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The mTORC1/S6K1 pathway regulates glutamine metabolism through the eIF4B-dependent control of *c-Myc* translation

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Abstract

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 catabolyzed to α-ketoglutarate (αKG), a TCA cycle intermediate, through two deamination reactions, the first requiring glutaminase (GLS) to generate glutamate, and the second reaction occurring via glutamate dehydrogenase (GDH) or transaminases [2]. Activation of the mTORC1 pathway was previously shown to promote the anaplerotic entry of glutamine to the tricarboxylic acid (TCA) cycle via GDH. Moreover, mTORC1 activation also stimulates the uptake of glutamine, but the mechanism is unknown [3]. It is generally thought that rates of glutamine utilization are limited by mitochondrial uptake via GLS, suggesting that in addition to GDH, mTORC1 could regulate GLS. Here, we demonstrate that mTORC1 positively regulates GLS and flux through this enzyme. We show that mTORC1 controls GLS levels through the S6K1-dependent regulation of c-Myc (Myc). Molecularly, S6K1 enhances Myc translation efficiency by modulating the phosphorylation of eukaryotic initiation factor eIF4B, which is critical to unwind its structured 5' untranslated region (5'UTR). Finally, our data show that the pharmacological inhibition of GLS is a promising target in pancreatic cancers expressing low levels of PTEN.

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Results and Discussion

The mTORC1 pathway regulates GLS1

mTORC1 positively regulates net glutamine flux into the TCA cycle thus suggesting that 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 WT or an active mutant (S16H) resulted in increased GLS protein levels that were reduced after mTORC1 inhibition (Figure 1A & 1B). Rapamycin potently decreased GLS levels after 6h 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).

Two different genes in distinct chromosomes code for mammalian GLS enzymes: the *Gls1* gene codes for kidney-type (K-type) isozymes, whereas the *Gls2* gene encodes liver-type (L-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 recently shown to regulate *Gls2* to drive glutamine metabolism, however no effects on *Gls1* were 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 was shown to positively stimulate the expression of genes involved in glutamine metabolism [6]. Moreover, Myc was 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 in *Tsc2*−/− MEFs compared to its WT counterpart, and were sensitive to rapamycin treatment (Figures 1A & 2B). These observations prompted us to test whether the modulation of GLS levels by mTORC1 occurs via Myc. Strikingly; we found the stable expression of Myc to abrogate the rapamycin-induced decrease of GLS (Figure 2C).

The mTORC1 substrate S6K1 controls Myc and GLS

Although early studies have linked mTORC1 to Myc [8], the mechanistic detail has 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 rapamycin-induced decrease in Myc and GLS levels (Figure 3B). The rapamycin-resistant S6K1 also significantly reduced 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 & S2B). PF-treatment or S6K1/2-knockdown increased intracellular glutamine levels (Figures 3D and 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 are comparable to those observed in cells after S6K inhibition (Figures S2D & 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' unstranslated region (5'UTR) [12]. Consistent with this, treatment of the translation inhibitor cycloheximide decreased Myc protein level, which is comparable to rapamycin treatment for 24 hrs. (Figure S3A). S6K1 promotes the translation of mRNAs with highly structured 5'UTR [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 decrease translation of *Myc* luciferase reporter while luciferase mRNA levels were not affected (Figures 3F and S3B). Rapamycin induced endogenous *Myc* mRNA to shift toward lighter polysomal fractions (Figure 3G) [14], demonstrating that *Myc* translation is decreased in conditions of mTORC1 inhibition. In contrast, distribution of *GLS* and *Actin* mRNAs, which do not contain highly structured 5'UTRs, were not affected by rapamycin treatment (Figure 3G).

S6K1-dependent phosphorylation of eIF4B on S422 results in increased association of eIF4B to eIF4A within the translation preinitiation complex [15] and subsequent enhancement of eIF4A helicase activity [16]. Importantly, the knockdown of either eIF4B or eIF4A resulted in reduced levels of GLS and Myc (Figures 3H and S3C). Consistently, upon overexpression of eIF4B, *Myc* mRNA moved toward heavier polysomal fractions, while knockdown of eIF4B resulted in *Myc* mRNA presence in lighter polysomal fractions (Figure S3D) [17]. To further evaluate the implication of S6K1/eIF4B on Myc, we used a phosphomimetic mutant of eIF4B (S422D). Strikingly, the mutation of this residue suppressed the rapamycin-induced decrease of GLS and Myc (Figure 3I).

