The mTORC1/S6K1 Pathway Regulates Glutamine Metabolism through the eIF4B-Dependent Control of c-Myc Translation

Alfredo Csibi,1,5,7 Gina Lee,1,6 Sang-Oh Yoon,1 Haoxuan Tong,3 Didem Ilter,1,5 Ilaria Elia,1,6 Sarah-Maria Fendt,4,5 Thomas M. Roberts,2 and John Blenis1,4,*

1Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA
2Department of Cancer Biology, Dana Farber Cancer Institute and Harvard Medical School, Boston, MA 02115, USA
3Department of Pharmacology, Meyer Cancer Center, Weill Cornell Medical College, New York, NY 10065, USA
4Flemish Institute of Biotechnology (VIB), Vesalius Research Center, Herestraat 49, 3000 Leuven, Belgium
5Department of Oncology, KU Leuven–University of Leuven, Herestraat 49, 3000 Leuven, Belgium
6Department of Oncology, KU Leuven–University of Leuven, Herestraat 49, 3000 Leuven, Belgium
7Correspondence: job2064@med.cornell.edu

Summary

Growth-promoting signaling molecules, including the mammalian target of rapamycin complex 1 (mTORC1), drive the metabolic reprogramming of cancer cells required to support their biosynthetic needs for rapid growth and proliferation [1]. Glutamine is catabolized to α-ketoglutarate (αKG), a tricarboxylic acid (TCA) cycle intermediate, through two deamination reactions, the first requiring glutaminase (GLS) to generate glutamate and the second occurring via GDH. Moreover, mTORC1 activation also stimulates the uptake of glutamine, but the mechanism is unknown [2]. Two different genes in distinct chromosomes code for mammalian GLS enzymes. The Gls1 gene codes for kidney-type isozymes, whereas the Gls2 gene encodes liver-type isozymes [4]. Only expression of Gls1, not Gls2, was detected in our cell system (Figure S1B). In addition, Gls1 mRNA levels were decreased upon rapamycin treatment in Tsc2+/− MEFS (Figure S1C). p53 has been shown recently to regulate Gls2 to drive glutamine metabolism. However, no effects on Gls1 have been described [5]. Consistently, the mTORC1-dependent regulation of GLS occurs independently of p53 (Figure S1D). A modulation of GLS levels by mTORC1 should also be reflected in the conversion of glutamine to glutamate. Rapamycin treatment increased the intracellular levels of glutamine (Figure 1F) [3]. Moreover, mTORC1 inhibition decreased glutamine flux in Tsc2−/− MEFS expressing an empty vector (EV) or TSC2 (Figure 1G).

The mTORC1 Pathway Regulates GLS1 via Myc

Oncogenic Myc has been shown to positively stimulate the expression of genes involved in glutamine metabolism [6]. Moreover, Myc has been shown to regulate GLS by repressing the transcription of miR-23a/b [7]. Consistent with this, the effective knockdown of Myc with two siRNAs (#25 and #26) in the human pancreatic cancer cell line BxPC3 correlated with reduced GLS protein levels (Figure 2A, lanes 5 and 6). Similar to GLS, Myc protein levels were dramatically higher with reduced GLS protein levels (Figure 2B). Finally, our data show that the pharmacological inhibition of GLS is a promising target in pancreatic cancers expressing low levels of PTEN.

Results and Discussion

The mTORC1 Pathway Regulates GLS1

mTORC1 positively regulates net glutamine flux into the tricarboxylic acid (TCA) cycle, therefore suggesting that glutaminase (GLS) is potentially regulated by mTORC1 [3]. To test this possibility, we assessed GLS protein levels in conditions of mTORC1 activation. We found that Tsc2 deficiency in mouse embryonic fibroblasts (MEFs) or the stable expression of Rheb wild-type (WT) or an active mutant (S16H) resulted in increased GLS protein levels that were reduced after mTORC1 inhibition (Figures 1A and 1B). Rapamycin potently decreased GLS levels after 6 hr of treatment (Figure 1C), consistent with reduced glutamine consumption at the same time point (Figure 1D). mTOR catalytic inhibitors tested, including LY294002 and BEZ235, also resulted in decreased GLS protein levels in MEFS (Figure 1E). Similar to mTOR inhibitors, the knockdown of components of the mTORC1 pathway, including raptor, Rheb, RagA, and mTOR itself, resulted in reduced GLS protein levels (Figure S1A available online).

