Asparagine sustains cellular proliferation and c-Myc expression in glutamine-starved cancer cells

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Abstract. During tumorigenesis, oncogene activation and metabolism rewiring are interconnected. Activated c-*Myc* upregulates several genes involved in glutamine metabolism, making cancer cells dependent on high levels of this amino acid to survive and proliferate. After studying the response to glutamine deprivation in cancer cells, it was found that glutamine starvation not only blocked cellular proliferation, but also altered c-Myc protein expression, leading to a reduction in the levels of the canonical c-Myc isoform and an increase in the expression of c-Myc 1, a c-Myc isoform translated from an in-frame 5' CUG codon. In an attempt to identify nutrients able to counteract glutamine deprivation effects, it was shown that, in the absence of glutamine, asparagine permitted cell survival and proliferation, and maintained c-Myc expression as in glutamine-fed cells, with high levels of canonical c-Myc and c-Myc 1 almost undetectable. In asparagine-fed cells, global protein translation was higher than in glutamine-starved cells, and there was an increase in the levels of glutamine synthetase (GS), whose activity was essential for cellular viability and proliferation. In glutamine-starved asparagine-fed cells, the inhibition of c-Myc activity led to a decrease in global protein translation and GS synthesis, suggesting an association between c-Myc expression, GS levels and cellular proliferation, mediated by asparagine when exogenous glutamine is absent.

Introduction

The high rates of growth and proliferation that characterize cancer cells require a very active anabolic metabolism to abundantly synthesize the different macromolecules constituting structural and functional cellular components (1,2). Oncogenic mutations, the increased usage of exogenous nutrients and the activation of specific metabolic pathways are all factors that reciprocally influence each other in the rewiring of cancer cell metabolism (3).

Elevated glucose uptake and high levels of glycolysis, despite the presence of oxygen, were established as features of cancer cells in the middle of the last century, when Otto Warburg described these phenomena in a seminal paper (4). Glycolytic intermediates are diverted towards anabolic pathways for the biosynthesis of nucleotides, amino acids and lipids, thus increasing the synthesis of macromolecule building blocks (5). Glutamine uptake and metabolism are also frequently boosted in cancer cells. Glutamine catabolism provides anaplerotic intermediates for the tricarboxylic acid cycle (6), as well as precursors for the biosynthesis of nucleotides, all the non-essential amino acids (NEAAs), glutathione (GSH), one of the major cellular antioxidant defenses, and ATP energy (7,8).

Since several oncogenes control the expression of metabolic genes, oncogenic alterations are important players in cancer cell metabolic reprogramming (9,10). Activated c-*Myc*, for example, upregulates genes involved in glucose or glutamine uptake and catabolism (11). In addition, the constitutive growth program driven by oncogenic c-*Myc* makes cells depend on exogenous nutrients to survive, in particular glutamine, despite it being a NEAA that cells can synthesize (12). Glutamine is synthesized from glutamate and ammonia by the enzyme glutamine synthetase (GS; also known as glutamate ammonia ligase, GLUL) (7). In some cancer cell lines, c-Myc can also indirectly upregulate GS expression, promoting the expression of a demethylase acting on its promoter (13). GS levels can also be post-transcriptionally regulated by glutamine levels (14).

c-Myc expression itself can be modulated by nutrient levels, both at the transcriptional level and also during translation (15-20). Hann *et al* (15) showed that cell growth at a high density, in exhausted culture medium or in the absence of methionine, altered c-Myc translation initiation. In these conditions, c-Myc synthesis was induced from an in-frame CUG translation initiation site located in the 5'-untranslated region, giving rise to a c-Myc isoform with 15 additional amino acids. To date, contrasting results have been obtained on the possible function of this non-canonical c-Myc isoform, known as c-Myc 1, with contradictory data showing either a cell growth inhibitory function of c-Myc 1 (21) or indistinguishable properties, compared with canonical c-Myc (22). Recently, Sato *et al* (23) showed that the two c-Myc isoforms

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had different transcriptional targets, with canonical c-Myc specifically promoting the expression of genes associated with cell growth and transformation (23).

Given the role of glucose and glutamine in cancer cell growth, these nutrients and their metabolism are under investigation as possible targets for cancer cell therapy (24-26). Hence, it is important to identify factors that can counteract the detrimental effects of nutrient shortage and thus prevent therapeutic results. Recently, it has been shown that asparagine can sustain cell survival in the absence of glutamine by suppressing C/EBP homologous protein induction and apoptosis following glutamine starvation (27). Moreover, asparagine has been found capable of supporting proliferation in the absence of glutamine, allowing a basal level of protein synthesis higher than in glutamine-deprivation conditions, particularly by increasing the synthesis of GS, and hence endocellular glutamine levels (28).

In our laboratory, we developed a cellular system (cen3tel) that recapitulates neoplastic transformation of human fibroblasts (29-33). The analysis of gene and microRNA expression of cells at different phases of the transformation process suggested the occurrence of metabolic reprogramming during transformation, which was particularly associated with c-Myc overexpression (30,32). The deep cellular and molecular characterization of these cells makes them a suitable and valuable tool with which to study cancer cell metabolism. In our previous study, the cellular response to glutamine or glucose deprivation in cen3tel cells was studied and it was shown that tumorigenic cells had become glutamine-addicted (34). In fact, in the absence of exogenous glutamine, cells underwent a prolonged growth arrest (72-96 h) and then began to die. By contrast, glucose deprivation led to cell death more quickly (within 48 h) and induced autophagy. In the present study, cen3tel cells and other cancer cell lines were used to study the relationship between nutrient deprivation and c-Myc expression, along with the role of asparagine in counteracting the detrimental effects of glutamine starvation. The results suggested an association between c-Myc expression and the asparagine capacity to allow cell proliferation in the absence of glutamine, possibly through a c-Myc-mediated increase in GS levels.

Materials and methods

Cell lines and culture. In the present study, cen3tel cells at ~1,000 population doublings (PDs) after telomerase immortalization were used. These cells are tumorigenic and metastatic in nude mice and carry a mutation in the *TP53* codon 161, which has been used to confirm the identity of the cen3tel cells used in the present study (30,35).

The following cells were used in the present study: Cen3tel, MDA-MB-231 (human metastatic breast cancer), U2OS (human osteosarcoma), A375MC2 [high metastatic melanoma cell line (36); a generous gift from Dr Richard Hynes, Howard Hughes Medical Institute, Center for Cancer Research, Cambridge, MA, USA), SW480 (human colon adenocarcinoma) and HeLa (human cervix carcinoma). Cells were cultured as previously described (34). Cells were counted using Burker chambers. Stock solutions of single NEAAs (Merck KGaA) were prepared by dissolving aspartic acid and glutamic acid in PBS (at a concentration of 25 and 50 mM, respectively), and alanine, asparagine and proline in distilled water (at a concentration of 150 mM). Solutions were then sterilized by filtration. To carry out nutrient deprivation experiments, cells were plated in complete medium [except for the experiments with methionine sulfoximine (MSO), see below] and when they had reached a density of ~2-3x10^5 cells/cm^2, they were processed as previously described (34). In the experiments in which cells were propagated in different culture conditions, cells were plated in complete medium and after 24 h they were incubated for an additional 24 h in the absence of glutamine and presence of the desired NEAAs. Then cells were collected, counted (first passage) and replated in a defined number in a medium with the same composition as that in which they were cultured. Cell harvesting was repeated three additional times every 48 or 72 h (second, third and fourth passage). For each culture condition, the number of cells obtained at each passage was used to calculate the number of PDs/day performed by the cells between each passage, which was considered as a measure of cell growth in the different media. Experiments were repeated at least three times with three replicates, unless otherwise specified.

Treatments. The MG132 (Merck KGaA) stock solution was prepared in DMSO at a concentration of 25 mM and kept at -20˚C. Cells were exposed to 25 μM MG132 during the last 2 or 4 h of incubation in the different nutrient conditions.

The puromycin (Merck KGaA) stock solution was prepared in sterile water at a concentration of 12 mM and was then sterilized by filtration and stored at -20˚C. During the last 10 min of incubation in the different nutrient conditions, cen3tel and MDA-MB-231 cells were incubated with 90 or 2.5 μM puromycin, respectively.

The MSO (Thermo Fisher Scientific, Inc.) stock solution was prepared in sterile water at a concentration of 200 mM, filtered and stored at -20˚C. MSO treatment was performed by plating cells directly in the different nutrient-containing media with or without 2 mM MSO. Cells were collected after 96 or 144 h of incubation. The medium was refreshed 96 h after incubation.

The 10074-G5 (Cayman Chemical Company) stock solution was prepared in DMSO at a concentration of 20 mM and stored at -20˚C. Cells were exposed to 10074-G5 during the entire incubation period in the different nutrient conditions.

The reduced GSH (Merck KGaA) stock solution was freshly prepared in DMEM for each experiment at a concentration of 100 mM. Twenty-four hours after plating, cells were incubated in the different nutrient conditions with or without 5 mM GSH (Merck KGaA) for a further 24 h. Growth was determined by calculating the number of PDs/day performed by each cell sample in the time interval between plating and cell harvesting.

