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mTORC1 Induces Purine Synthesis Through Control of the Mitochondrial Tetrahydrofolate Cycle

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Abstract

In response to growth signals, mTOR complex 1 (mTORC1) stimulates anabolic processes underlying cell growth. We found that mTORC1 increases metabolic flux through the *de novo* purine synthesis pathway in various mouse and human cells, thereby influencing the nucleotide pool available for nucleic acid synthesis. mTORC1 had transcriptional effects on multiple enzymes contributing to purine synthesis, with expression of the mitochondrial tetrahydrofolate (mTHF) cycle enzyme methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) being closely associated with mTORC1 signaling in both normal and cancer cells. *MTHFD2* expression and purine synthesis were stimulated by ATF4, which was activated by mTORC1 independent of its canonical induction downstream of eIF2 α phosphorylation. Thus, mTORC1 stimulates the mTHF cycle, which contributes one-carbon units to enhance production of purine nucleotides in response to growth signals.

The mTORC1 kinase integrates diverse growth signals to control nutrient-consuming biosynthetic processes, such as protein and lipid synthesis (1). mTORC1 also acutely stimulates the *de novo* synthesis of pyrimidine nucleotides through a posttranslational mechanism leading to increased intracellular pools of pyrimidines available for RNA and DNA synthesis (2, 3). Whether mTORC1 also influences the synthesis of purine nucleotides is unknown. Purines are enzymatically assembled on a 5-phosphoribosyl pyrophosphate (PRPP) molecule derived from the pentose phosphate pathway, with carbon and nitrogen moieties donated by non-essential amino acids and one carbon formyl units from the tetrahydrofolate (THF) cycle (Fig 1A).

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All authors reviewed manuscript and declare no competing financial interests.

To determine whether mTORC1 signaling affects *de novo* purine synthesis, we used targeted tandem mass spectrometry (LC-MS/MS) to measure relative flux of stable isotope-labeled glutamine (amide-¹⁵N), which is incorporated into the purine ring at two positions (Fig. 1A). mTORC1 activation in response to both genetic (*tuberous sclerosis complex 2* (*Tsc2*) null mouse embryo fibroblasts (MEFs)) or physiologic (insulin treatment of wild-type MEFs) stimuli resulted in increased amounts of doubly-labeled ¹⁵N-purine intermediates, and this labeling was attenuated in cells treated for 12 to 16 hours with the mTORC1 inhibitor rapamycin (Fig. 1B,D and fig. S1A-E). Unlike *de novo* pyrimidine synthesis, measured in the same metabolite extracts as the intermediate ¹⁵N-carbamoyl-aspartate (Fig 1C,E and fig. S1C)(2, 3), a shorter one-hour stimulation with insulin or treatment with rapamycin failed to respectively increase or decrease purine flux (Fig. 1B,D and fig. S1B,D). Similar results were obtained when flux from ¹³C-glycine into purine intermediates was measured (fig. S1F). mTORC1 activation through either loss of *Tsc2* or stimulation of cells with insulin increased flux through *de novo* purine synthesis into nucleic acids, as measured by ¹⁴C-glycine incorporation into RNA and DNA, without pronounced effects on the incorporation of an exogenously provided purine base (³H-adenine) (Fig. 1F,G and fig. S1G-J). Likewise, rapamycin decreased ¹⁴C-glycine flux into RNA in primary mouse hepatocytes and a panel of human cell lines (Fig. 1H).

The delayed timing of the respective inhibitory and stimulatory effects of rapamycin and insulin on purine synthesis, relative to that of pyrimidine synthesis (2, 3), suggested that mTORC1 might regulate this pathway through transcriptional mechanisms. Transcripts for specific enzymes within the purine pathway or essential supporting pathways, including the pentose phosphate pathway, serine synthesis, and the THF cycle (fig. S2A), were increased in *Tsc2*-deficient cells and sensitive to rapamycin treatment (Fig. 2A and fig. S2B-D). Amongst these genes, *methylenetetrahydrofolate dehydrogenase 2* (*Mthfd2*), encoding an enzyme in the mTHF cycle, showed the greatest mTORC1-dependent induction. Of those genes altered by mTORC1 signaling, *Mthfd2* was among the few that also showed corresponding changes in protein abundance, which were sensitive to both rapamycin and the mTOR kinase inhibitor Torin 1 (Fig 2B). MTHFD2 was reduced in cells treated with rapamycin for 8 h (fig. S3A), which was also sufficient to reduce *de novo* purine synthesis in these cells (Fig. 1F).