Inhibition of GLS reduces the growth of pancreatic cancer cells

Recent studies have demonstrated a major role for glutamine on 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 displayed higher basal phosphorylation of S6 (Figure 4A), 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 (Figure 4A). Higher GLS levels correlated with increased glutamine consumption in BxPC3 cells compared to AsPC-1

cells (Figure 4B). Given the importance of glutamine metabolism in driving the growth of cancer cells, we speculated that 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 BPTES in an anchorage-independent growth assay. We found BPTES to significantly reduce the ability of BxPC3 cells to grow in soft agar while not affecting the growth of AsPc-1 cells (Figure 4C & 4D). The growth of pancreatic ductal adenocarcinoma has been recently shown 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 was shown to play an important role for pancreatic cancer cell growth by maintaining the NADPH/$NADP^+$ 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 controls GLS by increasing the translation efficiency of the oncogene Myc mRNA. Molecularly; we showed that S6K1 regulates Myc by phosphorylating the initiation factor eIF4B (Figure 3). Our data extend our earlier model demonstrating that the phosphorylation of eIF4B promotes the association of eIF4B to eIF4A in the translation preinitiation complex (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 (IRES) 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 (Figures 3G and S3D) [14,17]. Although S6K1-mediated regulation of Myc protein stability has also been suggested [22,23], we did not see significant change of Myc level by MG132 treatment under the conditions used in our analysis (Figure S3E). In addition, exogenous Myc; which does not have the 5'UTR, was not significantly decreased by rapamycin (Figure 2C) compared to 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 demonstrated that oncogenic Myc positively regulates GLS levels post-transcriptionally 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]. Thus, 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.

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. Thus, 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 αKG to the TCA cycle via GDH [3], demonstrating that the activation of mTORC1 is involved in every aspect of glutamine anaplerosis.

Increasing evidence supports the 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 cancers [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 normal 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 kindly provided by Drs. Brendan Manning and David Kwiatkowski (Harvard Medical School). All the other cell lines (HT-29, BxPC3, MIAPaCa-2, AsPC-1, HEK293T) were obtained from ATCC. MEFs, HT-29 and HEK293T were cultured in DMEM. BxPC3, MIAPaCa-2 and AsPC-1cells were cultured in RPMI media (Mediatech). DMEM or RPMI were supplemented with 10% 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 (40mM HEPES [pH 7.4], 1mM EDTA, 120mM NaCl, 10mM β-glycerophosphate, 1mM NaF, 1mM Na_3VO_4, and 0.3% CHAPS) or in a low-salt lysis buffer (40mM HEPES [pH 7.4], 1mM EDTA, 10mM β-glycerophosphate, 1mM NaF, 1mM Na_3VO_4, 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 10,000rpm for 10 min at 4°C and 30μg of the lysates were used for immunoblotting. In brief, proteins were resolved by 8-12% SDS-PAGE followed by transfer onto 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/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 to 48 hours of culture in the presence of absence of drugs) using a Yellow Springs Instruments (YSI) 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 ± standard error of the mean (SEM) of at least three independent experiments performed in triplicates. An unpaired, 2-tail student *t*-test was used to determine differences between two groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The mTORC1 pathway positively regulates GLS and glutamine flux.
- mTORC1 controls the translation efficiency of Myc mRNA.
- S6K1 regulates Myc translation through eIF4B phosphorylation.
- Inhibition of GLS decreases the growth of pancreatic cancer cells.