When drugs were dissolved in DMSO, control cells were treated with the same DMSO concentration reached in drug-exposed samples.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using QIAzol reagent (Qiagen GmbH). cDNA was generated from 1 μg RNA using the QuantiTec Reverse Transcription Kit (Qiagen GmbH). Gene expression was quantified using SYBR®-Green

chemistry with a QuantiTect SYBR Green kit (Qiagen GmbH) using the QuantiTect Primer Assay specific for each target gene (Hs_MYC_1_SG; Hs_GLUL_1_SG, Hs_GUSB_1_SG; Qiagen GmbH). qPCR was performed on the Light Cycler 480 (Roche Diagnostics), using 96-well reaction plates (Roche Diagnostics). Raw data were normalized to the *GusB* housekeeping gene and the relative expression was calculated using the ΔΔCq method (37) and expressed as fold change (FC)=$2^{-\Delta\Delta Cq}$. qPCR analysis was carried out at least three times using different biological replicates.

Western blotting. Whole-cell lysates for western blot analysis were prepared using RIPA lysis buffer [1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.1% DOC, 1X protease inhibitor cocktail (Thermo Fisher Scientific, Inc.), and 1X phosphatase inhibitor cocktail (Roche Diagnostics)]. The following antibodies recognizing human proteins were used: Anti-c-Myc rabbit monoclonal (cat. no. ab32072; 1:10,000; Abcam), anti-puromycin mouse monoclonal (cat. no. MABE343; 1:10,000; Merck KGaA), anti-GS mouse monoclonal (cat. no. 610517; 1:250; BD Transduction Laboratories), anti-poly(ADP-ribose) polymerase 1 (PARP1) rabbit monoclonal (cat. no. ab191217; 1:1,000; Abcam), anti-poly(ADP-ribose) (PAR) chains mouse monoclonal (cat. no. sc-56198; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-vinculin mouse monoclonal (cat. no. 05-386; 1:5,000; Merck KGaA), anti-γ-tubulin mouse monoclonal (cat. no. T6557; 1:10,000; Merck KGaA), anti-β-actin mouse monoclonal (cat. no. A2066; 1:10,000; Merck KGaA). Incubations with primary antibodies were carried out overnight at 4˚C. Primary antibodies were probed by a secondary horseradish peroxidase-conjugated antibody (anti-mouse, cat. no. 115-035-146; anti-rabbit, cat. no. 111-035-144; 1:5,000; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescent reagent (Clarity™; Bio-Rad Laboratories, Inc.). The intensity of the band in each sample was quantified using QuantityOne software (Bio-Rad Laboratories, Inc.), corrected for the intensity of the corresponding band obtained with the anti-γ-tubulin, anti-vinculin, or anti-actin antibodies, and then normalized to the appropriate control sample.

Western blotting experiments were repeated at least three times and with independent biological replicates. Representative images of the results are shown in the figures.

Statistical analysis. Statistical analysis was performed using ANOVA single factor for comparison of means of multiple groups followed by Bonferroni post-hoc test, and two-tailed Student's t-test for comparison between two groups (Microsoft Excel, version 16.16.24 was used). $P<0.05$ was considered to indicate a statistically significant difference.

Results

c-Myc expression is altered in cells starved of glutamine and/or glucose. Given the profound effects of glutamine and or/glucose deprivation on cell growth and viability in cen3tel cells (34) and the association between c-*Myc* and metabolism, the expression of this oncogene was analyzed in the different starved samples.

The deprivation of each nutrient caused a rapid and specific change in c-Myc protein expression (Fig. 1A). In glucose-starved specimens, c-Myc levels were higher than in control cells 6-48 h after starvation and then declined (Fig. 1A), when cells started detaching from the culture dish and died (34).

Glutamine starvation led to a decrease in the levels of canonical c-Myc and the synthesis of the longer c-Myc isoform, c-Myc 1 (Fig. 1A), which was described by Hann *et al* (15) in cells grown in harsh culture conditions or in the absence of methionine. This suggested that, in the absence of glutamine, c-Myc synthesis can start from the cryptic CUG translation initiation site. c-Myc 1 expression began to appear 6 h after glutamine deprivation; 12 h after starvation, the two c-Myc isoforms were almost equally represented and together gave a c-Myc level similar to that observed in control cells (at 12, 16 and 20 h, the levels of the two isoforms together vs. c-Myc in control cells, was 0.8, 0.7 and 1.1, respectively; Fig. 1A).

Double-deprived cells had a mixed behavior (Fig. 1A); at 9-12 h after deprivation, c-Myc expression was higher than in control cells, but the c-Myc 1 band was also detectable. At subsequent incubation times, c-Myc levels were found to be diminished. Of note, at that stage, double-deprived cells had detached from the culture dish and started dying (34).

c-*Myc* expression was analyzed at the RNA level in samples starved of the different nutrients for 8-24 h by RT-qPCR (Fig. 1B). In all the conditions and at all the time points, c-*Myc* RNA levels were higher in starved cells compared with control cells, regardless of the protein level. In particular, in glutamine-starved cells, the higher RNA expression was not accompanied with a higher c-Myc protein level, indicating a translational impairment.

Characterization of c-Myc 1 expression in glutamine-starved cells. The synthesis of the two c-Myc isoforms in the absence of glutamine indicated a link between glutamine availability and c-Myc translation from the cryptic CUG initiation site, which, to the best of our knowledge, has not been described so far. Thus, the present study aimed to further characterize c-Myc 1 expression in the absence of glutamine. First, it was investigated whether c-Myc 1 was present in other cancer cell lines (namely MDA-MB-231, U2OS, A375MC2, SW480 and HeLa) cultured in the absence of glutamine. As shown in Fig. 2A, the association between glutamine deprivation and c-Myc 1 expression was not restricted to cen3tel cells. In fact, 24 h after glutamine starvation, c-Myc 1 was expressed in a nearly 1:1 ratio with canonical c-Myc in MDA-MB-231 and HeLa cells. In the other cell lines, c-Myc 1 expression was induced in the absence of glutamine, but this isoform represented a lower fraction of the total c-Myc compared with that in cen3tel, MDA-MB-231 and HeLa cells.

Using cen3tel cells, c-Myc 1 expression was further characterized in the absence of glutamine. It was shown that the increase in c-Myc 1 synthesis was reversible when glutamine levels were restored. In fact, c-Myc 1 became undetectable when cen3tel cells starved of glutamine for 24 h were fed with complete medium for 24 h (Fig. 2B). Glutamine supplementation also led to the recovery of cellular proliferation (Fig. 2B).

Since c-Myc undergoes a rapid turnover, being degraded by the proteasome, it was examined whether the two c-Myc isoforms were characterized by the same turnover. Thus,