Expression of MTHFD2 was broadly regulated by mTORC1 signaling in distinct settings. Insulin increased MTHFD2 mRNA and protein in a rapamycin-sensitive manner in wild-type MEFs (fig. S3B,C), and these were also decreased by rapamycin in primary mouse hepatocytes and various human cancer cell lines (Fig. 2C and fig. S3D). MTHFD2 is the most highly overexpressed metabolic enzyme in human cancers (4). Our data suggest that mTORC1, which is frequently activated in cancer (5), might contribute to increased MTHFD2 expression in tumors.

In 859 human breast cancer samples (6) elevated mTORC1 signaling, as scored by the abundance of phospho-S6, was associated with increased expression of *MTHFD2* and other mTHF cycle genes and, to a lesser extent, enzymes of the serine synthesis pathway. mTORC1 activation did not correlate with expression of cytosolic THF cycle genes (fig. S3E-G).

The cytosolic and mitochondrial THF cycles produce one-carbon formyl groups for various cellular processes, including *de novo* purine synthesis (Fig. 2D and fig. S2A) (7-11). To determine whether the mTORC1-mediated induction of MTHFD2 contributes to purine synthesis, we measured the effects of siRNA-mediated depletion of MTHFD2 on flux from ^{15}N -glutamine into purine intermediates. Indeed, MTHFD2 depletion lowered flux through *de novo* purine synthesis without affecting mTORC1 signaling (Fig. 2E and fig. S3H,I). Formate produced by the mTHF cycle can exit the mitochondria and be converted to the one-carbon donor N^{10} -formyl THF in the cytosol (Fig. 2D and fig. S2A). *Tsc2* null cells with activated mTORC1 displayed enhanced incorporation into RNA of radiolabeled carbon from multiple substrates specific for purine synthesis, including ^{14}C -serine, ^{14}C -glycine and ^{14}C -formate. Depletion of MTHFD2 decreased the incorporation from ^{14}C -serine and ^{14}C -glycine but did not influence incorporation of ^{14}C -formate (Fig. 2F and fig. S4A). These data suggest that MTHFD2 is required for the mTORC1-induced increase in purine synthesis because it increases formate production, with exogenous formate bypassing the need for this enzyme. This is further supported by the fact that MTHFD2 depletion consistently slowed proliferation in *Tsc2*^{-/-} MEFs, without substantial effects on cell size, and exogenous formate attenuated this effect (Fig. 2I and fig. S4B,C). MTHFD2 depletion also blocked ^{14}C -glycine incorporation into RNA in 5 human cell lines, and similar to mTORC1 depletion with Raptor siRNAs, loss of MTHFD2 slowed proliferation of these cells (fig. S4D,E).

We also measured effects of candidate transcription factors downstream of mTORC1 (12-15) on incorporation of ^{14}C -glycine into RNA and DNA. Of the six transcription factors tested, depletion of the sterol regulatory element binding proteins (SREBP1 and 2), c-Myc, or activating transcription factor 4 (ATF4) specifically attenuated the increase in *de novo* purine synthesis in *Tsc2*-deficient cells, without decreasing incorporation of ^3H -adenine (Fig. 3A and fig. S5A). Of these three transcription factors, only depletion of ATF4 decreased MTHFD2 transcripts and protein in *Tsc2*-deficient cells (Fig. 3B,C) and abolished MTHFD2 protein in wild-type MEFs stimulated with insulin and in HEK293E cells (fig. S5B,C). ATF4 depletion also decreased mRNA for another mTHF gene (*Shmt2*), as well as genes of the serine synthesis pathway (*Psat1* and *Psph*), with lesser effects on their protein products (Fig. 3C and fig. S5D) (16, 17). Consistent with the role of these enzymes in *de novo* purine synthesis, ATF4 depletion blocked the mTORC1-induced increase in flux from ^{15}N -glutamine into purine intermediates (fig. S5E).

Overexpression of an ATF4 cDNA lacking its 5'-untranslated region increased the abundance of MTHFD2 mRNA and protein and rendered its expression resistant to rapamycin (Fig. 3D,E), without effects on the cytosolic isoform, MTHFD1 (fig. S5F). Alignment of the human, mouse, and rat *MTHFD2* promoters revealed a highly conserved, consensus DNA-binding motif for ATF4 (fig. S5G). Chromatin immunoprecipitation (ChIP) assays demonstrated that ATF4 bound to the *MTHFD2* promoter, and ATF4 expression increased Pol II binding to this promoter (Fig. 3F). As controls, ATF4 bound the promoter of its established target *CHOP* but not *GAPDH*. Like loss of MTHFD2, depletion of ATF4 decreased flux from ^{14}C -glycine into RNA, but failed to block incorporation of ^{14}C -formate (Fig. 3G and fig. S5H). Thus, MTHFD2 is a transcriptional target of ATF4, and ATF4

promotes *de novo* purine synthesis, at least in part, through its regulation of formate production by the mTHF cycle.