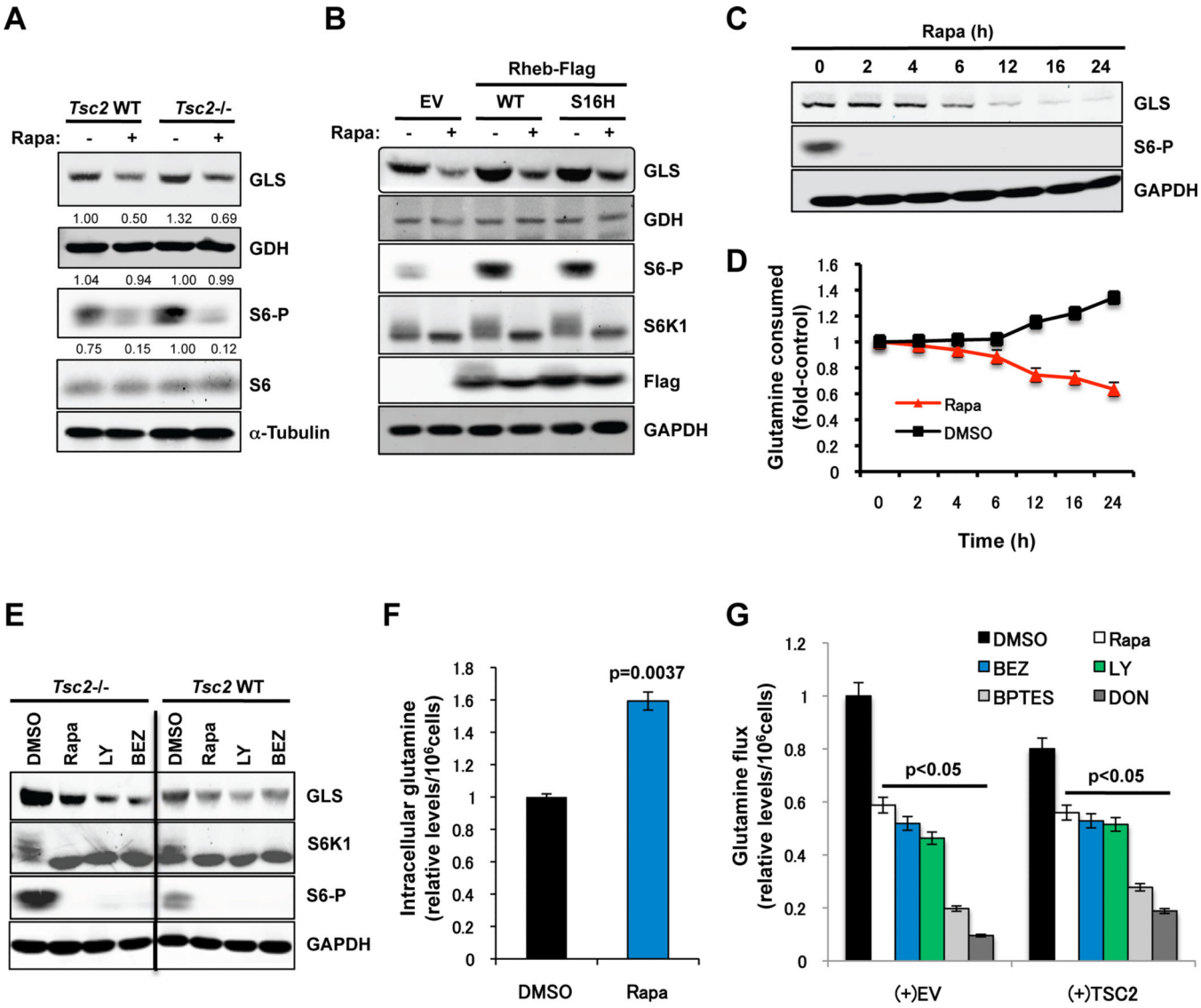

Figure 1. The mTORC1 pathway regulates GLS1

(A-C, E) GLS protein levels in whole cell lysates from: **(A)** *Tsc2* WT and *Tsc2*−/− MEFs treated with rapamycin for 8h; **(B)** HEK293T cells stably expressing Rheb WT, the mutant S16H Rheb or empty vector (EV) and treated with rapamycin for 24h; **(C)** *Tsc2*−/− MEFs treated with rapamycin at indicated time points; **(E)** *Tsc2* WT and *Tsc2*−/− MEFs treated with the indicated compounds for 8h. The concentrations of the compounds were: rapamycin 20ng/mL; LY294002 20μM, and BEZ235 10μM.

(D) Time course of glutamine consumption in *Tsc2*−/− MEFs incubated with or without 20ng/mL rapamycin for 24h. Each time data point is an average of triplicate experiments.

(F) Intracellular glutamine levels in *Tsc2*−/− MEFs treated with rapamycin for 24h.

(G) Glutamine flux in *Tsc2*−/− MEFs expressing an empty vector (EV) or re-expressing TSC2 treated with the indicated compounds for 24h. The concentrations of the compounds were: rapamycin 20ng/mL; LY294002 20μM, and BEZ235 10μM, BPTES 10μM, and DON 1mM.

The mean is shown; error bars represent SEM from at least three biological replicates. Numbers below the immunoblot image represent quantification normalized to the loading control.