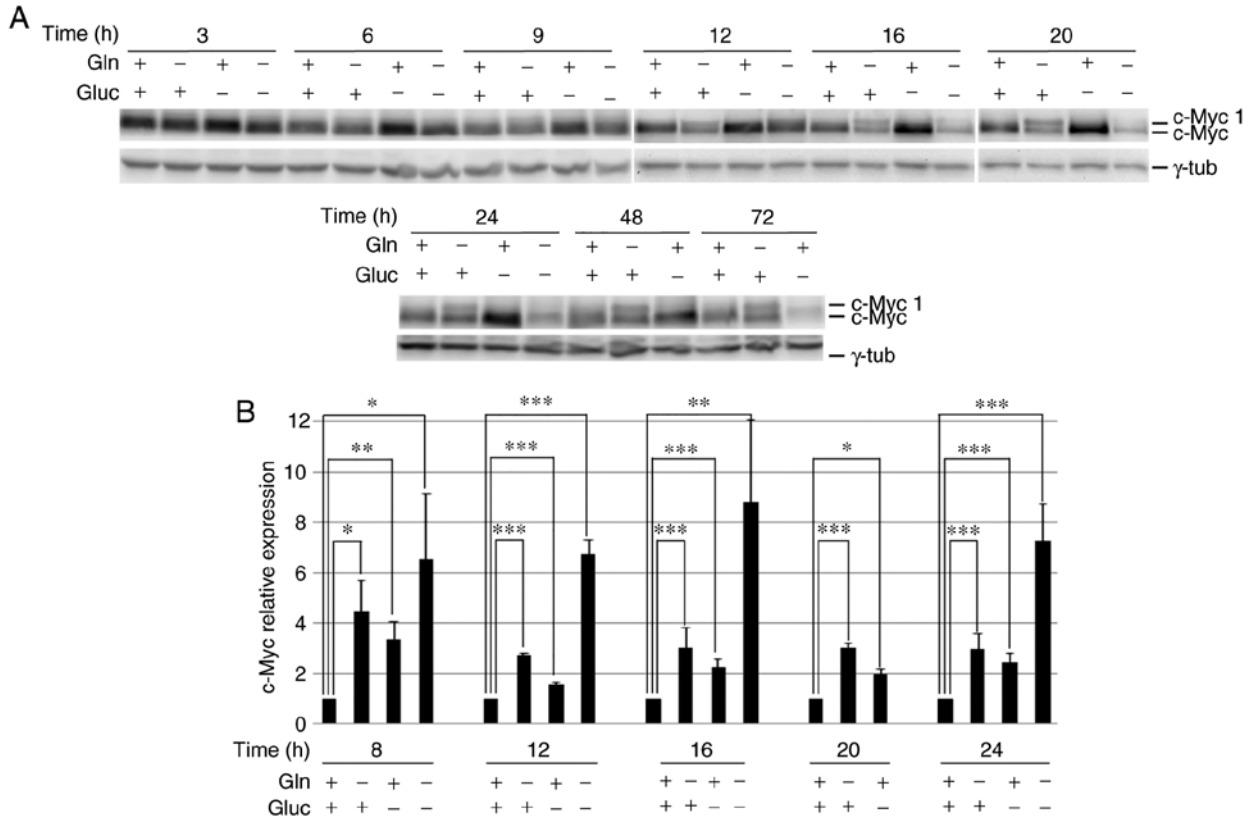

Figure 1. c-Myc expression in glucose- and/or glutamine-starved cells. (A) Western blot analysis of c-Myc expression in cen3tel glucose- and/or glutamine-starved cells for up to 72 h. γ-tubulin was used as the loading control. (B) RT-qPCR analysis of c-*Myc* expression in cen3tel glucose- and/or glutamine-starved cells for up to 24 h. For each time point, the expression of c-*Myc* in cells cultured in the different culture conditions is shown as Log_2FC relative to c-*Myc* expression in cells cultured in the presence of glutamine. Error bars: Standard deviation. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$. Gln, glutamine; Gluc, glucose; RT-qPCR, reverse transcription quantitative PCR; FC, fold change.

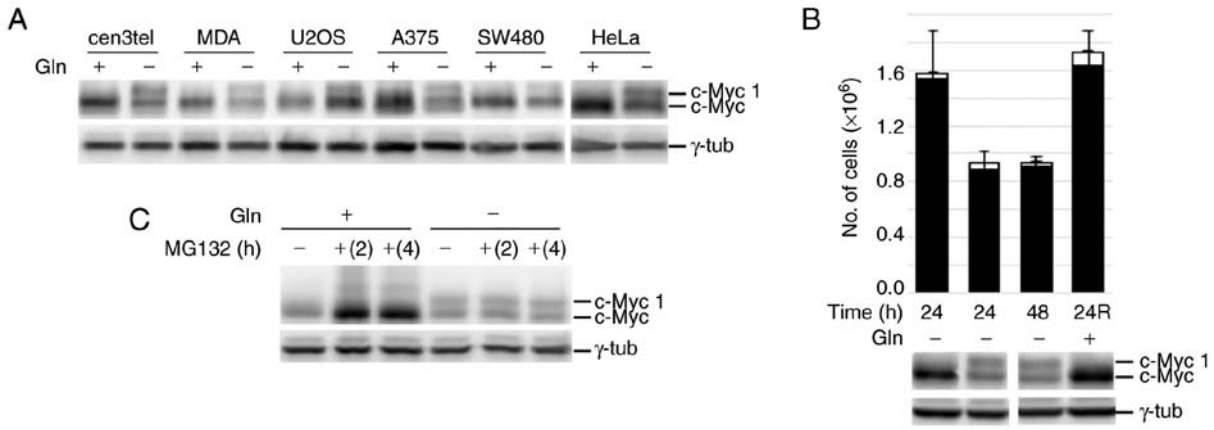

Figure 2. Characterization of c-Myc expression in glutamine-starved cells. c-Myc expression was analyzed by western blotting. In all the western blot panels, γ-tubulin was used as the loading control. (A) c-Myc expression in different cancer cell lines deprived of glutamine for 24 h. MDA, MDA-MB-231 cells. (B) Cell proliferation and c-Myc expression in cen3tel cells deprived of glutamine for 24 h and then re-fed with glutamine-containing medium for 24 h (24R in the figure). (C) c-Myc expression in cen3tel cells starved for glutamine for 24 h and exposed to 25 μM MG132 during the last 2 or 4 h of starvation. Cells cultured in the presence of glutamine were analyzed in parallel. Gln, glutamine.

24 h-glutamine-starved cen3tel cells were exposed to the MG132 proteasomal inhibitor for 2 or 4 h, and the same results were reported for both isoforms, that is, a compromised accumulation of both isoforms in the presence of the proteasomal inhibitor (Fig. 2C). This suggested that 24 h after glutamine deprivation, the synthesis and degradation of both c-Myc isoforms were impaired.

In glutamine-starved cells, asparagine is necessary and sufficient to maintain c-Myc expression just as in the presence of glutamine and allows cell survival and proliferation. Given that glutamine is a key precursor for the biosynthesis of NEAAs (7,8), it was analyzed whether single NEAAs, namely alanine, asparagine, aspartic acid, glutamic acid and proline (serine and glycine were not tested, as they are present in the culture medium), or a