Two different genes in distinct chromosomes code for mammalian GLS enzymes. The Gls1 gene codes for kidney-type isozymes, whereas the Gls2 gene encodes liver-type isozymes [4]. Only expression of Gls1, not Gls2, was detected in our cell system (Figure S1B). In addition, Gls1 mRNA levels were decreased upon rapamycin treatment in Tsc2+/− MEFS (Figure S1C). p53 has been shown recently to regulate Gls2 to drive glutamine metabolism. However, no effects on Gls1 have been described [5]. Consistently, the mTORC1-dependent regulation of GLS occurs independently of p53 (Figure S1D). A modulation of GLS levels by mTORC1 should also be reflected in the conversion of glutamine to glutamate. Rapamycin treatment increased the intracellular levels of glutamine (Figure 1F) [3]. Moreover, mTORC1 inhibition decreased glutamine flux in Tsc2−/− MEFS expressing an empty vector (EV) or TSC2 (Figure 1G).

The mTORC1 Substrate S6K1 Controls Myc and GLS

Although early studies have linked mTORC1 to Myc [8], the mechanistic details have not been described. We hypothesized that the mTORC1 downstream effector S6 Kinase 1 (S6K1) might modulate Myc. We found that inhibition of S6K1 with PF470861 (PF) or rapamycin resulted in decreased Myc transcriptional activity (Figure 3A). Moreover, expression of catalytically active S6K1 (S6K1-F5A/R3A/T389E) [9, 10] resulted in increased levels of both Myc and GLS and prevented a rapamycin-induced decrease in Myc and GLS levels (Figure 3B). The rapamycin-resistant S6K1 also significantly reduced a rapamycin-induced increase in intracellular glutamine...
levels (Figure 3C). In contrast, the knockdown of S6K1/2 or treatment with PF led to reduced levels of Myc and GLS (Figures S2A and S2B). PF treatment or S6K1/2 knockdown increased intracellular glutamine levels (Figure 3D; Figure S2C), which correlated with decreased glutamine uptake rates in S6K1/2-depleted cells (Figure 3E). Importantly, knockdown of Myc resulted in increased intracellular levels of glutamine that were comparable to those observed in cells after S6K inhibition (Figures S2D and S2E). Collectively, these show the biological significance of the S6K1/Myc-mediated regulation of GLS and glutamine metabolism.

Myc Is Regulated by S6K1 through eIF4B
Rapamycin treatment represses translation of Myc mRNA [11], which contains a secondary structure in its 5’ untranslated region (5’UTR) [12]. Consistent with this, treatment with the translation inhibitor cycloheximide decreased the Myc protein level, which was comparable with rapamycin treatment for 24 hr (Figure S3A). S6K1 promotes the translation of mRNAs with highly structured 5’UTRs [13], suggesting a potential regulation of Myc mRNA translation. To assess this possibility, we used a luciferase reporter containing the sequence of the 5’UTR of Myc. We found rapamycin and PF to significantly
S6K1 Regulates Glutaminase

Inhibition of GLS Reduces the Growth of Pancreatic Cancer Cells

Recent studies have demonstrated a major role for glutamine in supporting cancer cell metabolism, suggesting that the mTORC1-dependent regulation of GLS may be relevant for cancer cells. We measured GLS and Myc levels in three pancreatic cancer tumor cell lines, BxPC3, MIAPaCa-2, and AsPC-1. Both BxPC3 and MIAPaCa-2 cells displayed a higher basal expression of GLS and Myc (Figure S3D; [14]), consistent with lower levels of PTEN [18]. Levels of both GLS and Myc were higher in BxPC3 and MIAPaCa-2 cells and were reduced upon mTORC1 inhibition with rapamycin or BEZ235 treatment (Figure 4A). Interestingly, BEZ235 effects on GLS were more pronounced in BxPC3 cells compared with MIAPaCa-2 cells (Figure 4A). Higher GLS levels correlated with increased glutamine consumption in BxPC3 cells compared with MIAPaCa-2 cells (Figure 4B). Given the importance of glutamine metabolism in driving the growth of cancer cells, we speculated that the inhibition of glutamine metabolism might reduce the growth of pancreatic cells with increased mTORC1 signaling. To test this idea, we used the GLS-1 inhibitor bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide 3 (BPTES) in an anchorage-independent growth assay. We found BPTES to significantly reduce the ability of BxPC3 cells to grow in soft agar but not to affect the growth of AsPC-1 cells (Figures 4C and 4D). The growth of pancreatic ductal adenocarcinoma has been shown recently to be sensitive to GLS-1 inhibition [19]. The addition of the TCA cycle intermediate oxaloacetate (OAA) was able to rescue BxPC3 cell growth upon GLS inhibition (Figure 4C). OAA has been shown to play an important role in pancreatic cancer cell growth by maintaining the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)/nicotinamide adenine dinucleotide phosphate (NADP⁺) ratio [19]. Consistently, we observed that the antioxidant N-acetylcysteine (NAC) rescued the decreased growth of BxPC3 cells cultured in the presence of BPTES (Figure 4D). Together, these data indicate that the mTORC1-mediated regulation of GLS is critical for glutamine anaplerosis, redox homeostasis, and pancreatic cancer cell growth.

