; **, $P < 0.01$.

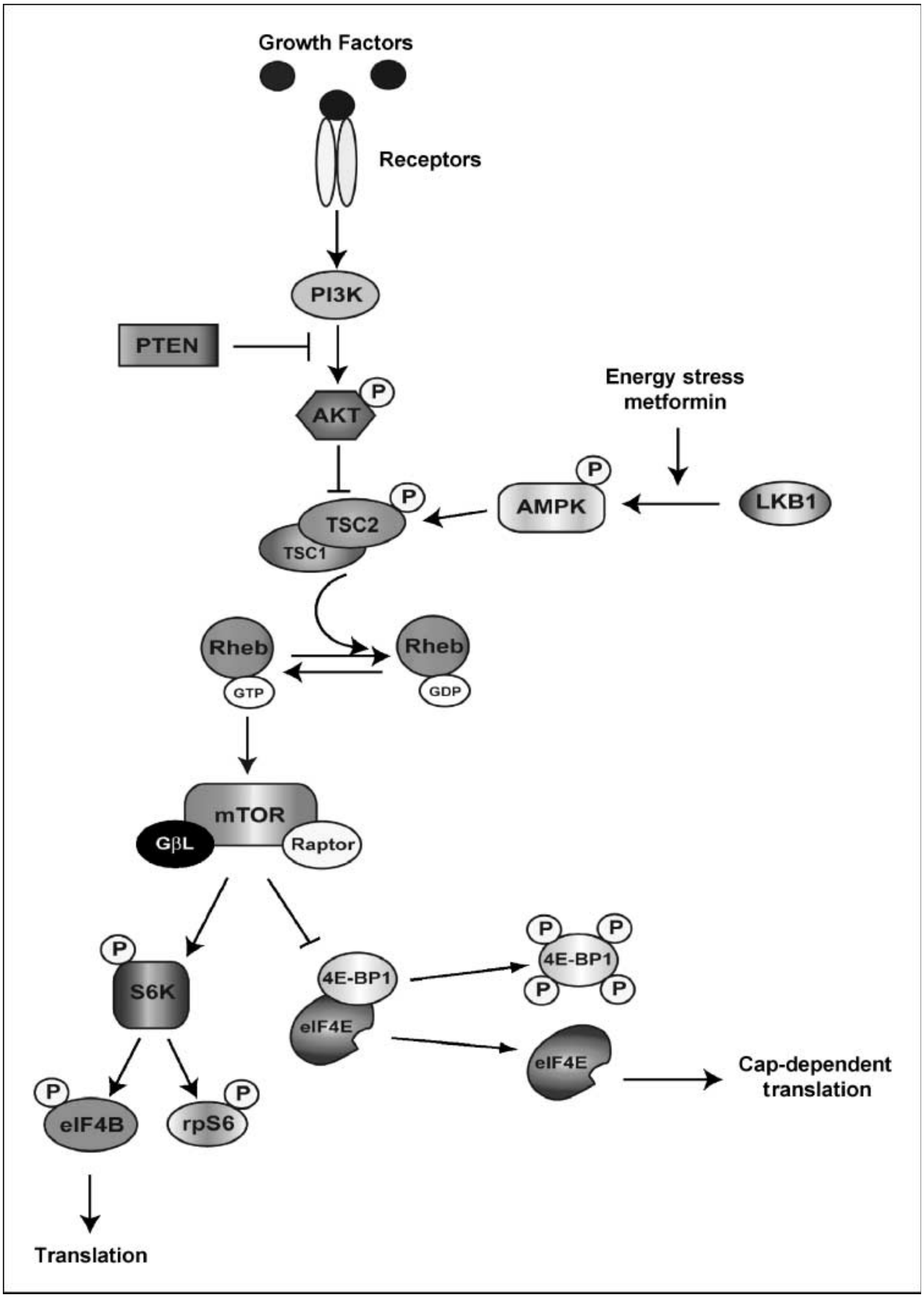

Figure 6. Regulation of mTOR signaling by LKB1 and AMPK. Metformin activates AMPK via LKB1, leading to the phosphorylation of TSC2. The activated TSC1/TSC2 complex exerts GAP activity toward the small GTPase Rheb, converting it to its GDP-bound form that is not able to activate mTOR. Consequently, mTOR signaling is reduced, leading to the dephosphorylation of S6K and 4E-BP1, and decreased mRNA translation initiation. In the absence of LKB1 or TSC2, metformin has no effect on mTOR, and translation continues unabated.

AMPK activation and mTOR inhibition represents a mechanism of action for the reduction of cancer cell growth by metformin and potentially other AMPK activators.

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