See also Figure S1.

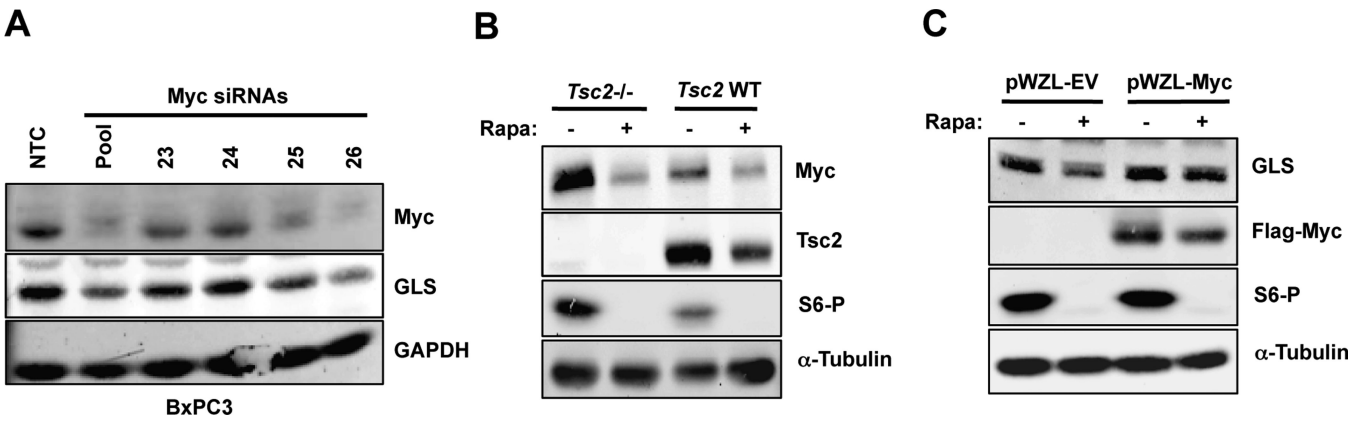

Figure 2. The mTORC1 pathway regulates GLS1 via Myc

(A-C) GLS and Myc protein levels in whole cell lysates from: **(A)** BxPC3 cells transfected with a non-targeting control siRNA (NTC) or four independent siRNAs against Myc for 72h; **(B)** *Tsc2* WT and *Tsc2*−/− MEFs treated with rapamycin 20ng/mL for 8h; **(C)** *Tsc2*−/− MEFs stably expressing Myc or empty vector (EV), and treated with rapamycin 20ng/mL for 24h.