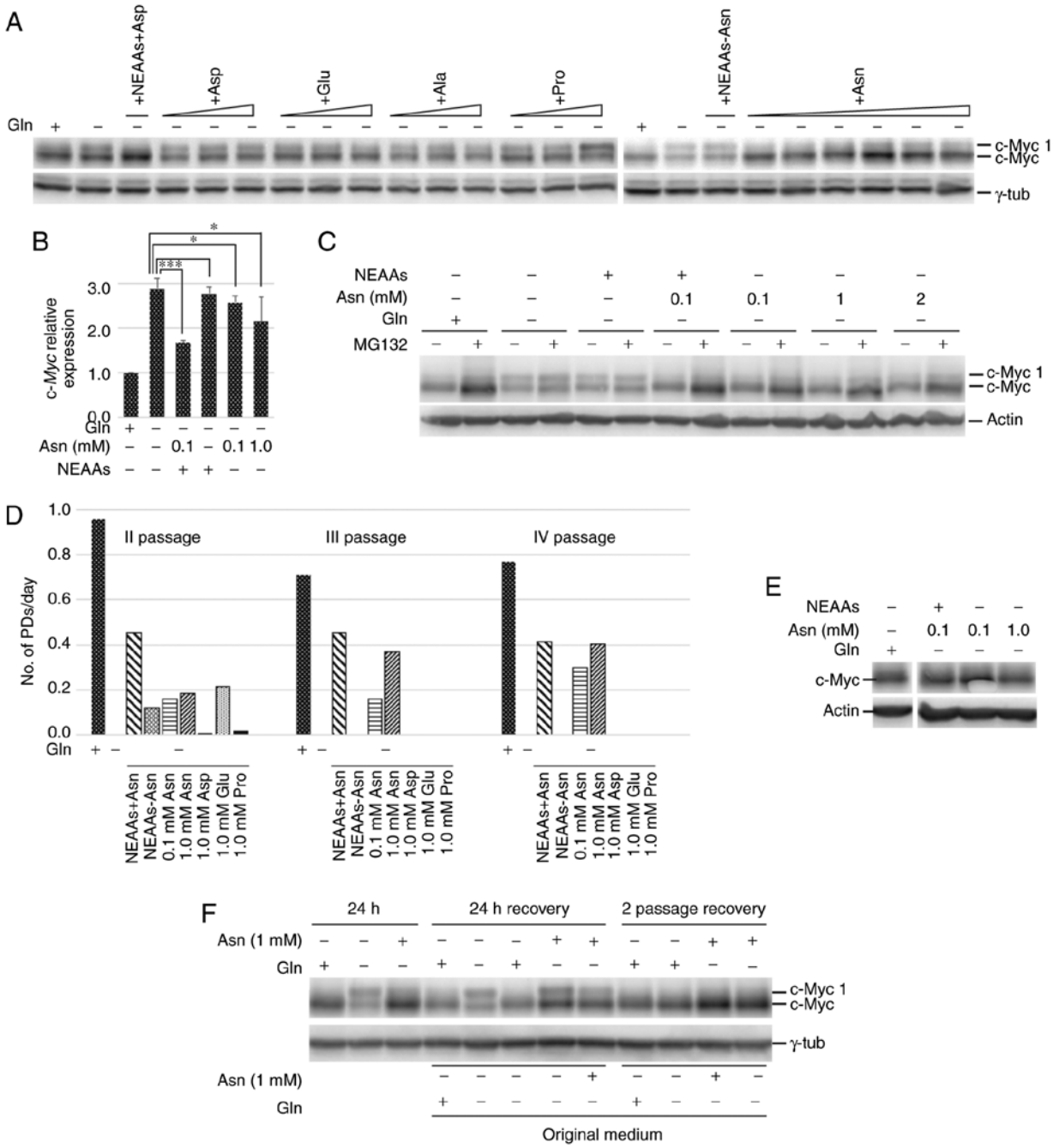

Figure 3. Asparagine is necessary and sufficient to maintain c-Myc expression as in the presence of glutamine and allows cell survival and proliferation. NEAAs, a pool of alanine, aspartic acid, glutamic acid and proline at a concentration of 0.1 mM each, with or without 0.1 mM asparagine. In the western blots, γ-tubulin or actin were used as the loading control. (A) Western blot analysis of c-Myc expression in cen3tel cells cultured for 24 h in the indicated medium; for each NEAA, increasing concentrations were tested, for alanine, aspartic acid, glutamic acid and proline, concentrations were 0.1, 0.5 and 1.0 mM, for asparagine 0.1, 0.5, 1.0, 1.5 and 2.0 mM. (B) RT-qPCR of c-*Myc* expression in cen3tel cells cultured for 24 h in the indicated medium. The expression of c-*Myc* in cells cultured in the different culture conditions is shown as Log_2FC relative to c-*Myc* expression in cells cultured in the presence of glutamine. Error bars: Standard deviation. $^{*}P<0.05$, $^{***}P<0.005$. (C) Western blot analysis of c-Myc expression in cells cultured for 24 h in the indicated medium and incubated with 25 μM MG132 during the last 2 h of culture. (D) Cen3tel cell propagation in different media. In the histogram, each column represents the number of PDs/day performed by the cells in the different culture conditions, starting from the second passage. An exemplificative experiment is shown. (E) Western blot analysis of c-Myc expression in cells propagated in the absence of glutamine and in the presence of 0.1 or 1.0 mM asparagine for four passages. (F) Western blot analysis of c-Myc expression in cen3tel cells incubated for 24 h in the medium indicated above the lanes (first three lanes) and then fed with different media for 24 h (lanes, 24 h recovery) or for two passages (lanes, 2 passage recovery). The incubation medium during the recovery period is indicated above the lanes, the original medium in which each cell sample was incubated for the first 24 h is indicated below the lanes. Gln, glutamine; Ala, alanine; Asn, asparagine; Asp, aspartic acid; Glu, glutamic acid; Pro, proline; NEAAs, non-essential amino acids; RT-qPCR, reverse transcription-quantitative PCR.

pool of them, could compensate for the absence of glutamine in the culture medium for both c-Myc synthesis and cell growth.

As shown in Fig. 3A, in cen3tel cells incubated with either alanine, aspartic acid, glutamic acid or proline, the same pattern of c-Myc expression as that in glutamine-starved cells was observed. By contrast, following asparagine supplementation, the pattern of c-Myc expression was the same as that in control cells, with the expression of c-Myc 1 almost undetectable

and high levels of canonical c-Myc (~2-3 times higher than in glutamine-starved cells). Asparagine was effective at a concentration of 0.1 mM, which is close to the physiological concentration of this amino acid (38). The relevance of asparagine on c-Myc expression was confirmed by the observation that in glutamine-starved cells incubated in medium supplemented with the pool of the 5 NEAAs, c-Myc was synthesized as in the presence of asparagine only, while in cells grown with a pool of NEAAs devoid of asparagine, c-Myc was expressed as in glutamine-starved cells. Thus, asparagine is necessary and sufficient to maintain canonical c-Myc synthesis in the absence of glutamine and to promote c-Myc translation from the canonical initiation site. The same result was obtained in MDA-MB-231 cells, which expressed high levels of c-Myc 1 in the absence of glutamine, but not in the presence of asparagine (Fig. S1A).

As far as c-*Myc* transcription is concerned, in cen3tel cells incubated in the presence of asparagine and in the absence of glutamine, there were increased levels of c-*Myc* RNA compared with control cells, but decreased levels compared with glutamine-starved cells (Fig. 3B). When cells were incubated with a pool of the 4 NEAAs (alanine, aspartic acid, glutamic acid and proline), no changes in c-*Myc* RNA levels were observed compared with glutamine-starved cells, while, when asparagine was added to the pool, c-*Myc* transcription was decreased (Fig. 3B). These results suggested that, in the absence of glutamine but presence of asparagine, c-Myc expression was still upregulated at the transcriptional level and, being the RNA efficiently translated, gave rise to high levels of canonical c-Myc.

In glutamine-starved cen3tel cells, asparagine supported a c-Myc turnover comparable to that observed in control cells, while a pool of alanine, aspartic acid, glutamic acid and proline did not allow c-Myc accumulation in the absence of glutamine when the proteasome was inhibited by MG132 (Fig. 3C).

Considering the effect of NEAAs on c-Myc expression, it was further analyzed whether NEAAs, and in particular asparagine, had an influence on cell growth in the absence of glutamine. As previously mentioned, glutamine-starvation at 24-72 h led to cen3tel cell growth arrest. When cells were incubated for these periods of time in the absence of glutamine and in the presence of NEAAs, either singly or in pools, no significant differences were detected in the cell number between glutamine-starved and NEAA-supplemented cells (Fig. S2). Longer-term experiments were therefore performed, in which cells were propagated in the different experimental conditions for four passages. As shown in Fig. 3D, cells grown in the absence of glutamine or in glutamine-free media supplemented with proline or aspartic acid did not grow between the first and the second passages. At the third passage, most cells were floating in the medium. In the other culture conditions, cells showed an increase in cell number relative to seeding, but decreased numbers compared with the control cells. At the subsequent passages (III and IV), only asparagine supplementation (either alone or in combination with the other NEAAs) allowed cell growth in the absence of glutamine, although at a lower rate compared with cells grown in glutamine-containing medium. Therefore, asparagine is necessary and sufficient for cell growth in the absence of glutamine.

The analysis of c-Myc expression in cells collected at the fourth passage confirmed that glutamine-starved cells cultured in the presence of asparagine expressed high levels of canonical c-Myc, similar to cells propagated in complete medium (Fig. 3E).

Finally, it was examined whether asparagine was able to rescue canonical c-Myc expression in cells that had been starved for glutamine. As expected, 24 h after glutamine deprivation (Fig. 2B and 3F), the addition of glutamine allowed a complete recovery of canonical c-Myc expression in cen3tel cells; cells re-fed with medium without glutamine continued to exhibit an equal expression of the two c-Myc isoforms, while cells re-fed with asparagine still expressed c-Myc 1, but showed an increase in canonical c-Myc levels (Fig. 3F). In fact, the ratios between the intensities of the c-Myc and c-Myc 1 bands were 1.1 in the sample re-fed without glutamine, and 2.1 in that re-fed with asparagine. When cells re-fed with asparagine were maintained in culture for two passages, the pattern of c-Myc expression became indistinguishable from that observed in cells always maintained in medium with glutamine or asparagine, or re-fed with glutamine following glutamine deprivation (Fig. 3F).

Thus, in glutamine-starved cells, asparagine allows high levels of canonical c-Myc synthesis, cell survival and proliferation, although at lower rates compared with the presence of glutamine.

GSH prevents PARP1 activation in glutamine-starved asparagine-supplemented cells. In our previous study, glutamine deprivation was shown to lead to PARP1 activation, with high levels of PARP1 auto-(poly-ADP) ribosylation (PARylation) and protein PARylation (34). In fact, compared with cells grown in the presence of glutamine, in cells deprived of glutamine for 24 h, the anti-PARP1 antibody revealed a reduced intensity of the PARP1 band and an increased series of higher molecular weight bands (Fig. 4A); this was paralleled by an intense signal highlighted by the antibody against PAR chains, which confirmed the activation of PARP1 in these cells (Fig. 4A). Herein, PARP1 was also found to be activated when glutamine-starved cen3tel cells were supplemented with asparagine; in fact, in these cells, the signals obtained with either the PARP1 antibody or the anti-PAR chain antibody were comparable to those obtained in glutamine-starved cells (Fig. 4A). This suggested that asparagine was not sufficient to prevent cellular stress, due to the absence of glutamine, which could contribute to the lower proliferation rate observed in cells cultured without glutamine but in the presence of asparagine.

Given that glutamine is essential for GSH synthesis (7,8), it was examined whether the addition of GSH to the culture medium could decrease PARP1 activation in the absence of glutamine. Indeed, it was found that GSH supplementation reduced both the PARP1 smeared signal and protein PARylation (Fig. 4B) and increased cellular proliferation (Fig. 4C). This suggested that oxidative stress plays a role in blocking cell growth in the absence of glutamine, and that asparagine alone is not able to counteract this phenomenon.