In this study, we identified GLS as a downstream effector of mTORC1/S6K1 signaling involved in energy metabolism. We demonstrated that S6K1 positively regulates GLS by increasing the translation efficiency of the oncogene Myc mRNA. Molecularly, we showed that S6K1 regulates Myc by phosphorylating the initiation factor eIF4F (Figure 3). Our data extend our earlier model demonstrating that the phosphorylation of eIF4B promotes the association of eIF4B to eIF4A in the PIC [15]. This interaction enhances eIF4A helicase activity and Myc mRNA translation as a result of improving the ability of the PIC to transit to the translation start site and/or by exposing a possible internal ribosome entry site hidden within the structured 5’UTR [20, 21]. In this way, mTORC1/S6K1-regulated helicase stimulation is increasing the number of ribosomes engaged in Myc mRNA translation. Consistently, changes in Myc mRNA distribution, from heavier to lighter polysomes, by rapamycin or by eIF4B knockdown, were observed and are consistent with previous polysome profiles (Figure 3G; Figure S3D) [14, 17]. Although S6K1-mediated regulation of Myc protein stability has also been suggested [22, 23], we did not see a significant change in Myc level with MG132 treatment under the conditions used in our analysis (Figure S3E). In addition, exogenous Myc, which does not have the 5’UTR, was not decreased significantly by rapamycin (Figure 2C) compared with endogenous Myc with its 5’UTR (Figure 2B). Collectively, these data are consistent with the model that mTORC1/S6K1 controls Myc expression by modulating its mRNA translation initiation efficiency.

Previous studies have demonstrated that oncogenic Myc positively regulates GLS levels posttranscriptionally by repressing miR-23a/b expression [7]. Importantly, inhibition of mTORC1 by rapamycin increased miR-23a/b levels (Figure S3F), linking mTORC1 regulation of GLS expression through Myc-mediated miR-23a/b control. Interestingly, we observed the regulation of GLS expression by mTORC1/S6K1 both at the protein and mRNA levels. However, Gls transcription was not significantly affected by Myc knockdown (Figure S3G), which is consistent with previous work showing that Gls mRNA levels do not respond to alterations in Myc levels in P493-6 cells [7]. Therefore, although the Myc-mediated control of GLS expression plays a major role downstream of mTORC1, mTORC1 may have other mechanisms to regulate Gls mRNA levels.
Figure 3. The mTORC1 Substrate S6K1 Controls GLS through Myc mRNA Translation

(A) Normalized luciferase light units of *Tsc2*^−/−^ MEFs stably expressing a Myc-responsive firefly luciferase construct (Myc–Luc) or vector control (pCignal Lenti-TRE Reporter). Myc transcriptional activity was measured after treatment with rapamycin (20 ng/ml) or PF4708671 (10 μM) for 8 hr.

(B) GLS and Myc protein levels in whole cell lysates from HEK293T cells expressing HA-S6K1-CA (F5A-R3A-T389E) or EV treated with rapamycin (20 ng/ml) for 24 hr. HA, hemagglutinin.

(C and D) Intracellular glutamine levels of *Tsc2*^−/−^ MEFs stably expressing S6K-CA (F5A/R5A/T389E, mutating either the three arginines or all residues within the RSPRR motif to alanines shows the same effect; [10]) or empty vector and treated with rapamycin (20 ng/ml) or DMSO for 48 hr (C) or transfected with NTC siRNA or siRNA against both S6K1/2 (D). 24 hr posttransfection, cells transfected with NTC siRNA were treated with PF4708671 (10 μM) or DMSO for 48 hr.

(E) Glutamine consumption of *Tsc2*^−/−^ MEFs transfected with NTC siRNA or siRNA against both S6K1/2. 72 hr posttransfection, media were collected, and levels of glutamine in the media were determined.

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Through the regulation of GLS, S6K1 is directly linked, for the first time, to glutamine uptake and metabolism. Interestingly, two recent studies demonstrated that S6K1 controls the synthesis of nucleotides [24, 25], a process that requires glutamine-derived nitrogen. Therefore, the combination of these studies and the data presented here reveal the existence of a positive feedback mechanism through which mTORC1/S6K1 signaling promotes glutamine uptake via GLS, providing building blocks to create the genetic material required for cell growth. Besides providing nitrogen for protein and nucleotide synthesis, glutamine serves as fuel for growth and proliferation [26]. Interestingly, mTORC1 also stimulates anaplerotic entry of glutamine-derived α-ketoglutarate to the TCA cycle via glutamate dehydrogenase [3], demonstrating that the activation of mTORC1 is involved in every aspect of glutamine anaplerosis. Increasing evidence supports a major role of glutamine metabolism in driving tumor growth. For example, the knockdown or inhibition of GLS impairs growth of multiple cancer cells, including prostate, glioma, lymphoma, and pancreatic cancer cells [7, 19, 27, 28]. Along these lines, we observed reduced anchorage-independent growth of pancreatic tumor cells expressing higher levels of GLS as a result of mTORC1 hyperactivation upon GLS-1 inhibition with BPTES. This finding may have significant therapeutic implications, given that clinical-grade GLS inhibitors are being developed [29] and because increased glutamine metabolism is not critical for normally differentiated cells. The use of GLS inhibitors may provide greater efficacy with fewer and less toxic side effects.