ATF4 DNA-binding elements are overrepresented in the promoters of mTORC1-regulated genes (12), suggesting that mTORC1 might activate ATF4. *Tsc2*-deficient cells had increased ATF4 that was lowered by 2 to 4 hours of rapamycin treatment (Fig. 4A). Loss of TSC2 function in mouse neurons, through conditional deletion of exon 3 (18), activated mTORC1 signaling and increased both ATF4 and its target MTHFD2 in the brain (Fig. 4B). Treatment of a panel of cancer cell lines, primary mouse hepatocytes, or HEK293E cells with rapamycin for 4 hours or less decreased ATF4 (Fig. 4C and fig. S6A,B). The shorter-term effects of mTORC1 inhibition with rapamycin or activation with insulin did not correspond with changes in ATF4 transcripts (fig. S6B,C), but prolonged stimulation or inhibition of mTORC1 caused transcriptional changes (fig. S6D) (16). Treatment of cells with the proteasome inhibitor MG132 increased ATF4 but did not affect its regulation by mTORC1 (Fig. 4D). Therefore, the primary mechanism of regulation of ATF4 by mTORC1 is neither through transcription nor stability, nor does it appear to influence nuclear shuttling of ATF4 (fig. S6E). The insulin-stimulated increase in ATF4 was blocked in cells upon inhibition of translation with cycloheximide (Fig. 4E), but was unaffected by inhibition of transcription with actinomycin D (fig S6F). Thus, mTORC1 appears to increase ATF4 abundance by promoting its translation.

The translation of ATF4 is well established to be regulated downstream of eIF2 α phosphorylation on Ser51 in response to cellular stresses, including amino acid deprivation and endoplasmic reticulum (ER) stress (19-21). Unlike growth factor-induced ATF4, the upregulation of ATF4 upon amino acid starvation or exposure to the ER stress-inducing agent tunicamycin was largely resistant to rapamycin (Fig. 4F). The induction of ATF4 by tunicamycin was abolished in a Ser51Ala knock-in mutant of eIF2 α (A/A) (21), but the insulin-stimulated, rapamycin-sensitive increase in ATF4 was similar between these cells and their wild-type (S/S) counterparts (Fig. 4G). These data reveal a regulatory input from mTORC1 to ATF4 that is independent of its established mechanism of regulation by cellular stress.

This study adds purine synthesis to the list of anabolic processes induced by mTORC1 in both normal and cancer cells (1). In response to growth signals, mTORC1 activates ATF4, which stimulates expression of MTHFD2 and other enzymes of the serine synthesis and mTHF cycle, thereby increasing production of formyl units required for *de novo* purine synthesis (Fig. 4H).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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CA197459 (B.D.M.). I.B.-S. and G.H. conceived, performed, and analyzed all experiments, and prepared manuscript. S.J.H.R performed TCGA analysis. J.M.A. performed LC-MS/MS experiments. B.D.M. directed research, reviewed all data, and prepared manuscript.

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One Sentence Summary

mTORC1 signaling stimulates an increase in *de novo* purine synthesis through an ATF4-dependent transcriptional induction of the mitochondrial tetrahydrofolate cycle.

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Fig. 1. mTORC1 stimulates *de novo* purine synthesis

(A) Schematic of the *de novo* purine synthesis pathway.

(B,C) Normalized peak areas of ¹⁵N-labeled intermediates of purine (B) and pyrimidine (C) synthesis, measured by targeted LC-MS/MS, from serum-deprived Tsc2^{+/+} and Tsc2^{-/-} MEFs treated with vehicle or rapamycin (20 nM) for 1 hour or 12 hours and labeled (20 min) with ¹⁵N-glutamine.

(D,E) Metabolite abundance from wild-type MEFs treated as in (B,C), but stimulated with insulin (500 nM) for 1 hour or 16 hours.

(F,G) Relative incorporation of radiolabel from ¹⁴C-glycine or ³H-adenine into RNA and DNA from serum-deprived Tsc2^{+/+} and Tsc2^{-/-} MEFs treated with vehicle or rapamycin (8 hours, 20 nM) (F) or wild-type MEFs stimulated with insulin (6 hours, 100 nM) with or without rapamycin.

(H) The given cells were labeled as in (F).