In asparagine-supplemented glutamine-starved cells, global protein synthesis and GS levels are higher than in cells cultured in the absence of glutamine. Since asparagine is mainly used for protein synthesis in mammalian cells (39), it was analyzed whether asparagine could have

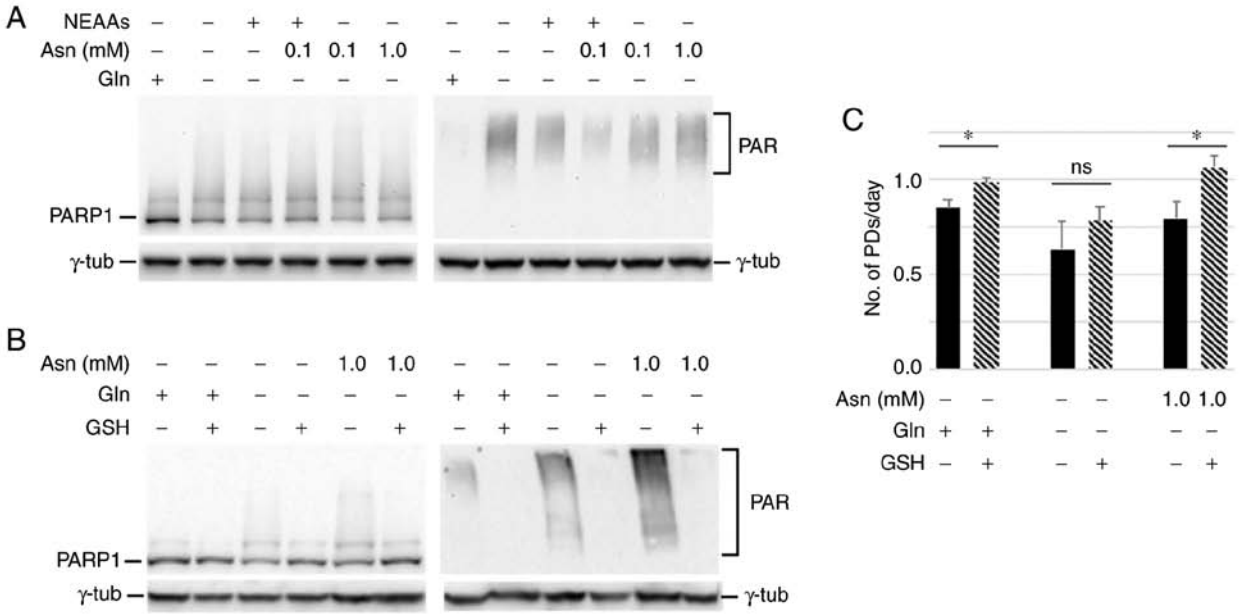

Figure 4. GSH rescues PARP1 activation in glutamine-starved asparagine-supplemented cells. NEAAs, a pool of alanine, aspartic acid, glutamic acid and proline at a concentration of 0.1 mM each. (A) Western blot analysis of PARP1 and protein PARylation with an antibody against PAR chains in cells incubated in the indicated medium for 24 h. (B) GSH was used at 5 mM during the entire incubation period. γ-tubulin was used as the loading control. (C) Cell growth in the indicated media plus or minus GSH. Each column represents the number of PDs/day performed by the cells in the different culture conditions between plating and harvesting. Error bars, standard deviations of two independent experiments. *P<0.05; ns, not significant. Gln, glutamine; Asn, asparagine; GSH, glutathione; NEAAs, non-essential amino acids; PARP1, poly(ADP-ribose) polymerase 1; PAR, poly(ADP-ribose); PDs, population doublings.

a positive effect on global protein synthesis in cells starved for glutamine, which could in turn positively affect cell survival and proliferation. Evaluating the incorporation of puromycin, a tyrosyl-tRNA mimetic in cen3tel cells incubated in different culture media (Fig. 5A), it was found that a 24 h glutamine starvation period decreased protein synthesis to ~50% compared with control cells, while asparagine in the absence of glutamine allowed a higher level of protein synthesis; however, this was still lower than in control cells (60-65% of control cells). Similar levels of protein synthesis were detected both 24 h after incubation of glutamine-starved cells in the presence of asparagine and following propagation of the starved cells in asparagine-containing medium for three passages. These results were consistent with the observation that glutamine deprivation led to a modest increase in eIF2α phosphorylation, compared with control cells, which was not detected following asparagine supplementation (Fig. 5B). The higher level of protein synthesis in glutamine-deprived cells fed with asparagine, compared with those starved for glutamine, was also observed in MDA-MB-231 cells (Fig. S1B, lanes 1, 3, 5 and 7). Thus, asparagine likely allows cells to maintain a level of protein translation in the absence of glutamine that is compatible with cell survival and proliferation.

Since glutamine can be synthesized in mammalian cells by the enzyme GS, it was analyzed whether the higher translational ability of glutamine-deprived asparagine-fed cen3tel cells could have an effect on the expression of GS. High levels of this enzyme could sustain glutamine synthesis, and hence cell survival and proliferation, when asparagine is supplied to glutamine-starved cells. As shown in Fig. 5C-E, GS was barely detectable in cen3tel cells cultured in the presence of glutamine. This result was not surprising, since it is known that glutamine itself can regulate GS levels, directing the enzyme towards ubiquitination and proteasomal degradation (14). In glutamine-starved cells, GS levels were slightly higher than in control cells (Fig. 5C and D), but GS expression was markedly increased when glutamine-starved cells were incubated with asparagine, regardless of asparagine concentration (Fig. 5C). Asparagine was sufficient and necessary to increase GS expression; in fact, no increase in GS levels was observed when glutamine-starved cells were incubated with a pool of alanine, aspartic acid, glutamic acid and proline, or with each single amino acid, while an increase was observed when asparagine was added to the pool (Fig. 5D). Of note, the propagation of glutamine-starved cells in the presence of asparagine boosted GS expression in a concentration-independent manner (Fig. 5E). The same type of modulation of GS expression was observed in MDA-MB-231 cells cultured in the absence of glutamine and in the presence of asparagine (Fig. S1C, lanes 1, 3, 5 and 7).

To determine whether GS was required for glutamine-starved cell survival and proliferation in the presence of asparagine, cen3tel cells were treated with the GS inhibitor MSO (2 mM). Cells were seeded in the presence of the inhibitor and collected either 96 or 144 h after plating. As shown in Fig. 5F, MSO did not have any effect on cells grown in the presence of glutamine-containing medium, while glutamine-starved cells died independently of the presence of the inhibitor. Glutamine-starved asparagine-supplemented cells survived and proliferated, as expected, but died in the presence of MSO, indicating that both GS expression and activity were essential for cell survival and growth in the absence of glutamine.

When GS levels were analyzed in cells exposed to MSO (Fig. 5G), it was found that the protein accumulated in cells cultured with glutamine. It can be speculated that this occurred