**Experimental Procedures**

**Cell Lines and Culture**

Tsc2+/−, p53+/−, Tsc2 WT, p53−/−, Tsc1−/−, p53−/−, and Tsc1−/− p53+/− MEFs were provided by Drs. Brendan Manning and David Kwiatkowski (Harvard Medical School). All the other cell lines (HT-29, BxPC3, MIAPaCa-2, and HEK293T) were obtained from the ATCC. MEFs, HT-29, and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), BxPC3, MIAPaCa-2, and AsPC-1 cells were cultured in RPMI medium (Mediatech). DMEM or RPMI medium were supplemented with 10% fetal bovine serum (FBS) (dialyzed for deprivation experiments, Gibco). All extra energetic additives that are often added to some DMEM formulations, such as sodium pyruvate and succinate, were excluded.

**Cell Lysis and Immunoblotting**

Cells washed once with cold PBS were solubilized on ice either in a regular lysis buffer (40 mM HEPES [pH 7.4], 1 mM EDTA, 120 mM NaCl, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na3VO4, and 0.3% 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) or in a low-salt lysis buffer (40 mM HEPES [pH 7.4], 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na3VO4, and 0.3% CHAPS) supplemented with protease inhibitors (250 μM PMSF, 5 μg/ml Pepstatin A, 10 μg/ml Leupeptin, and 5 μg/ml Aprotinin). Cleared cell lysates were obtained by centrifugation at 10,000 rpm for 10 min at 4°C, and 30 μg of the lysates was used for immunoblotting. In brief, proteins were resolved by 8%–12% SDS-PAGE, followed by transfer onto a nitrocellulose membrane. Primary antibodies were incubated overnight at 4°C. Infrared fluorescent IRDye secondary antibodies were used to develop band intensities in the LI-COR

(F) Normalized luciferase light units of Tsc2 WT MEFS transfected with the pDL-N reporter construct containing the 5’ UTR of Myc under the control of Renilla luciferase. Firefly luciferase was used as an internal control. 48 hr posttransfection, cells were treated with rapamycin (20 ng/ml) or PF4708671 (10 μM) for 8 hr.

(G) Relative levels of Myc, Glu, and Actin mRNA in each polysomal gradient fraction. mRNA levels were measured by quantitative PCR and normalized to the 5S rRNA level. HEK293T cells were treated with rapamycin (20 ng/ml) for 24 hr, and polysomes were fractionated on sucrose density gradients. The values are averaged from two independent experiments performed in duplicate, and the error bars denote SEM (n = 4).

(H and I) GLS and Myc protein levels in whole cell lysates from Tsc2+/− MEFS transfected with NTC siRNA or two independent siRNAs against elf4B for 72 hr (H) and Tsc2−/− MEFs stably expressing elf4B WT, mutant S422D, or E1V and treated with rapamycin for 24 hr (I). The mean is shown. Error bars represent the SEM from at least three biological replicates. The asterisk denotes a nonspecific band. The numbers below the immunoblot image represent quantification normalized to the loading control. See also Figures S2 and S3.
Biosciences/Odyssey system. Band intensities were quantified using Adobe Photoshop CS3 extended software.

Glutamine Consumption and Glutamine Flux
Glutamine concentrations were measured in fresh and spent medium (after 24–48 hr of culture in the presence or absence of drugs) using a Yellow Springs Instruments 7100. Glutamine levels were normalized to cell number. The media used for these experiments did not contain pyruvate and were supplemented with 10% dialyzed FBS. Net glutamine flux was calculated from glutamine uptake rates and glutamate secretion rates.

Statistics
Data were expressed as average ± SEM of at least three independent experiments performed in triplicates. An unpaired, two-tailed Student’s t test was used to determine differences between two groups.

Supplemental Information
Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.08.007.

Author Contributions
A.C., G.L., and J.B. conceived and designed the functional studies. A.C. and G.L. performed the cellular experiments. S.O.Y. helped develop the poly-U translation assay. A.C., G.L., and J.B. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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