(B-H) Data presented as mean ± S.D. of biological triplicates and are representative of at least two independent experiments. *P<0.05 by two-tailed Student's t test.

Fig. 2. MTHFD2 is induced downstream of mTORC1 and is required for *de novo* purine synthesis

(A) Heat map of relative gene expression in serum-deprived MEFs treated 15 hours with vehicle or rapamycin (20 nM). Transcripts listed from highest to lowest fold increase in *Tsc2*^{-/-} relative to *Tsc2*^{+/+} MEFs for each category.

(B) Immunoblots from cells treated as in (A), but also with Torin 1 (250 nM) treatment.

(C) MTHFD2 transcript (graphs) and protein (immunoblots) abundance in the given cell lines treated with vehicle or rapamycin (20 nM, 16 hours).

(D) Schematic of serine synthesis, cytosolic and mitochondrial THF pathways, and their relation to *de novo* purine synthesis.

(E) Normalized peak areas of ¹⁵N-labeled purine intermediates, measured by targeted LC-MS/MS, from *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs 48 hours after transfection with *Mthfd2* siRNAs or non-targeting controls (siCtl) and labeled (20 min) with ¹⁵N-glutamine.

(F) Relative incorporation of radiolabel from ¹⁴C-serine, ¹⁴C-glycine, or ¹⁴C-formate (8 hour labeling) into RNA from *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs treated as in (E).

(G) Relative cell number 96 hours after transfecting *Tsc2*^{-/-} MEFs as in (E), with growth in low (1%) serum with or without formate (1 mM) for final 60 hours.

(C,E,F,G) Data graphed as mean ± S.D. of biological triplicates and are representative of at least two independent experiments. *P<0.05 by two-tailed Student's t test.

Fig. 3. ATF4 is required for mTORC1 to induce MTHFD2 expression and purine synthesis

(A) Relative incorporation of radiolabel from ^{14}C -glycine or ^3H -adenine (8h labeling) into RNA and DNA from *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs transfected with the indicated siRNAs is shown relative to that from *Tsc2*^{+/+} MEFs with control siRNAs.

(B) Relative *Mthfd2* transcript amounts from cells transfected as in (A).

(C) Immunoblots of proteins in *Tsc2*^{-/-} MEFs transfected as in (A).

(D) Immunoblots from HEK293E cells expressing empty vector (Vec) or Flag-ATF4 (ATF4) treated, where indicated, with rapamycin (15h, 20nM). (C,D) Biological duplicates shown.

(E) *MTHFD2* transcript abundance from cells transfected as in (D). **P* < 0.05 by two-tailed Student's *t* test.

(F) Cells transfected as in (D) were subjected to ChIP with control IgG, anti-Flag, or anti-Pol II antibodies. Bound promoter regions for the given genes were quantified and shown normalized to control IgG.

(G) Relative incorporation of radiolabel from ^{14}C -glycine or ^{14}C -formate (8h labeling) into RNA from *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs transfected with the indicated siRNAs is shown as in (A).

(A,B,E-G) Data are mean $\pm$ S.D. of biological triplicates and are representative of at least two independent experiments.

Fig. 4. mTORC1 activates ATF4 independent of cellular stress responses

(A) ATF4 abundance in MEFs deprived of serum (15 hours) and treated with rapamycin (20 nM).

(B) Immunoblots of brain lysates from mice with neuron-specific deletion of *Tsc2* exon 3 (c Δ 3/c Δ 3) compared to wild-type.

(C) Amounts of ATF4 in cancer cell lines treated 4 hours with rapamycin (20 nM).

(D) Amounts of ATF4 in MEFs deprived of serum (15 hours), treated with vehicle or MG132 (2 μ M), with or without rapamycin (20 nM), for 30 min before insulin stimulation (4 hours).

(E) Immunoblots of proteins in MEFs treated with insulin (4 hours, 500 nM) and, for the final hour, cycloheximide (10 μ M) or rapamycin (20 nM).

(F) Immunoblots of proteins from MEFs grown in dialyzed serum and deprived of amino acids (6 hours) or treated with tunicamycin (6 hours, 2 μ g/ml) with or without rapamycin (20 nM).

(G) Immunoblots of proteins from eif2 α WT (S/S) or Ser51Ala knock-in (A/A) MEFs deprived of serum (16 hours), treated with rapamycin (30 min, 20 nM), then stimulated with insulin (4 hours, 500 nM). (Right) Cells grown in serum were treated with tunicamycin (4 hours, 2 μ g/ml). (A,B,D,G) Biological duplicates are shown.

(H) Model of findings. See text.