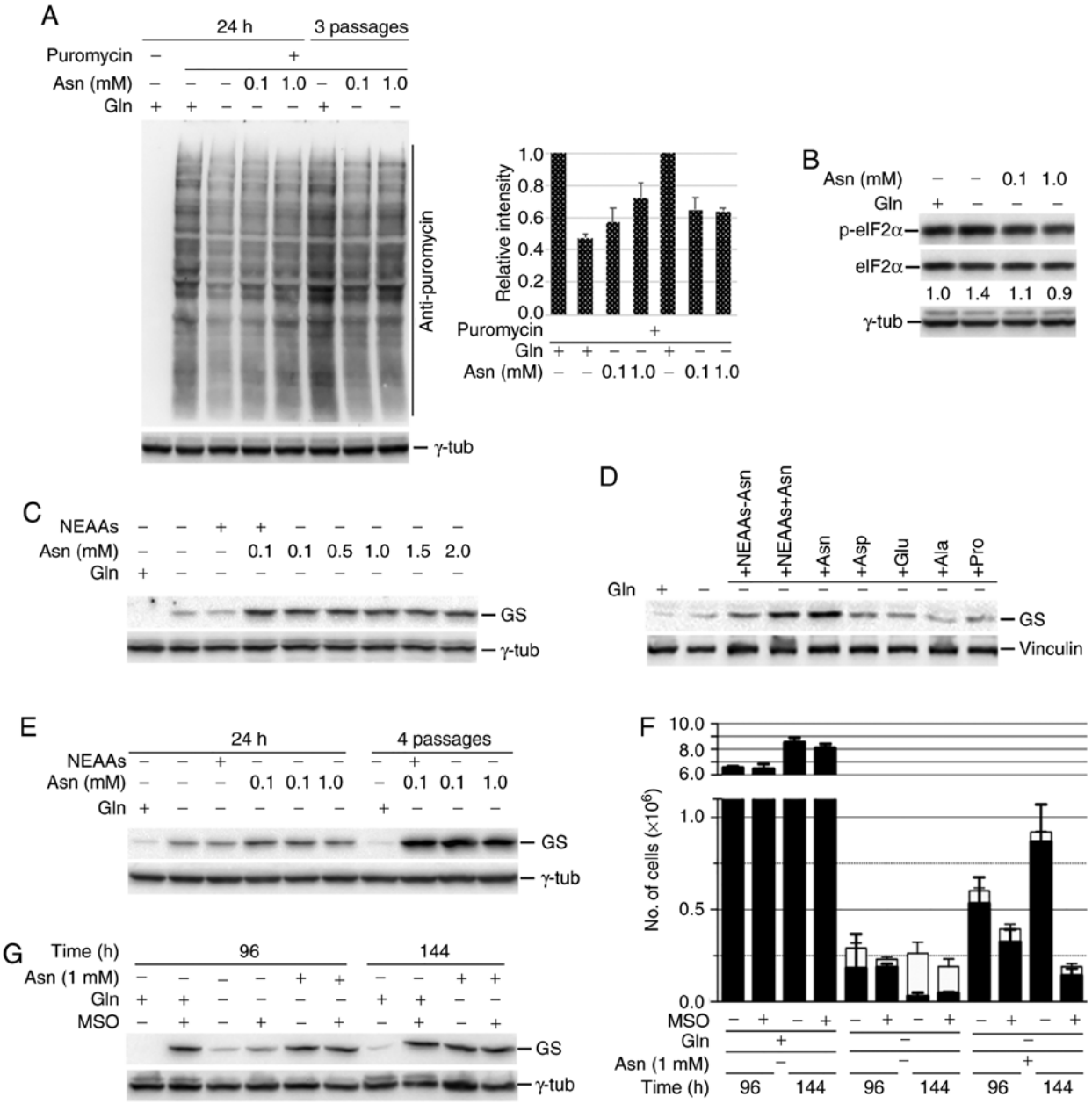

Figure 5. Asparagine allows high levels of global protein synthesis and GS expression in glutamine-starved cen3tel cells. NEAAs, a pool of alanine, aspartic acid, glutamic acid and proline at a concentration of 0.1 mM each, with or without 0.1 mM asparagine. (A) Western blot analysis of global protein synthesis in cen3tel cells cultured for either 24 h in the medium indicated or for 3 passages in the presence of glutamine or asparagine. Puromycin (90 μM) was added during the last 10 min before sample collections. Puromycin incorporation was revealed using an anti-puromycin antibody. The plot shows the intensity of the signal in each sample relative to cells grown in the presence of glutamine (average values from two independent experiments; error bars, standard deviations). (B) Western blot analysis of eIF2α phosphorylation in cen3tel cells cultured in the indicated medium. (C-E) Western blot analysis of GS expression in cen3tel cells. (C and D) Cells were incubated in the presence of different NEAAs or pool of NEAAs for 24 h or (E) cultured either for 24 h in the medium indicated or for 4 passages in the presence of glutamine or asparagine. (F) Cen3tel cell growth following incubation in the medium indicated for 96 or 144 h with or without the GS inhibitor MSO (2 mM). Error bars, standard deviation. (G) Western blot analysis of GS expression in the samples cultured with or without MSO. In the western blots, γ-tubulin or vinculin were used as the loading controls. GS, glutamine synthetase; Gln, glutamine; Asn, asparagine; NEAAs, non-essential amino acids; MSO, methionine sulfoxamine.

because the binding between MSO and GS (40) prevented GS ubiquitination and degradation by the proteasome in the presence of glutamine. In glutamine-deprived cells treated with MSO, GS levels remained low, probably due to the fact that the impairment of translation occurring in these conditions did not allow high levels of GS synthesis.

Possible connection between c-Myc, global protein synthesis and GS expression in glutamine-starved asparagine-fed cells. The results presented so far indicated that, in the presence of asparagine, glutamine-starved cells survive, proliferate, express almost undetectable levels of c-Myc 1, high levels of canonical c-Myc and GS, with the activity of this enzyme being required for their survival. Given that c-Myc can positively regulate *GS* expression through promoter demethylation (13), *GS* transcripts were analyzed by RT-qPCR in cen3tel cells incubated with different nutrients. As shown in Fig. 6A, the same levels of *GS* RNA were found in cells cultured either in

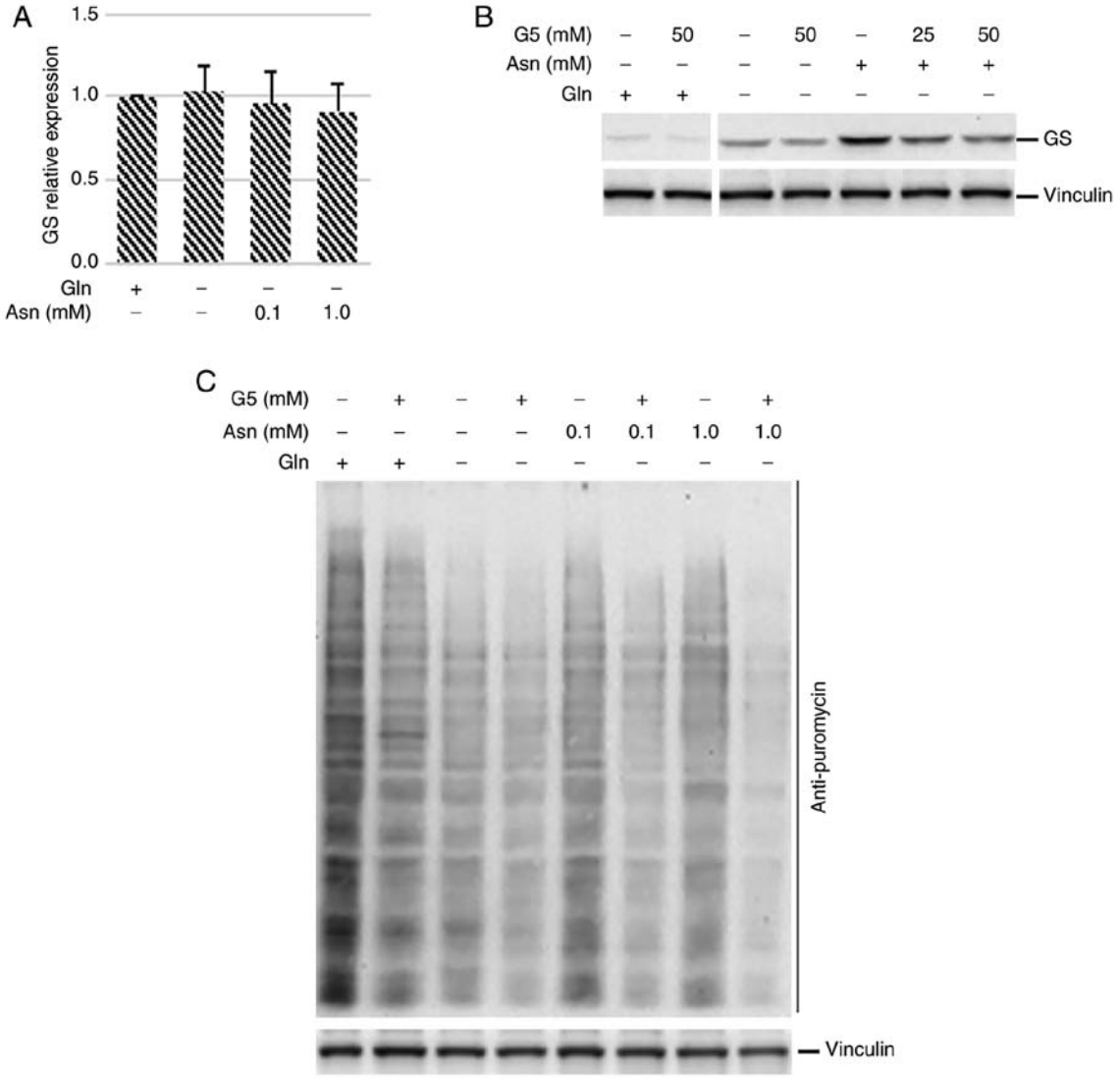

Figure 6. c-Myc inhibition leads to a decrease in GS levels in glutamine-starved asparagine-fed cells. (A) RT-qPCR analysis of *GS* expression in cen3tel cells cultured for 24 h in the indicated medium. The expression of *GS* in cells cultured in the different culture conditions is presented as a Log_2FC relative to *GS* expression in cells grown in the presence of glutamine. Error bars, standard deviation. (B) Western blot analysis of GS expression in cen3tel cells grown for 24 h in the medium indicated with or without the c-Myc inhibitor 10074-G5 (50 μM; indicated in the figure as G5). (C) Western blot analysis of global protein synthesis in cen3tel cells cultured for either 24 h in the medium indicated with or without 10074-G5 (50 μM). Puromycin (90 μM) was added during the last 10 min before sample collections. Puromycin incorporation was revealed with an anti-puromycin antibody. In the western blots, vinculin was used as the loading control. GS, glutamine synthetase; Gln, glutamine; Asn, asparagine; RT-qPCR, reverse transcription-quantitative PCR; FC, fold change.

the presence or absence of glutamine, or in the absence of glutamine but presence of asparagine. However, when cells were exposed to 10074-G5, a compound that blocks c-Myc-Max interaction, and hence c-Myc transcriptional activity (41), an evident decrease in GS protein expression levels was observed in glutamine-starved cells supplemented with asparagine, both in cen3tel and MDA-MB-231 cells (Figs. 6B and S1C). This suggested that c-Myc could control GS expression indirectly, at levels other than transcription.

Since c-Myc is involved in protein synthesis regulation at multiple levels, it was investigated whether c-Myc inhibition with 10074-G5 had an effect on global protein translation, which could account for the reduction in GS expression. Indeed, 10074-G5 treatment reduced global protein synthesis both in cen3tel and MDA-MB-231 cells incubated in different culture conditions (Figs. 6C and S1B). This suggested that in glutamine-starved cells fed with asparagine, c-Myc could contribute to the maintenance of translation, which may in turn allow the expression of GS, and hence glutamine synthesis.

Discussion

Oncogene activation and nutrient availability are crucial factors for cancer cell hyperproliferation. c-Myc is a key player in the metabolism of glucose and glutamine, which are avidly used by cancer cells. Glutamine is an essential amino acid for several tumors, particularly those bearing constitutively active c-*Myc* (12). In fact, oncogenic c-*Myc* causes cancer cells to become addicted to exogenous glutamine and glutamine withdrawal leads to cell growth arrest and cell death (6,42,43).