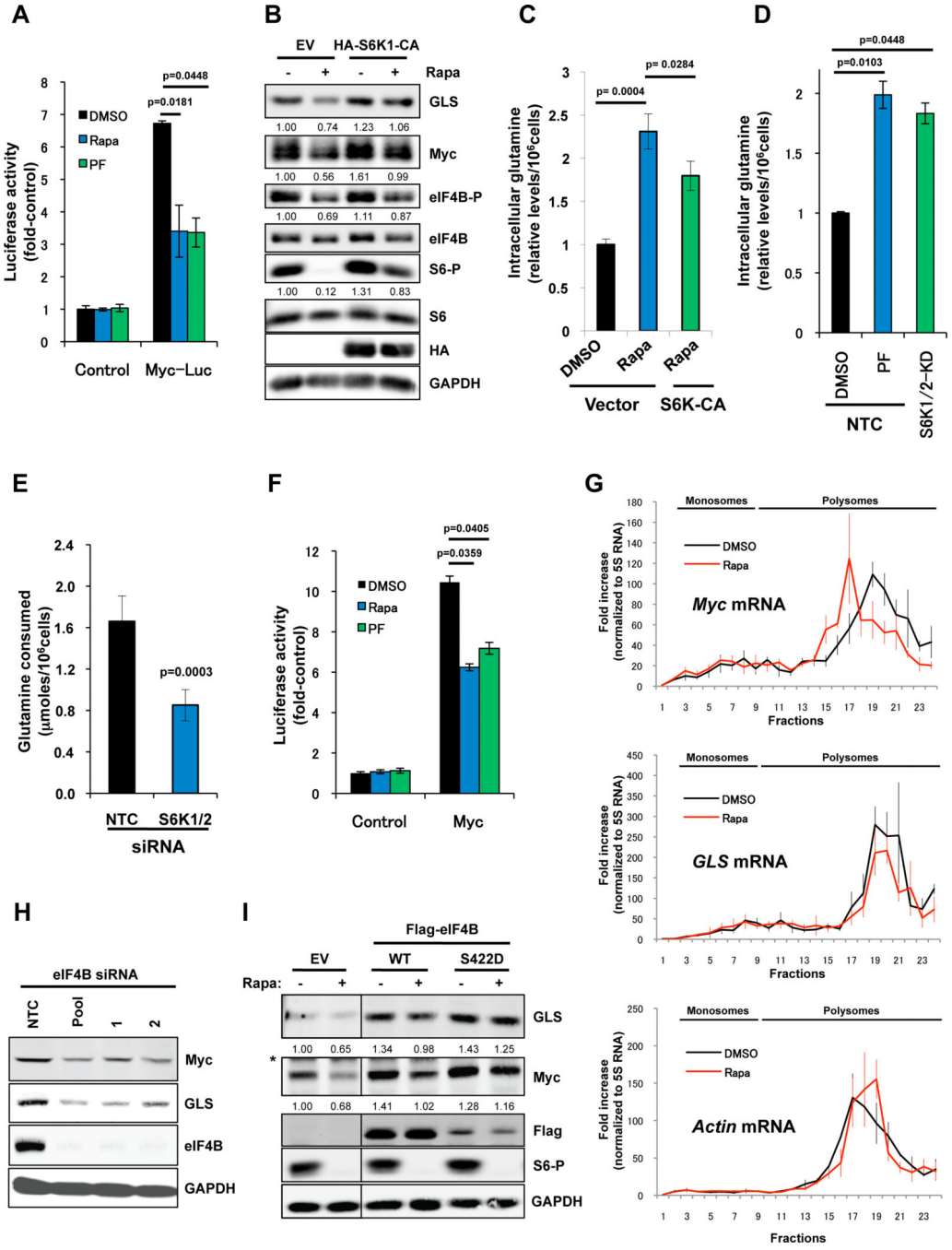

Figure 3. The mTORC1 substrate S6K1 controls GLS through Myc mRNA translation

(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 20ng/mL or PF4708671 10μM for 8h.

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

(C-D) Intracellular glutamine levels of *Tsc2*−/− MEFs: **(C)** stably expressing S6KCA (F5A/R5A/T389E; mutating either the three arginines or all the residues within the RSPRR motif to alanines shows same effect) [10] or empty vector, and treated with rapamycin 20ng/mL or DMSO for 48h; **(D)** transfected with a non-targeting control siRNA (NTC) or siRNA against both S6K1/2. 24h post-transfection, cells transfected with NTC siRNA were treated with PF4708671 10μM or DMSO for 48h.

(E) Glutamine consumption of *Tsc2*−/− MEFs transfected with a non-targeting control siRNA (NTC) or siRNA against both S6K1/2. 72h post-transfection, media was collected and levels of glutamine in the media were determined.

(F) Normalized luciferase light units of *Tsc2* WT MEFs transfected with the pDLN reporter construct containing the 5'UTR of Myc under the control of renilla luciferase. Firefly luciferase was used as an internal control. 48h post-transfection, cells were treated with rapamycin 20ng/mL or PF4708671 10μM for 8h.

(G) Relative levels of *Myc*, *Gls* and *Actin* mRNA in each polysomal gradient fraction. mRNA levels were measured by qPCR and normalized to 5S rRNA level. HEK293T cells were treated with rapamycin 20ng/mL for 24h, and polysomes were fractionated on sucrose density gradients.

(H-I) GLS and Myc protein levels in whole cell lysates from: **(H)** *Tsc2*−/− MEFs transfected with a non-targeting control siRNA (NTC) or two independent siRNAs against eIF4B for 72h; **(I)** *Tsc2*−/− MEFs stably expressing eIF4B WT, mutant S422D or empty vector (EV) and treated with rapamycin for 24h.

The mean is shown; error bars represent SEM from at least three biological replicates. The asterisk (*) denotes a nonspecific band. Numbers below the immunoblot image represent quantification normalized to the loading control.

See also Figure S2 and S3.