The expression of c-*Myc* in cancer cells can be influenced by nutrient levels. Herein, glucose deprivation was shown to lead to c-*Myc* overexpression in cen3tel cells, similar to the results obtained from various cancer cell lines in previous

studies (16,17). In different cancer cell types, glutamine deprivation was found to be associated with either c-*Myc* upregulation or downregulation (16,20). In the present study, glutamine starvation caused an alteration in c-Myc protein expression, which, to the best of our knowledge, has not yet been described in these culture conditions. In cen3tel cells and various cancer cell lines cultured without glutamine, a decrease in the expression of the canonical c-Myc isoform was observed, together with the synthesis of the longer c-Myc isoform, c-Myc 1. In cen3tel cells, the decline in canonical c-Myc and synthesis of c-Myc 1 paralleled cell growth arrest. Furthermore, it was demonstrated that asparagine was the only NEAA that, in the absence of glutamine, rescued c-Myc expression and allowed cell survival and proliferation, suggesting a possible link between glutamine deprivation, aberrant c-Myc expression and cell growth arrest.

In glutamine-starved cells, c-Myc 1 was already detectable 6 h after deprivation and was expressed in a ~1:1 ratio with the canonical c-Myc isoform after 24 h. This change in c-Myc expression was specific to glutamine deprivation; in fact, it did not occur in the absence of glucose only, but appeared shortly after deprivation of both glucose and glutamine.

The functional role of the two c-Myc isoforms is debated (21-23). While Blackwood *et al* (22) did not find variations in the transcriptional activity of c-Myc 1 and canonical c-Myc, Hann *et al* (21) showed that the overexpression of c-Myc 1, but not that of canonical c-Myc, significantly inhibited cell growth. In line with these results, Sato *et al* (23) demonstrated that the overexpression of the canonical c-Myc isoform in cancer cells promoted cell proliferation and colony formation at much higher levels than the longer isoform, and gene ontology analysis of the genes upregulated by canonical c-Myc revealed an enrichment in oncogenic and cell-cycle-related pathways. In the present study, the decreased expression of canonical c-Myc and the presence of the longer isoform were found to be associated with an arrest in cell proliferation, supporting the hypothesis that c-Myc 1 cannot take over the functions of canonical c-Myc, since the total level of the c-Myc proteins was almost the same in control and glutamine-starved cells. Indeed, it was found that asparagine was the only NEAA that used as supplement for glutamine-starved cells allowed a high expression of canonical c-Myc with a net decrease in c-Myc 1 levels and, simultaneously, cell survival and proliferation. The administration of the NEAAs alanine, aspartic acid, glutamic acid or proline in glutamine-starved cells did not alter c-Myc expression compared with glutamine deprivation, and did not allow cellular proliferation, thus strengthening the idea that high levels of canonical c-Myc are important for cellular viability and growth. Asparagine was also able to rescue c-Myc expression in cells that had been previously starved for glutamine.

In cells deprived of glutamine for 24 h, the addition of the proteasomal inhibitor MG132 did not lead to the accumulation of any c-Myc isoform, indicating that, at that stage of starvation, both c-Myc synthesis and c-Myc degradation were impaired. When asparagine was given to glutamine-starved cells, c-Myc turnover appeared to occur as in the presence of glutamine, confirming the importance of asparagine for c-Myc homeostasis.

Asparagine is synthesized by the enzyme asparagine synthetase from aspartate and glutamine, which provides the ammonium group (7). Thus, in the absence of glutamine, asparagine cannot be synthesized and cells must rely on exogenous asparagine to survive. When glutamine is absent, asparagine behaves as an essential amino acid (27,28), with the present results supporting this finding. Asparagine is not catabolized in mammalian cells and is mainly used in protein synthesis (39). Indeed, it was found here that asparagine supplementation allowed an increase in global translation, compared to glutamine-deprived cells and, in particular, an increase in the levels of glutamine synthetase (GS), the enzyme deputed to glutamine synthesis.

GS plays a fundamental role in cellular physiology; in particular, when extracellular glutamine is scarce, it allows the biosynthesis of endogenous glutamine, which in turn provides the atoms required for the biosynthesis of multiple different macromolecules. Indeed, *GS* has been found to be upregulated in several cancers (13,44,45). As also shown by Pavlova *et al* (28), this study confirmed that GS activity is required for cellular viability in glutamine-deprived asparagine-fed cells. In fact, its inhibition led to cell death in these culture conditions. In glutamine-fed cells, MSO did not have any effect on cellular viability, as expected. Asparagine was the only NEAA capable of inducing an increase in GS levels in the absence of glutamine. This increase was associated with GS ability to sustain cellular viability and proliferation, confirming that the high levels of this enzyme are important for cell survival when exogenous glutamine is not available. The observation that MSO led to the accumulation of GS in glutamine-fed cells, but not in glutamine-deprived cells, reinforces the hypothesis that the impairment in protein translation observed in the absence of glutamine also prevents GS synthesis, which can instead occur following asparagine supplementation.

However, cells maintained without glutamine but in the presence of asparagine proliferated at a lower rate than cells maintained in the presence of glutamine, suggesting that the high levels of GS observed in these conditions did not provide sufficient glutamine to support all the biosynthetic pathways in which this NEAA is involved. Indeed, asparagine was not able to relieve the stressful conditions induced by glutamine deprivation, as indicated by the high levels of PARP1 auto-PARylation and protein-PARylation, which were still present when glutamine-starved cells were fed with asparagine. The observation that GSH administration in asparagine-fed cells rescued cell proliferation and PARP1 activation suggested that asparagine cannot support the synthesis of adequate levels of GSH in the absence of glutamine, and thus cannot prevent high levels of oxidative stress, which could impair cell growth.

The present results revealed two main effects of asparagine in cells deprived of glutamine: Asparagine allows a high level of canonical c-Myc synthesis, as well as cell survival and proliferation boosting GS levels. By inhibiting c-Myc activity using 10074-G5, which prevents c-Myc/Max interaction (41), a decrease in GS levels was shown in glutamine-starved asparagine-fed cells, suggesting that c-Myc can play a role in regulating GS levels. However, this was not a transcriptional regulation, as no differences in *GS* transcript levels were observed between cells fed with glutamine, without glutamine or with asparagine in the absence of glutamine. Given that c-Myc is also known to indirectly control translation at various levels, including the expression of translational factors and ribosomal proteins (46,47), and indeed c-Myc inhibition by 10074-G5 was found to lead to

a decrease in global protein synthesis, it can be envisaged that the high levels of the canonical c-Myc isoform found in glutamine-starved cells cultured in the presence of asparagine could sustain translation and GS synthesis in particular, which leads to the biosynthesis of glutamine, which can in turn sustain cellular viability.

In conclusion, these results indicate that glutamine deprivation can have an effect on c-Myc translation. Even though further experiments will be required to better elucidate the roles of c-Myc and c-Myc 1 in the response to nutrient starvation, the observed correlation between the decrease in the expression of canonical c-Myc, the increase in c-Myc 1 levels and growth arrest in glutamine-deprived cells suggests that these phenomena can be interconnected. The finding that, in the absence of glutamine, asparagine allows both a high expression of canonical c-Myc and cellular proliferation strengthens this hypothesis. Future experiments testing these observations *in vivo* could provide relevant information for the development of cancer therapies targeting metabolism.

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Authors' contributions

IC, CP and DB performed the experiments and analyzed the data. IC and CM conceived the research concept and designed the experiments and wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

References

1. DeBerardinis RJ, Lum JJ, Hatzivassiliou G and Thompson CB: The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. Cell Metab 7: 11-20, 2008.
2. Zhu J and Thompson CB: Metabolic regulation of cell growth and proliferation. Nat Rev Mol Cell Biol 20: 436-450, 2019.
3. Pavlova NN and Thompson CB: The emerging hallmarks of cancer metabolism. Cell Metab 23: 27-47, 2016.
4. Warburg O: On the origin of cancer cells. Science 123: 309-314, 1956.
5. Ganapathy-Kanniappan S: Molecular intricacies of aerobic glycolysis in cancer: Current insights into the classic metabolic phenotype. Crit Rev Biochem Mol Biol 53: 667-682, 2018.
6. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, Nissim I, Daikhin E, Yudkoff M, McMahon SB and Thompson CB: Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proc Natl Acad Sci USA 105: 18782-18787, 2008.
7. Altman BJ, Stine ZE and Dang CV: From Krebs to clinic: Glutamine metabolism to cancer therapy. Nat Rev Cancer 16: 619-634, 2016.
8. Zhang J, Pavlova NN and Thompson CB: Cancer cell metabolism: The essential role of the nonessential amino acid, glutamine. EMBO J 36: 1302-1315, 2017.
9. Pinweha P, Rattanapornsompong K, Charoensawan V and Jitrapakdee S: MicroRNAs and oncogenic transcriptional regulatory networks controlling metabolic reprogramming in cancers. Comput Struct Biotechnol J 14: 223-233, 2016.
10. Tarrado-Castellarnau M, de Atauri P and Cascante M: Oncogenic regulation of tumor metabolic reprogramming. Oncotarget 7: 62726-62753, 2016.
11. Dejure FR and Eilers M: MYC and tumor metabolism: Chicken and egg. EMBO J 36: 3409-3420, 2017.
12. Dang CV: A Time for MYC: Metabolism and therapy. Cold Spring Harb Symp Quant Biol 81: 79-83, 2016.
13. Bott AJ, Peng IC, Fan Y, Faubert B, Zhao L, Li J, Neidler S, Sun Y, Jaber N, Krokowski D, *et al*: Oncogenic Myc induces expression of glutamine synthetase through promoter demethylation. Cell Metab 22: 1068-1077, 2015.
14. Arad G, Freikopf A and Kulka RG: Glutamine-stimulated modification and degradation of glutamine synthetase in hepatoma tissue culture cells. Cell 8: 95-101, 1976.
15. Hann SR, Sloan-Brown K and Spotts GD: Translational activation of the non-AUG-initiated c-myc 1 protein at high cell densities due to methionine deprivation. Genes Dev 6: 1229-1240, 1992.
16. Sun L, Song L, Wan Q, Wu G, Li X, Wang Y, Wang J, Liu Z, Zhong X, He X, *et al*: cMyc-mediated activation of serine biosynthesis pathway is critical for cancer progression under nutrient deprivation conditions. Cell Res 25: 429-444, 2015.
17. Wu S, Yin X, Fang X, Zheng J, Li L, Liu X and Chu L: c-MYC responds to glucose deprivation in a cell-type-dependent manner. Cell Death Discov 1: 15057, 2015.
18. Wong WJ, Qiu B, Nakazawa MS, Qing G and Simon MC: MYC degradation under low O2 tension promotes survival by evading hypoxia-induced cell death. Mol Cell Biol 33: 3494-3504, 2013.
19. Effenberger M, Bommert KS, Kunz V, Kruk J, Leich E, Rudelius M, Bargou R and Bommert K: Glutaminase inhibition in multiple myeloma induces apoptosis via MYC degradation. Oncotarget 8: 85858-85867, 2017.
20. Dejure FR, Royla N, Herold S, Kalb J, Walz S, Ade CP, Mastrobuoni G, Vanselow JT, Schlosser A, Wolf E, *et al*: The MYC mRNA 3'-UTR couples RNA polymerase II function to glutamine and ribonucleotide levels. EMBO J 36: 1854-1868, 2017.
21. Hann SR, Dixit M, Sears RC and Sealy L: The alternatively initiated c-Myc proteins differentially regulate transcription through a noncanonical DNA-binding site. Genes Dev 8: 2441-2452, 1994.
22. Blackwood EM, Lugo TG, Kretzner L, King MW, Street AJ, Witte ON and Eisenman RN: Functional analysis of the AUG- and CUG-initiated forms of the c-Myc protein. Mol Biol Cell 5: 597-609, 1994.
23. Sato K, Masuda T, Hu Q, Tobo T, Gillaspie S, Niida A, Thornton M, Kuroda Y, Eguchi H, Nakagawa T, *et al*: Novel oncogene 5MP1 reprograms c-Myc translation initiation to drive malignant phenotypes in colorectal cancer. EBioMedicine 44: 387-402, 2019.
24. Hay N: Reprogramming glucose metabolism in cancer: Can it be exploited for cancer therapy? Nat Rev Cancer 16: 635-649, 2016.
25. Fung MKL and Chan GC: Drug-induced amino acid deprivation as strategy for cancer therapy. J Hematol Oncol 10: 144, 2017.
26. Matés JM, Di Paola FJ, Campos-Sandoval JA, Mazurek S and Márquez J: Therapeutic targeting of glutaminolysis as an essential strategy to combat cancer. Semin Cell Dev Biol 98: 34-43, 2020.

27. Zhang J, Fan J, Venneti S, Cross JR, Takagi T, Bhinder B, Djaballah H, Kanai M, Cheng EH, Judkins AR, *et al*: Asparagine plays a critical role in regulating cellular adaptation to glutamine depletion. Mol Cell 56: 205-218, 2014.
28. Pavlova NN, Hui S, Ghergurovich JM, Fan J, Intlekofer AM, White RM, Rabinowitz JD, Thompson CB and Zhang J: As extracellular glutamine levels decline, asparagine becomes an essential amino acid. Cell Metab 27: 428-438.e5, 2018.
29. Mondello C, Chiesa M, Rebuzzini P, Zongaro S, Verri A, Colombo T, Giulotto E, D'incalci M, Franceschi C and Nuzzo F: Karyotype instability and anchorage-independent growth in telomerase-immortalized fibroblasts from two centenarian individuals. Biochem Biophys Res Commun 308: 914-921, 2003.
30. Zongaro S, de Stanchina E, Colombo T, D'Incalci M, Giulotto E and Mondello C: Stepwise neoplastic transformation of a telomerase immortalized fibroblast cell line. Cancer Res 65: 11411-11418, 2005.
31. Belgiovine C, Frapolli R, Bonezzi K, Chiodi I, Favero F, Mello-Grand M, Dei Tos AP, Giulotto E, Taraboletti G, D'Incalci M and Mondello C: Reduced expression of the ROCK Inhibitor Rnd3 is associated with increased invasiveness and metastatic potential in mesenchymal tumor cells. PLoS One 5: e14154, 2010.
32. Ostano P, Bione S, Belgiovine C, Chiodi I, Ghimenti C, Scovassi AI, Chiorino G and Mondello C: Cross-analysis of gene and miRNA genome-wide expression profiles in human fibroblasts at different stages of transformation. OMICS 16: 24-36, 2012.
33. Bono B, Ostano P, Peritore M, Gregnanin I, Belgiovine C, Liguori M, Allavena P, Chiorino G, Chiodi I and Mondello C: Cells with stemness features are generated from in vitro transformed human fibroblasts. Sci Rep 8: 13838, 2018.
34. Chiodi I, Picco G, Martino C and Mondello C: Cellular response to glutamine and/or glucose deprivation in *in vitro* transformed human fibroblasts. Oncol Rep 41: 3555-3564, 2019.
35. Belgiovine C, Chiesa G, Chiodi I, Frapolli R, Bonezzi K, Taraboletti G, D'Incalci M and Mondello C: Snail levels control the migration mechanism of mesenchymal tumor cells. Oncol Lett 12: 767-771, 2016.
36. Xu L, Begum S, Hearn JD and Hynes RO: GPR56, an atypical G protein-coupled receptor, binds tissue transglutaminase, TG2, and inhibits melanoma tumor growth and metastasis. Proc Natl Acad Sci USA 103: 9023-9028, 2006.
37. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
38. Stegink LD, Filer LJ Jr, Brummel MC, Baker GL, Krause WL, Bell EF and Ziegler EE: Plasma amino acid concentrations and amino acid ratios in normal adults and adults heterozygous for phenylketonuria ingesting a hamburger and milk shake meal. Am J Clin Nutr 53: 670-675, 1991.
39. Ubuka T and Meister A: Studies on the utilization of asparagine by mouse leukemia cells. J Natl Cancer Inst 46: 291-298, 1971.
40. Freikopf A and Kulka RG: Specificity of the glutamine-binding site involved in the regulation of glutamine-synthetase activity in hepatoma tissue-culture cells. Eur J Biochem 56: 483-492, 1975.
41. Yap JL, Wang H, Hu A, Chauhan J, Jung KY, Gharavi RB, Prochownik EV and Fletcher S: Pharmacophore identification of c-Myc inhibitor 10074-G5. Bioorg Med Chem Lett 23: 370-374, 2013.
42. Yuneva M, Zamboni N, Oefner P, Sachidanandam R and Lazebnik Y: Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. J Cell Biol 178: 93-105, 2007.
43. Qing G, Li B, Vu A, Skuli N, Walton ZE, Liu X, Mayes PA, Wise DR, Thompson CB, Maris JM, *et al*: ATF4 regulates MYC-mediated neuroblastoma cell death upon glutamine deprivation. Cancer Cell 22: 631-644, 2012.
44. Tardito S, Oudin A, Ahmed SU, Fack F, Keunen O, Zheng L, Miletic H, Sakariassen PØ, Weinstock A, Wagner A, *et al*: Glutamine synthetase activity fuels nucleotide biosynthesis and supports growth of glutamine-restricted glioblastoma. Nat Cell Biol 17: 1556-1568, 2015.
45. Bott AJ, Shen J, Tonelli C, Zhan L, Sivaram N, Jiang YP, Yu X, Bhatt V, Chiles E, Zhong H *et al*: Glutamine anabolism plays a critical role in pancreatic cancer by coupling carbon and nitrogen metabolism. Cell Rep 29: 1287-1298.e6, 2019.
46. Biffo S, Manfrini N and Ricciardi S: Crosstalks between translation and metabolism in cancer. Curr Opin Genet Dev 48: 75-81, 2018.
47. Singh K, Lin J, Zhong Y, Burčul A, Mohan P, Jiang M, Sun L, Yong-Gonzalez V, Viale A, Cross JR, *et al*: c-MYC regulates mRNA translation efficiency and start-site selection in lymphoma. J Exp Med 216: 1509-1524, 2019.