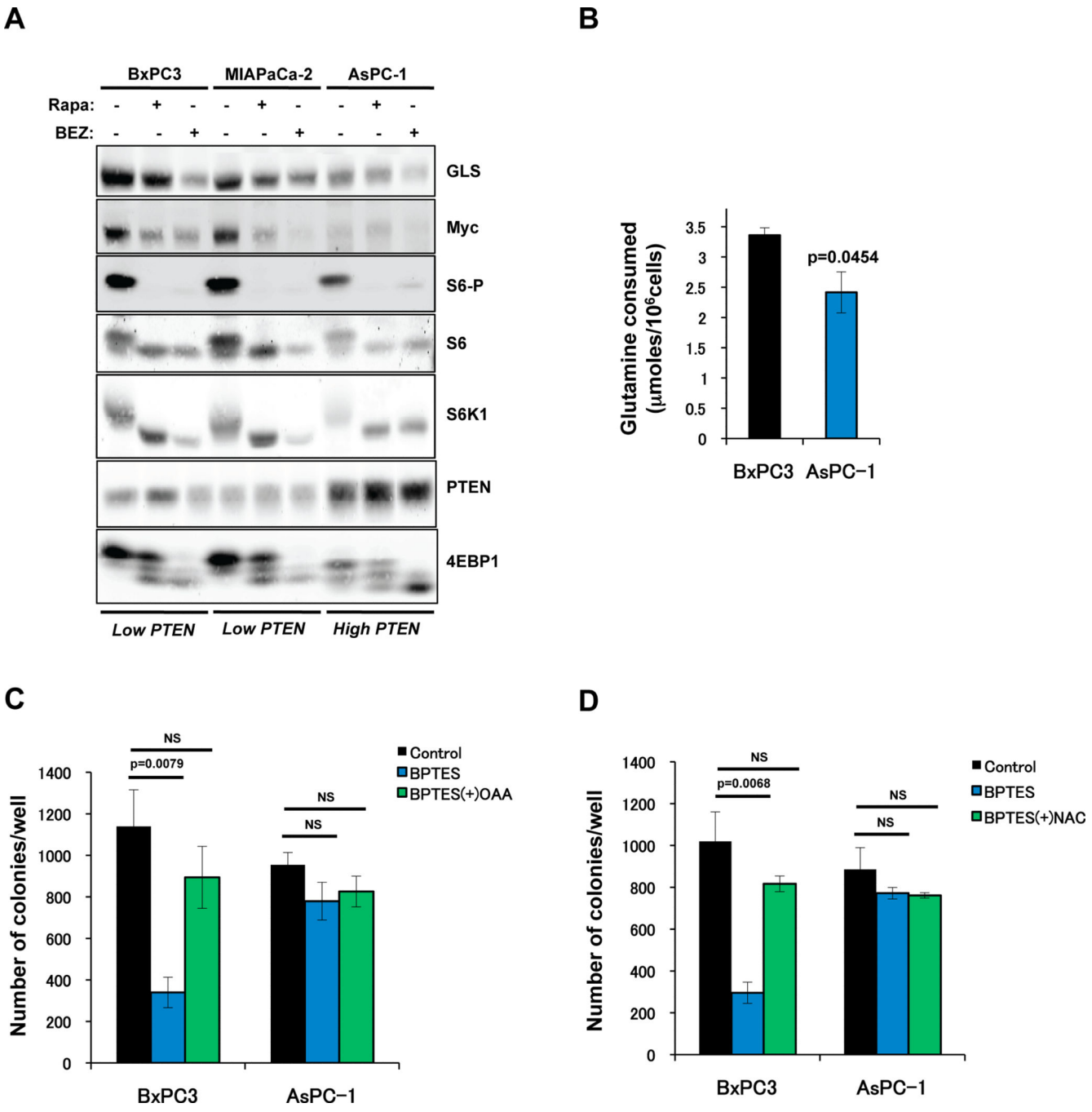

Figure 4. Inhibition of GLS reduces the growth of pancreatic cancer cells

(A) GLS and Myc protein levels in whole cell lysates from BxPC3, MIAPaCa-2 or AsPC-1 cells treated with rapamycin 20ng/mL or BEZ235 1μM for 24h.

(B) Glutamine consumption of BxPC3 or AsPC-1 cells 48h after plating.

(C-D) Soft agar assays with: **(C)** BxPC3 or AsPC-1 cells treated with BPTES (10μM), and the combination of BPTES (10μM)+OAA (2mM); **(D)** BxPC3 or AsPC-1 cells treated with BPTES, and the combination of BPTES (10μM)+NAC (10mM).

The mean is shown; error bars represent SEM from at least three biological replicates.